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Regulation of muscle cell differentiation and growth by nutrients and exercise

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List of abbreviations

ADP, adenosine diphosphate

AMPK, adenosine monophosphate-activated protein kinase

ARE-mRNAs, AU-rich element-messenger RNAs

ATP, adenosine triphosphate

BCAA, branched-chain amino acid

BMP, bone morphogenetic protein

Cdk, cyclin-dependent kinase

DNA, desoxyribonucleic acid

DYRK, dual-specificity tyrosine-phosphorylated and -regulated kinase

eEF2, eukaryotic elongation factor 2

eEF2K, eukaryotic elongation factor 2 kinase

eIF2, eukaryotic initiation factor 2

eIF2B, eukaryotic initiation factor 2B

eIF4A, eukaryotic initiation factor 4A

eIF4E, eukaryotic initiation factor 4E

eIF4F, eukaryotic initiation factor 4F

eIF4G, eukaryotic initiation factor 4G

ERK1/2, extracellular signal-regulated kinase 1/2

FGF, fibroblast growth factor

FOXO, forkhead box gene, group O

GDP, guanosine diphosphate

GDF8, growth and differentiation factor 8

GEF, guanosine exchange factor

GSK3, glycogen synthase kinase 3

GTP, guanosine triphosphate

HGF, hepatocyte growth factor

IEG, immediate early genes

IGF, insulin-like growth factor

IGFI-R, insulin-like growth factor I receptor

IL6, interleukin 6

IRS, insulin receptor substrate

JNK, c-Jun NH_2 terminal

kDa, kilodalton

MAFbx, muscle atrophy F-box

MAPK, mitogen-activated protein kinase

MEF2, myocyte enhancer factor 2

MEK, extracellular signal-regulated kinase kinase

MGF, mechano growth factor

MHC, myosin heavy chain

MK2, MAPK-activated protein kinase-2

MKK, mitogen-activated protein kinase kinase

MKKK, mitogen-activated protein kinase kinase kinase

Mnks, mitogen-activated protein kinase signal-integrating kinases

MRF, myogenic regulatory factor

mTOR, mammalian target of rapamycin

MURF1, muscle ring finger 1

NFAT, nuclear factor of activated T-cells

NFκB, nuclear factor kappa B

PCNA, proliferating cell nuclear antigen

PGC1α, PPARγ coactivator 1 alpha

PI3K, phosphatidylinositol 3 kinase

PKA, protein kinase A

PKB, protein kinase B

PKC, protein kinase C

PPARγ, peroxisome proliferator-activated receptor gamma

PP1, protein phosphatase 1

PP2A, protein phosphatase 2A

$p70^{s6k}$, 70kDa ribosomal protein S6 kinase

$p90^{RSK}$, 90kDa ribosomal protein S6 kinase

Rheb, Ras homolog enriched in brain

Raptor, regulatory associated protein to mTOR

Rictor, rapamycin-insensitive companion of mTOR

RNA, ribonucleic acid

RNA-BP, RNA-binding protein

S6, ribosomal protein S6

TGFβ, transforming growth factor beta

TNFα, tumor necrosis factor alpha

TLR, toll-like receptor

TSC1/2, tumor sclerosis complex 1/2

4E-BP1, eukaryotic initiation factor 4E-binding protein

5'-TOP mRNA, 5' terminal oligopyrimidine messenger RNA

Chapter 1

Muscle cell differentiation and growth

1. Introduction

The development of muscle tissue, or myogenesis, involves a sequence of complex cellular changes including commitment of cells to a muscle lineage, withdrawal of precursor myoblasts from the cell cycle, cell fusion to form multinucleated cells and expression of muscle-specific genes (Fig. 1.1). These different events are thought to be regulated independently but are coordinated to produce multinucleated, elongated myofibres containing large amounts of muscle- and fibre type-specific forms of proteins such as actin, myosin and creatine kinase (Hesketh & Whitelaw, 1992).

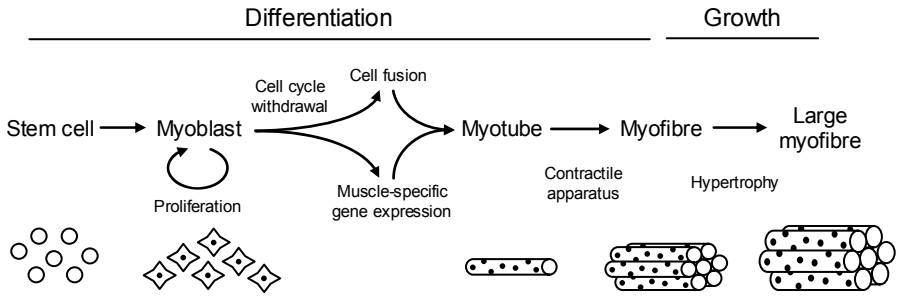

Fig. 1.1. *Sequence of events in myogenic cell development and growth* (Adapted from Hesketh & Whitelaw, 1992).

In this first chapter, a summary of the current knowledge on the regulation of muscle cell differentiation and growth will be presented as well as the influence of nutrients and contractile activity on both processes. As some notions of the regulation of protein synthesis and degradation will help to understand muscle cell differentiation, the latter will be developed after having described the process of muscle growth.

2. Muscle cell growth

During life, muscle mass undergoes many changes in terms of quantity and quality. Skeletal muscle is a dynamic tissue able to hypertrophy and to atrophy according to the nutrient state and the physical activity. Muscle mass accretion or loss results from net protein balance. If protein synthesis exceeds protein breakdown, proteins will accumulate and muscle mass will increase. Inversely, if protein synthesis is less than breakdown, loss of muscle mass will occur. To understand how muscle mass is affected, it is important to clarify the regulation of both protein synthesis and breakdown.

2.1. Protein synthesis

2.1.1. From gene to protein (Fig. 1.2)

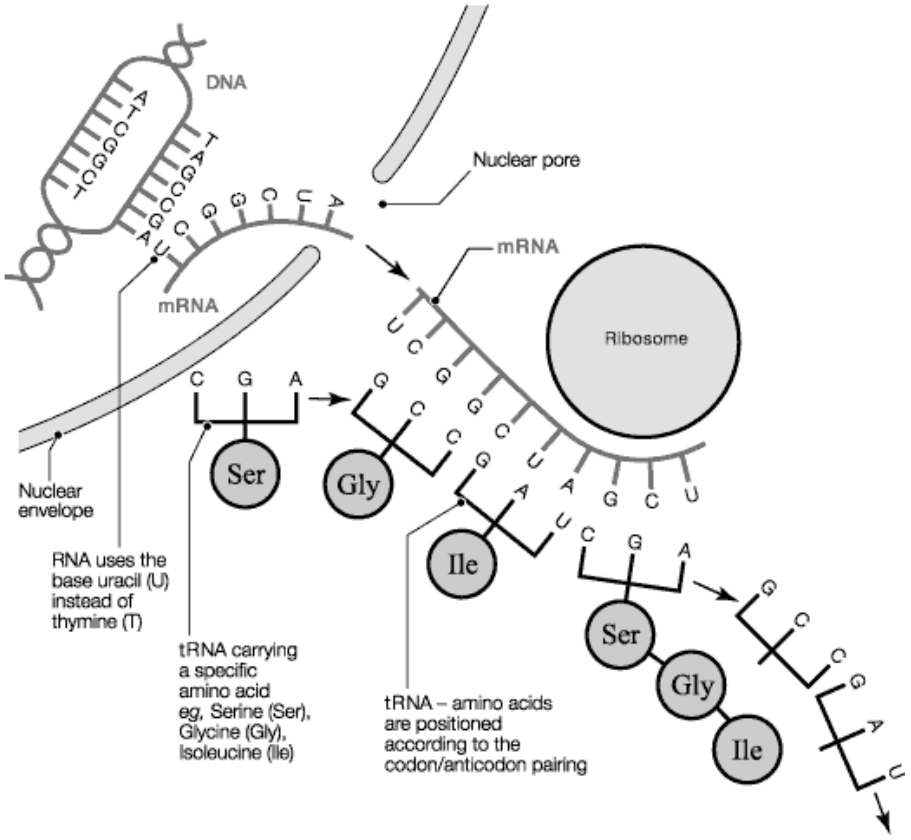

Fig. 1.2. *Schematic representation of transcription and translation* (Givens & Reiss, 1996).

Transcription

Before the synthesis of a protein begins, the corresponding RNA molecule is produced by RNA transcription. One strand of the DNA double helix is used as a template by the RNA polymerase to synthesize a messenger RNA (mRNA). This mRNA migrates from the nucleus to the cytoplasm. During this step, mRNA goes through different types of maturation including the splicing where the non-coding sequences are eliminated. The coding mRNA sequence can be described as a unit of three nucleotides called a codon.

Translation

The ribosome binds to the mRNA at the start codon AUG that is recognized only by the initiator methionyl-tRNA. The ribosome proceeds to the elongation phase. During this stage, complexes, composed of an amino acid linked to tRNA, sequentially bind to the appropriate codon in mRNA by forming complementary base pairs with the tRNA anticodon. The ribosome moves from codon to codon along the mRNA. Amino acids are added one by one, translated into polypeptidic sequences dictated by DNA and represented by mRNA. At the end, a release factor binds to the stop codon, terminating translation and releasing the polypeptide from the ribosome.

2.1.2. Protein synthesis regulation

Any component of gene expression can potentially be regulated: chemical and structural modifications of DNA or chromatin, transcription and post-transcriptional modifications, mRNA stability, translational and post-translational modifications.

Transcription

Regulation of transcription controls when transcription occurs and how much RNA is created. Transcription of a gene by RNA polymerase can be regulated by at least five mechanisms (Raven *et al.*, 2004):

1. Specificity factors alter the specificity of RNA polymerase for a given promoter or set of promoters, making it more or less likely to bind to them.
2. Repressors bind to non-coding sequences on the DNA strand that are close to or overlapping the promoter region, impeding the progress of RNA polymerase along the strand, thus impeding the expression of the gene.
3. Basal transcription factors position RNA polymerase at the start of a protein-coding sequence and then release the polymerase to transcribe the mRNA.
4. Activators enhance the interaction between RNA polymerase and a particular promoter, favouring the expression of the gene. Activators do this by increasing the attraction of RNA polymerase for the promoter, through interactions with subunits of the RNA polymerase or by changing the structure of the DNA.
5. Enhancers are sites on the DNA helix that are bound to by activators in order to loop the DNA bringing a specific promoter to the initiation complex.

The regulation of most genes is regulated primarily at the level of transcription rather than translation. This transcriptional regulation is mediated by transcription factors, which generally simultaneously bind DNA and RNA polymerase, as well as other agents necessary for the transcription process. Transcription factors, and their cofactors, can be regulated through reversible structural alterations such as phosphorylation or inactivated through mechanisms such as proteolysis. Specific muscular transcription factors, namely the myogenic regulatory factors (MRFs), have been discovered (Puri & Sartorelli, 2000) and will be detailed in the next section.

Transcription is initiated at the promoter site as an increase in the amount of an active transcription factor binds a target DNA sequence. Other proteins, known as scaffolding proteins bind other cofactors and hold them in place. DNA sequences far from the point of initiation, known as enhancers, can aid in the assembly of this transcription machinery. The latter will not be further detailed since the current work will essentially report data on the translational machinery, which will be described in details in the next paragraph.

Frequently, extracellular signals induce the expression of immediate early genes (IEGs) such as c-fos, c-jun, or AP-1 (activating protein-1). These are in and of themselves transcription factors or components thereof, and can further influence gene expression.

Post-transcription and mRNA stability

Two classes of short RNA molecules, small interfering RNA (siRNA) and microRNA (miRNA), have been identified as sequence-specific post-transcriptional regulators of gene expression (Tang, 2005).

Pri-microRNA transcripts are first processed into ~70-nucleotide pre-miRNAs with imperfect stem-loop by Drosha inside the nucleus. Pre-miRNAs are transported to the cytoplasm by Exportin 5 and are processed into miRNA:miRNA* duplexes by Dicer, a multidomain enzyme of the RNase III family (Fig. 1.3).

Dicer also processes long double-stranded RNA (dsRNA) molecules into small interfering RNA (siRNA) duplexes. The nascent siRNAs and miRNAs are double-stranded duplexes. These duplexes need to be unwound before they can be assembled into an RNA-induced silencing complex (RISC). Only one strand (~21-25 nucleotides) of the miRNA:miRNA* duplex or the siRNA duplex is preferentially assembled into the RISC, which subsequently

acts on its target by translational repression or mRNA cleavage, depending, at least in part, on the level of complementarity between the small RNA and its target (He & Hannon, 2004).

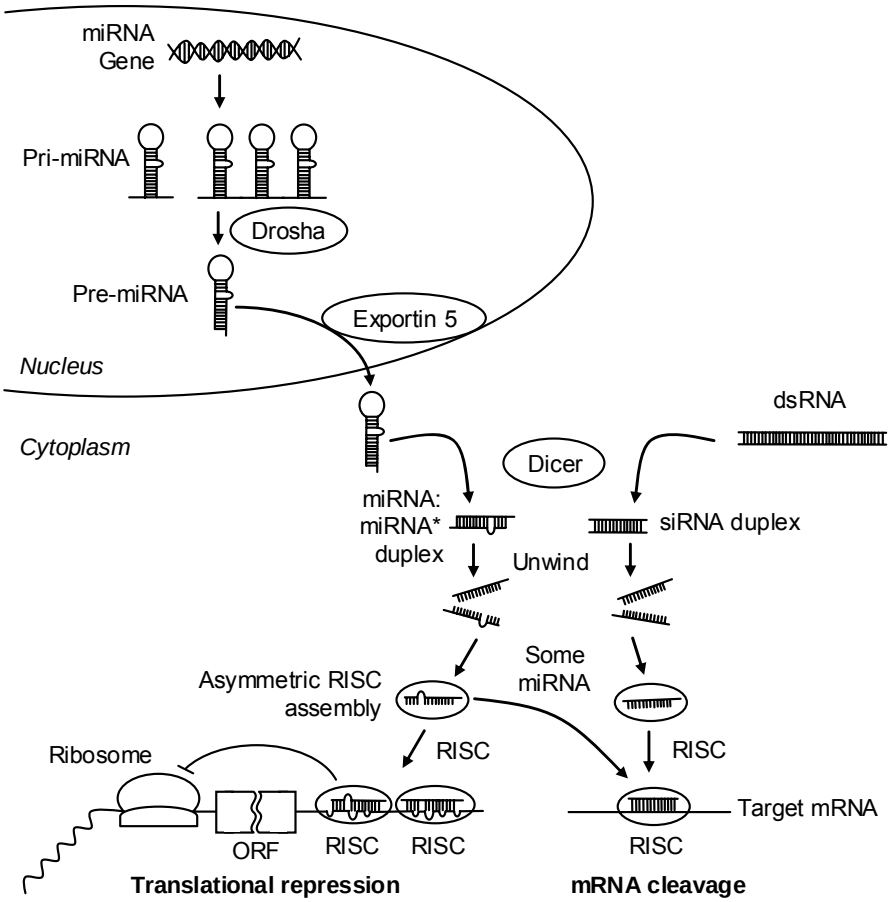

Fig. 1.3. *Model for the biogenesis and post-transcriptional suppression of microRNAs and small interfering RNAs.* ORF, open reading frame. (Adapted from He & Hannon, 2007)

In vitro and in vivo biochemical studies have shown that a siRISC can function as a miRISC to repress translation of the target mRNA; similarly, a miRISC can function as a siRISC to cleave the target mRNA. This functional interchangeability between a siRISC and miRISC argues that siRISCs and miRISCs are highly similar (Zeng *et al.*, 2003) although

evidence suggests that they are distinct types of complex. siRNA and miRNA programmed RISCs have distinct targeting functions in cells. Many endogenous miRNAs and their RISCs are genetically programmed to regulate gene expression and thus are important for the growth and development of an organism (Rhoades *et al.*, 2002). This point will be discussed in the third section of this chapter. By contrast, siRNAs are produced from dsRNA that are often synthesized in vitro and in vivo from viruses or repetitive sequences introduced by genetic engineering (Hannon, 2002; Pfeffer *et al.*, 2004). In addition, dsRNA can be produced from endogenously activated transposons (Tabara *et al.*, 1999). In summary, siRNAs have been proposed to function in antiviral defense, silencing mRNAs that are overproduced or translationally aborted and guarding the genome form disruption by transposons.

Translation

The translation rate may be regulated by rapid changes within a few minutes. In this case, it implicates the activity or association of components of the translational machinery, which are primarily mediated by changes in the states of phosphorylation of translation factors and specific RNA-binding proteins. Over the longer term, hours to days, the control of protein synthesis involves changes in the cellular levels of translation factors and ribosomes (Proud, 2007).

The process of translation is divided into three stages: initiation, elongation and termination. Each stage requires translation factors that transiently associate with the ribosome. These factors are called eukaryotic initiation (eIFs), elongation (eEFs) and termination or release factors (eRFs). The regulation of the translation phase mainly occurs at the level of the initiation and elongation (Fig. 1.4).

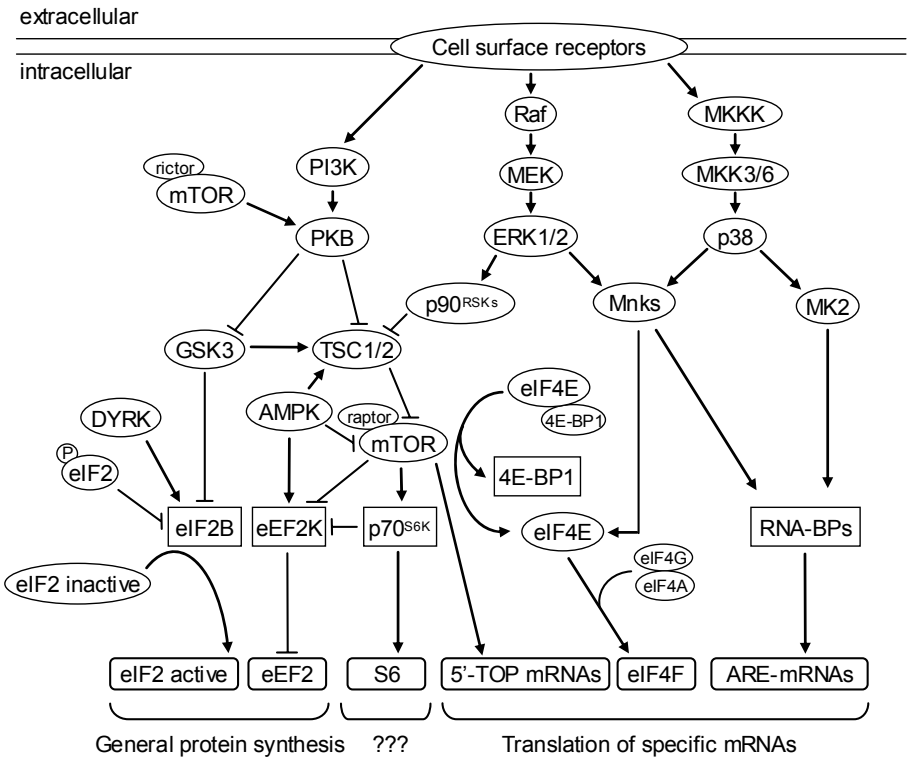

Fig 1.4. *Overview of the control of translation by signal transduction pathways*. MKKK, mitogen-activated protein kinase kinase kinase; MKK, mitogen-activated protein kinase kinase; MEK, extracellular signal-regulated kinase kinase; ERK1/2, extracellular signal-regulated kinase 1/2; PI3K, phosphatidylinositol 3 kinase; PKB, protein kinase B; mTOR, mammalian target of rapamycin; raptor, regulatory associated protein of mTOR; rictor, rapamycin-insensitive companion of mTOR; $p90^{RSK}$, 90kDa ribosomal protein S6 kinase; Mnks, mitogen-activated protein kinase signal-integrating kinases; MK2, mitogen-activated protein kinase-activated protein kinase-2; GSK3, glycogen synthase kinase 3; TSC1/2, tumor sclerosis complex 1/2; AMPK, adenosine monophosphate-activated protein kinase; raptor, regulatory associated protein to mTOR; eIF4E, eukaryotic initiation factor 4E; 4E-BP1, eukaryotic initiation factor 4E-binding protein; eIF4G, eukaryotic initiation factor 4G; eIF4A, eukaryotic initiation factor 4A; eIF4F, eukaryotic initiation factor 4F; $p70^{s6k}$, 70kDa ribosomal protein S6 kinase; S6, ribosomal protein S6; eEF2K, eukaryotic elongation factor 2 kinase; eEF2, eukaryotic elongation factor 2; DYRK, dual-specificity tyrosine-phosphorylated and -regulated kinase; eIF2, eukaryotic initiation factor 2; eIF2B, eukaryotic initiation factor 2B; RNA-BPs, RNA-binding proteins; ARE-mRNAs, AU-rich element-mRNAs; 5'-TOP mRNAs, 5' terminal oligopyrimidine mRNAs (Adapted from Proud, 2007).

The translation machinery

The initiation stage of translation involves the recruitment of the 40S ribosomal subunit to the mRNA and the identification of the start codon. Several major events are involved in this process, including the recruitment of methionyl-tRNA to the 40S subunit. **eIF2** (eukaryotic initiation factor 2) is a heterotrimeric GTP-binding protein which binds methionyl-tRNA only in its GTP-bound state (Fig. 1.5). The GTP is hydrolysed during the initiation process and eIF2 leaves the 40S subunit as inactive eIF2·GDP. Regeneration of active eIF2·GTP requires a GEF (guanosine exchange factor) called **eIF2B**, a heteropentamer (α-ε) (Hinnebusch, 2000). The alpha subunit of eIF2 is subject to regulatory phosphorylation. When phosphorylated, it acts as a competitive inhibitor of eIF2B, blocking recycling of eIF2 and inhibiting translation initiation (Proud, 2007).

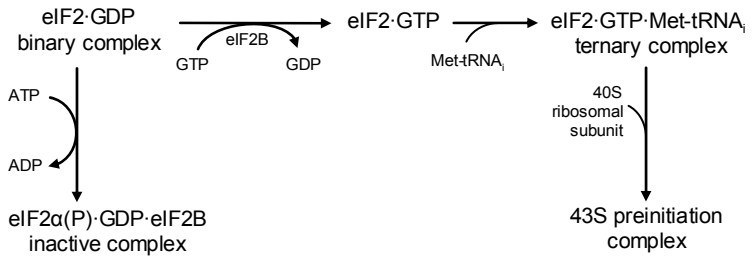

Fig 1.5. *Control of eIF2* (Adapted from Webb & Proud, 1997).

Another initiation factor playing a key role in the regulation of protein synthesis is **eIF4E** (eukaryotic initiation factor 4E) (Fig. 1.6). It binds the 5'-cap structure of the mRNA. The 5' cap is a specially altered nucleotide end to the 5' end of precursor mRNA. The process of 5' capping in the nucleus is vital to creating mature mRNA which is then able to undergo translation. eIF4E may be phosphorylated by Mnk1/2 (mitogen-activated protein kinase signal-integrating kinases 1/2) resulting in a decreased affinity for capped RNA (Joshi *et al.*, 1995). eIF4E also binds a number of protein partners including eIF4G, a multidomain scaffold protein which interacts with other

components of the translational machinery such as eIF4A. The eIF4A·eIF4E·eIF4G complex is usually referred to eIF4F (Mader *et al.*, 1995). The biological activity of eIF4E can be regulated by a set of **4E-BPs** (eIF4E-binding proteins). By binding eIF4E, the 4E-BPs block its interaction with eIF4G, thereby making eIF4E unavailable for initiation complex formation (Mader *et al.*, 1995; Gingras *et al.*, 1999). The regulation of eIF4E by 4E-BPs is described in chapter 2.

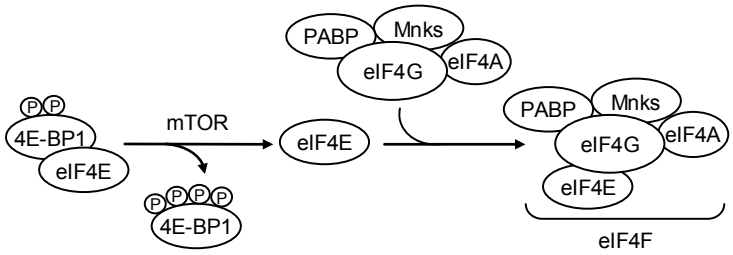

Fig. 1.6. *Control of eIF4F initiation complex* (Adapted from Proud, 2007).

Peptide-chain elongation requires two eEFs. **eEF1** binds GTP and recruits aminoacyl-tRNAs to the A-site of the ribosome to match the codon located in that site. It is actually eEF1A that does this, whereas eEF1B acts as the GEF for eEF1A (Proud, 2007). **eEF2** is a monomer that binds GTP and mediates the translocation step of elongation where the ribosome moves by one codon relative to the mRNA and the peptidyl-tRNA moves from the A- to the P-site (Jorgensen *et al.*, 2006). The phosphorylation of eEF2 impairs the interaction of eEF2 with the ribosome, thus inactivating it (Carlberg *et al.*, 1990).

Phosphorylation is catalysed by a highly specific enzyme, **eEF2 kinase**, which is dependent upon calcium and calmodulin for activity (Herbert & Proud, 2006) and is inhibited by several kinases like $p70^{s6k}$ (p70 ribosomal S6 kinase) (Wang *et al.*, 2001b), $p90^{RSK}$ (p90 ribosomal S6 kinase) (Wang *et al.*, 2001b) and p38 MAPK (Knebel *et al.*, 2001). It is of note that the

majority of the energy used in translation is consumed during elongation. The ability of calcium ions to activate eEF2 kinase and inhibit eEF2 may lead to a reduction in the rate of elongation to decrease energy utilization in contracting muscle, which requires energy (Proud, 2007).

Certain ribosomal proteins are phosphorylated within cells. The best-known of these is S6 which is phosphorylated by **S6Ks** (S6 kinases). In addition to S6, other substrates for S6Ks have been identified, eIF4B (Raught *et al.*, 2004; Holz *et al.*, 2005) and eEF2 kinase (Wang *et al.*, 2001b). See chapter 2 for more details.

Upstream signalling to the translational machinery (Fig. 1.4)

The **PI3K** (phosphatidylinositol 3 kinase) plays a key role in signalling downstream of a wide variety of receptors, including those for insulin and insulin-like growth factors. Activation of PI3K activates effectors such as **PKB** (protein kinase B also termed Akt). One substrate of PKB is **GSK3** (glycogen synthase kinase 3). PKB phosphorylates both GSK3 isoforms (α and β) at their N-termini, inhibiting their activity against certain substrates, including eIF2Bε (Welsh & Proud, 1993) which needs to be phosphorylated by members of the **DYRK** (dual-specificity tyrosine-phosphorylated and -regulated kinase) group of enzymes before being phosphorylated by GSK3 (Woods *et al.*, 2001). The phosphorylation of eIF2B by GSK3 inhibits its activity (Welsh *et al.*, 1998). Other regulatory inputs seem to be important to activate eIF2B like those from amino acids (Campbell *et al.*, 1999) and from ERK (extracellular signal-regulated kinase) signalling (Quevedo *et al.*, 2000). The input from ERK to the control of eIF2B appears to be mediated via activation of PP1 (protein phosphatase 1) (Quevedo *et al.*, 2000). The N-terminal site in GSK3 can also be phosphorylated by $p90^{RSK}$ which lies downstream of ERK signalling (Quevedo *et al.*, 2003).

Another substrate of PKB is **mTOR** (mammalian target of rapamycin). Since the regulation of mTOR by **TSC1/TSC2** (tumor sclerosis complex 1 and 2), **Rheb** (Ras homolog enriched in brain) and **AMPK** (AMP-activated protein kinase) as well as the activation of its downstream targets, **p70^{s6k}** and **4E-BP1**, will be extensively described in the second chapter of this work, they will not be further detailed at this level.

In addition to the PI3K pathway, the **ERK** and the **p38** MAPK pathways also play a role in the control of the translational machinery. ERK activates members of the **p90^{RSKs}** group of kinases (RSK1-RSK4, ribosomal S6 kinases 1-4), which phosphorylate several translation factors or their regulators. The p90^{RSKs} may provide a link between Ras/Raf/MEK/ERK signalling and the activation of mTOR since they can phosphorylate TSC2 at a C-terminal site (Rolfe *et al.*, 2005) and RSK-mediated phosphorylation of TSC2 leads to activation of mTOR. Moreover, p90^{RSK} phosphorylate at least two other proteins that are involved in translational control, eEF2k and p70^{s6k} (Wang *et al.*, 2001b).

p38 MAPK activates **MK-2** (or MAPKAPK-2, MAPK-activated protein kinase-2), which regulates the stability of mRNAs, probably via the phosphorylation of ARE (AU-rich element)-binding proteins (Dean *et al.*, 2004).

Recent data suggest that **Mnks** may be important in controlling mRNA function at post-transcriptional levels, such as RNA stability or translation (Buxade *et al.*, 2005). Mnk 1 and 2 are activated by phosphorylation by either ERK1/2 or by p38 (Fukunaga & Hunter, 1997; Waskiewicz *et al.*, 1997). They bind through a region in the N-termini to the C-terminus of eIF4G and mediate eIF4E phosphorylation in vivo (Pyronnet *et al.*, 1999; Ueda *et al.*, 2004). The role of the phosphorylation of eIF4E in mammals remains unclear. By weakening the interaction of eIF4E with the cap,

phosphorylation of eIF4E may allow the eIF4F complex to detach from the 5'-cap during scanning, perhaps accelerating this process or allowing a second initiation complex to bind to the mRNA. Alternatively, phosphorylation of eIF4E may allow it to disengage from mRNAs that are already being translated in order to bind to others that have arrived recently in the cytoplasm or have perhaps been translationally derepressed to allow their translation (Proud, 2007).

The initiation and elongation stages of translation are controlled by many regulatory inputs but their quantitative importance for the overall regulation of protein synthesis is not yet established and may vary between cell types and between stimuli. The fourth part of this introduction will present the role of nutrients and exercise in the regulation of translation and protein synthesis. But, as mentioned above, net protein balance results from both protein synthesis and protein degradation. The next section will thus present the 3 main proteolytic pathways in skeletal muscle.

2.2. Protein degradation

If detailed information is available on the mechanisms that regulate protein synthesis in muscle, much less is known about proteolysis. Current findings suggest that protein degradation is supported by three major proteolytic systems in skeletal muscle (Fig. 1.7): the calcium-dependent calpain (Zeman *et al.*, 1985), the lysosomal protease/cathepsins (Bird *et al.*, 1980) and the ubiquitin-proteasome systems (Fagan *et al.*, 1987). Evidence has accumulated that these three systems work as partners during muscle proteolysis rather than independently.

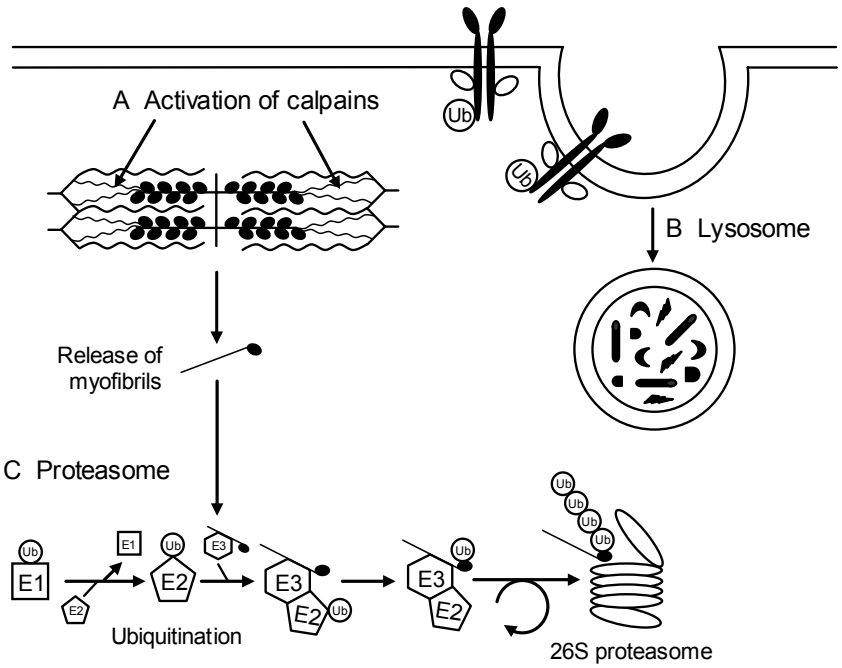

Fig. 1.7. *The proteolytic systems implicated in muscle atrophy:* the calcium-dependent calpain (A), the lysosomal protease (B) and the ubiquitin-proteasome systems (C) (Adapted from Jackman & Kandarian, 2004).

2.2.1. Calcium-dependent calpain system

At least half of total muscle protein is myofibrillar protein. The bulk of myofibrillar proteolysis involves the ubiquitin-proteasome pathway, but the latter cannot degrade intact myofibrils (Taillandier *et al.*, 1996; Solomon *et al.*, 1998). Titin, vinculin, nebulin and other proteins involved in the assembly and scaffolding of myofibrillar proteins are known calpain substrates (Huang & Forsberg, 1998), whereas calpains are unable to degrade the myofibrillar proteins actin and myosin (Thompson & Palmer, 1998). These findings have led to the idea that calpains are involved in myofibrillar disassembly, the earliest event in muscle-specific protein degradation.

Recently, caspases, proteins involved in apoptosis that have specific proteolytic activity, have been shown to have a role in muscle atrophy (Du *et al.*, 2004). Similarly to calpains, caspases have been proposed to have a role in the initial step in myofibrillar proteolysis by cleavage of actomyosin, making myofibrillar proteins available for ubiquitination (Jackman & Kandarian, 2004).

2.2.2. Lysosomal system

Cathepsins likely do not degrade cytosolic nor myofibrillar proteins, but rather membrane proteins, including receptors, ligands, channels and transporters (Mayer, 2000). The membrane-associated proteins degraded by lysosomal pathways will not contribute significantly to the bulk of total muscle protein resulting from catabolism. However, they may represent key proteins involved in the atrophied muscle phenotype (Jackman & Kandarian, 2004).

It seems that the lysosomal and ubiquitin-proteasomal systems also work together to degrade specific protein substrates (Hershko & Ciechanover, 1998). Several receptors and ion channels are ubiquitinated and then degraded by either the lysosomal or the proteasomal system. The signal that determines which of these pathways is used is the type of ubiquitin modification that occurs. If a protein is ubiquitinated by a polyubiquitin chain, then it is degraded by the proteasome. If the protein substrate is mono- or diubiquitinated, it is degraded by internalization and transport to the lysosome. It is not recognized by the proteasome because of the lack of the polyubiquitin chain (Jackman & Kandarian, 2004). In summary, monoubiquitination of membrane associated proteins leads to degradation by the lysosomal pathway and polyubiquitination to proteasomal degradation.

2.2.3. Ubiquitin-proteasome system

Covalent attachment of a polyubiquitin chain to the substrate

The process of substrate polyubiquitination involves a cooperative interaction of at least three classes of proteins termed E1 (ubiquitin activating), E2 (ubiquitin conjugating) and E3 (ubiquitin ligating) enzymes (Glickman & Ciechanover, 2002). Recently, attention has been paid to the increased expression of ubiquitin protein ligases in muscle disuse and atrophy, because they have the greatest tissue and substrate specificity in comparison with other proteins involved in ubiquitinating protein substrates. The two most studied are the ubiquitin ligases atrogin-1 or MAFbx (muscle specific F-box) and MuRF1 (muscle ring finger 1) which are both upregulated with atrophy (Bodine *et al.*, 2001). Muscle atrophy also leads to increased levels of Nedd4 and Mdm2, two other ubiquitin ligases that have specificity for IGF-IR (insulin-like growth factor 1 receptor) (Rotin *et al.*, 2001; Girnita *et al.*, 2003).

Specific recognition of the polyubiquitin degradation signal and subsequent breakdown of the targeted protein by the 26S proteasome

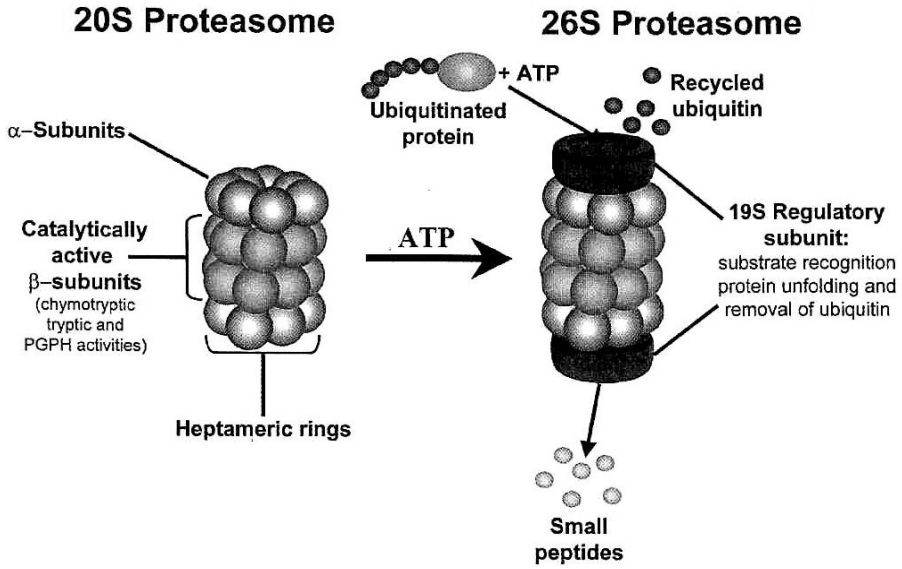

Fig. 1.8. *Structure of the proteasome machinery* (http://www.benbest.com/lifeext/aging.html, 11/09/2007).

The 26S proteasome is formed by the association of the 20S proteasome with two 19S regulatory complexes (Fig. 1.8). The 20S proteasome is the core of the proteolytic machinery. This barrel-shaped particle is organised as a pile of four rings, each ring containing seven subunits. The non-catalytic α-subunits form the two outer rings and the catalytic β-subunits form the two inner rings. The β-subunit active sites are located inside the cylinder so that the proteasome is a self-compartmentalising protease, as substrates must enter the catalytic chamber delimited by the β-rings to be degraded into peptides. The 20S proteasome contains at least two chymotrypsin-, two trypsin- and two caspase-like active sites. 19S complexes bind to both outer α-rings of the 20S proteasome. The α-rings contain ATPase subunits that provide energy for assembly of the 26S proteasome, gating of the 20S proteasome channel, unfolding and injection of protein substrates into the catalytic chamber, proteolysis and energy for peptide release.

2.2.4. Protein degradation regulation

Protein degradation in muscle functions in maintaining normal physiological homeostasis and is required for muscle atrophy in various pathological states. The intracellular events that connect extracellular signals to the molecular control of protein degradation are incompletely understood, but likely involve interacting signal transduction networks rather than isolated pathways (Fig. 1.9).

The control of the degradation rate *per se* is likely accomplished in two distinct ways: in the short-term, by controlling the enzymatic activities of proteases and/or the access of substrates to these proteases, and in the long-term by controlling, at both transcriptional and translational levels, the synthesis of the proteases and their accessory enzymes (Szewczyk & Jacobson, 2005).

Several expression screening studies, designed to identify markers of the atrophy process, have identified two genes the expression of which increased significantly in multiple models of skeletal muscle atrophy: the E3 ubiquitin ligases MuRF1 and MAFbx, the latter being also called Atrogin-1 (Bodine *et al.*, 2001; Gomes *et al.*, 2001; Dehoux *et al.*, 2003). MuRF1 encodes a protein which contains three domains: a RING-finger domain (Borden & Freemont, 1996), which is required for ubiquitin ligase activity (Kamura *et al.*, 1999); a "B-box", the function of which is unclear, and a "coiled-coil domain", which may be required for the formation of heterodimers between MuRF1 and a related protein, MuRF2 (Centner *et al.*, 2001). MuRF1 has been suggested to act by degrading components of the contractile apparatus, as it can induce the ubiquitination of the cardiac form of troponin I (Kedar *et al.*, 2004) and bind to the myofibrillar protein titin (Centner *et al.*, 2001). MAFbx/Atrogin-1 contains an F-box domain. F-box containing E3 ligases usually bind a substrate only after that substrate has first been post-translationally modified, for example by phosphorylation (Jackson & Eldridge, 2002). Recently, substrates have been suggested for MAFbx, including MyoD (Tintignac *et al.*, 2005) and calcineurin (Li *et al.*, 2004).

Activation of Akt/PKB was shown to be sufficient to block the atrophy-associated increases in MAFbx and MuRF1 transcription (Stitt *et al.*, 2004), suggesting that this kinase plays a key role in the regulation of muscle atrophy. The mechanism by which Akt/PKB inhibited MAFbx and MuRF1 upregulation was demonstrated to involve the FOXO (forkhead box gene, group O) family of transcription factors (Lee *et al.*, 2004; Stitt *et al.*, 2004). When phosporylated, FOXO transcription factors are present in the sarcoplasm, but they translocate to the nucleus upon dephosphorylation. The translocation and activity of FOXO transcription factors is required for upregulation of MuRF1 and MAFbx (Glass, 2005).

In addition to regulating FOXO transcription factors, Akt/PKB can promote cell survival by inhibiting proteins that mediate apoptosis (Fig. 1.9). The first of these substrates to be identified was the Bcl2 family member BAD, which promotes apoptosis by interacting with Bcl-XL on the mitochondrial membrane. Phosphorylation of BAD by Akt/PKB enables it to interact with 14-3-3 proteins, which prevent it from binding to Bcl-XL and thereby suppresses apoptosis (Downward, 1999).

Akt/PKB could also directly phosphorylate and inhibit the caspase protease 8, as it has a putative Akt/PKB phosphorylation site (Lawlor & Alessi, 2001). Caspase 3 could be indirectly inhibited by Akt/PKB through phosphorylation of Bax (Yamaguchi & Wang, 2001).

Inhibition of GSK3 following its phosphorylation by Akt/PKB has also been suggested to play a role in inhibiting protein degradation (Evenson *et al.*, 2005), but the dowstream effectors of GSK3 remain to be defined.

Muscle wasting can be induced by several cytokines, most notably the pro-inflammatory cytokine TNFα (tumor necrosis factor α) (Beutler *et al.*, 1985). TNFα binding to its receptor induces the activation of the Rel/NFκB (nuclear factor kappa B) family of transcription factors (von Haehling *et al.*, 2002). NFκB activation was shown to be required for cytokine-induced loss of skeletal muscle proteins (Ladner *et al.*, 2003). Since NFκB in muscle is activated by disuse (Hunter *et al.*, 2002) or sepsis (Penner *et al.*, 2001), it might play a role in the pathogenesis of these conditions. Activation of NFκB is controlled by the IκB kinase complex (IKK). Upon phosphorylation by IKK, IκB is ubiquitinated and targeted to the proteasome for degradation, resulting in the activation of NFκB (Yaron *et al.*, 1998). It has been shown that activation of the NFκB pathway in skeletal muscle resulted in an upregulation of MuRF1 but not MAFbx. Given these data, a linear IKK/NFκB/MuRF1 signalling pathway was proposed (Cai *et al.*, 2004).

It is noteworthy that MAFbx/Atrogin-1 was not perturbed upon NFκB activation. The trigger for upregulation of MAFbx has recently been determined to be p38. It was shown that TNFα acts to stimulate expression of MAFbx, and that this upregulation could be blocked by pharmacologic inhibitors of p38 (Li *et al.*, 2005). Whether or not p38 has any cross-effect on MuRF1 upregulation, or on the NFκB pathway, has yet to be determined.

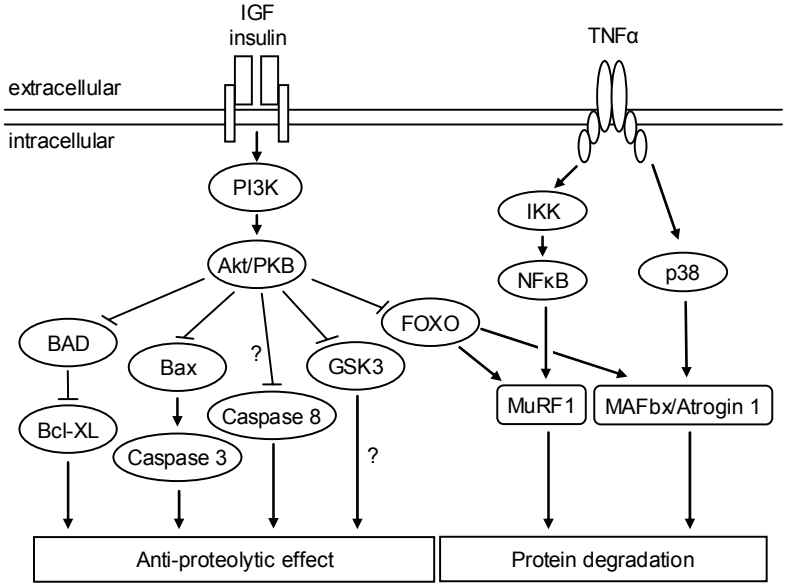

Fig. 1.9. *Summary of the signalling regulating protein degradation in skeletal muscle.* The activation of Akt/PKB leads to anti-proteolytic effects through the activation or inactivation of a series of intermediates. On the other hand, the activation of the NFκB and the p38 pathways stimulates the transcription of MuRF1 and MAFbx/Atrogin-1 resulting in an increased protein breakdown.

Other signal transduction pathways and molecules have been suggested to play a role in the regulation of muscle protein degradation, such as myostatin (Zimmers *et al.*, 2002), mTOR (Latres *et al.*, 2005), JNK (Childs *et al.*, 2003) and calcineurin (Dupont-Versteegden *et al.*, 2002). They will not be discussed any further here, since their potential involvement in protein degradation is not yet clearly established.

3. Muscle cell differentiation

3.1. Satellite cells

Satellite cells are a population of muscle-derived stem cells responsible for myofibre development and renewal (Mauro, 1961). They are located outside the sarcolemma and under the basal lamina of the muscle fibre (Campion, 1984). Normally, in resting skeletal muscles, satellite cells are generally in a non-proliferative, quiescent state, but they have the ability to re-enter the cell cycle to generate new muscle fibres or to provide new myonuclei during postnatal growth (Schultz & McCormick, 1994). Activation, proliferation and fusion of this population of cells is required by a myofibre when undergoing myofibrillar protein growth to maintain a constant nuclear/cytoplasmic ratio. Activation of satellite cells is thus the first step of one mechanism by which muscle hypertrophy may occur (Fig. 1.10).

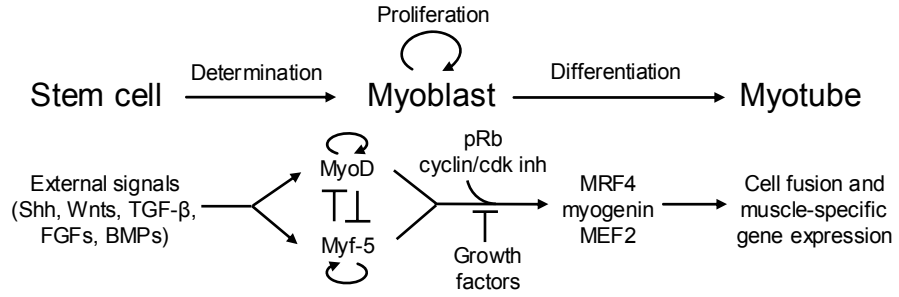

Fig. 1.10. *Role of the myogenic regulatory factors in myogenic cell determination and differentiation.* Shh, sonic hedgehog; TGF-β, transforming growth factor-beta-like molecules; FGFs, fibroblast growth factors; BMPs, bone morphogenic proteins; pRb, retinoblastoma protein; cdk inh, cyclin-dependent kinase inhibitors (Adapted from Hesketh & Whitelaw, 1992).

3.2. Myogenic regulatory factors

The myogenic regulatory factors (MRFs) represent a family of four members (MyoD, Myf-5, myogenin and MRF4, also called Myf-6). These are critical for the appropriate determination, development and maintenance of the skeletal muscle lineage (Fig. 1.10). The MRFs belong to the basic helix-

loop-helix (bHLH) superfamily of transcription factors. The HLH domain is responsible for the dimerization of these factors with the ubiquitously expressed E–proteins, and the basic domain is responsible for DNA binding (Murre *et al.*, 1989). Heterodimers bind to the consensus E-box DNA sequence motif found in the promoters of many muscle specific genes such as desmin (Li & Capetanaki, 1993), troponin I (Lin *et al.*, 1991) and myosin light chain (Wentworth *et al.*, 1991).

Sequential activation of MRFs in cultured myogenic cells suggests that these factors play distinct, but overlapping, roles in the control of myogenesis. Quiescent cells express no detectable levels of MRFs. MyoD and/or Myf-5 are the first MRFs to be expressed in activated muscle satellite cells, whereas myogenin and MRF4 transcript accumulation is only observed when cells start differentiating (Smith *et al.*, 1994). MyoD and Myf-5 are thus important for myogenic determination whereas myogenin and MRF4 are necessary for terminal differentiation and lineage maintenance (Perry & Rudnick, 2000). Decrease in growth factor concentration represents a cue for myoblasts to exit the cell cycle and undergo terminal differentiation. As myoblasts exit the cell cycle, expression of cyclin/cdk (cyclin-dependent kinase) inhibitors and retinoblastoma protein (pRb) are upregulated (Walsh & Perlman, 1997). It is clear that cell cycle regulation and terminal differentiation are in fine balance.

3.3. Myocyte enhancer factor 2

Along with the MRFs, the myocyte enhancer factor 2 (MEF2) family of transcription factors play a key role in myogenesis (Black & Olson, 1998) (Fig. 1.10). MEF2 proteins are members of the MADS (MCM1, agamous, deficiens, serum response factor) box-containing family of transcription factors. The MEF2 family consists of four members, MEF2A-D. Structurally, MEF2 proteins are composed of amino terminal MEF and

MADS domains which are responsible for dimerization and DNA binding. The carboxyl terminal domain is thought to be important for gene activation and kinase responsiveness. Homo- and heterodimers bind an A (adenine)/T (thymine) rich DNA sequence element which is found in the promoters of many muscle-specific genes (Black & Olson, 1998).

Several lines of evidence suggest that MEF2 and MRFs synergistically activate gene expression. MEF2 expression is initiated after the onset of differentiation suggesting these factors are involved during later stages of terminal differentiation (Naya & Olson, 1999). At the level of gene expression, full activation of MRF4 and myogenin promoters require both MRF and MEF2 proteins (Edmondson *et al.*, 1994).

3.4. Insulin-like growth factors

Insulin-like growth factors (IGFs) are of special importance in muscle growth and development. IGF-I and IGF-II are structurally related to insulin. They are highly similar small single chain peptides of 70 and 67 amino acids respectively, and approximate 7.5 kDa (Daughaday & Rotwein, 1989). Four domains, designated A, B, C and D, have been identified in the IGF molecules; the A and B domains being homologous to the A and B chains of insulin.

IGFs are growth factors that circulate at high levels in the blood stream (Furlanetto *et al.*, 1977). Although the main source of IGF-I in the serum is the liver, many other tissues, such as skeletal muscle, synthesize it and are sensitive to its action. Regulation of hepatic IGF-I production is mostly mediated by growth hormone and insulin, whereas the synthesis of IGF-II is relatively growth hormone-independent. In addition to growth hormone, developmental factors (Pollak *et al.*, 2004), nutrition (Thissen *et al.*, 1994) and exercise (Kraemer *et al.*, 2000) modify hepatic IGF-I production.

In humans, IGF-I is the product of a single gene composed of 6 exons. Although there is only one mature peptide, there are three IGF-I precursors (IGF-IEa, IGF-IEb and IGF-IEc) (Goldspink & Yang, 2001) and many IGF-I transcripts resulting from different promoter usage, alternative splicing and various polyadenylation sites (Hall *et al.*, 1992). IGF-I transcripts encoding Ea peptides are widely expressed in all tissues and may be considered as the autocrine/paracrine form of IGF-I. Transcripts encoding Eb peptides predominate in the liver and are more associated with endocrine action (LeRoith & Roberts, 1991). IGF-IEc transcript, also called mechanical growth factor (MGF), is generated specifically in the muscles subjected to stretch and damage (McKoy *et al.*, 1999).

The IGF binding proteins (IGF-BPs) represent a family of 6 members (IGF-BP 1-6), with the common property of binding IGF-I and IGF-II in all biological fluids (Clemmons, 2001). They are responsible for the transport and control of IGF efflux from the vascular space, increasing the half-life of circulating IGFs and regulating their clearance. IGF-BPs modulate the interaction of IGFs with their receptor, thereby controlling the biological actions of IGFs (Jones & Clemmons, 1995).

The effects of IGF-I and IGF-II on a target cell are mediated by two types of IGF receptors (type 1 and type 2 IGF receptors) and the insulin receptor (Rechler & Nissley, 1985). Although IGF binding has been shown for both IGF receptors, most biological actions of IGF-I and IGF-II on muscle cells appear to be mediated by type 1 IGF receptor (Ewton *et al.*, 1987). Type 1 IGF receptor resembles the insulin receptor, since both have 40% homology and a similar heterotetrameric subunit structure (Ullrich *et al.*, 1986).

IGFs are unique among growth factors in that they stimulate both proliferation and differentiation of muscle cells in culture. It has been shown that IGF action occurs through two phases (Florini *et al.*, 1996). This involves an initial mitogenic respone in which intracellular mediators of proliferation such as cyclin D mRNA and c-fos mRNA are up-regulated and mediators of the myogenic response such as myogenin mRNA are suppressed (Rosenthal & Cheng, 1995). The first phase is then followed by a strong myogenic response characterized by the expression of the muscle-specific transcription factor myogenin and the fusion of myoblasts into myotubes. Activation of the IGF-I receptor can induce the activation of the MAPK, PI3K (Coolican *et al.*, 1997) and calcineurin (Semsarian *et al.*, 1999) pathways. Whereas the mitogenic effects of IGF-I are primarily mediated by the Ras/Raf-1/MAPK pathway, the myogenic effects are mediated by the PI3K/$p70^{s6k}$ pathway (Coolican *et al.*, 1997).

3.5. Myostatin

Myostatin, or GDF-8 (growth and differentiation factor-8), was identified through sequence identity with members of the BMP (bone morphogenetic protein)/TGFβ (transforming growth factor β) superfamily (McPherron et al., 1997). Myostatin is a negative regulator of skeletal muscle development. It binds with high affinity to the serine/threonine kinase activin type IIB receptor, which initiates signalling through a smad2/3 (small mother against decapentaplegic 2/3)-dependent pathway (Lee & McPherron, 2001).

Myostatin is synthesized as a 376 amino acid precursor protein containing a signal sequence, a N-terminal propeptide domain, and a C-terminal domain considered as the active molecule (McPherron et al., 1997). Myostatin is secreted in a latent form by binding to its propeptide; proteolytic processing between the propeptide domain and the C-terminal domain produces an N-terminal propeptide and the mature form of myostatin. In serum, as well as

in skeletal muscle, myostatin can be found bound to several proteins that are able to modulate its activation, secretion or receptor binding. In serum, in addition to the propeptide, FLRG (follistatin-related gene) (Hill *et al.*, 2002) and GASP-1 (growth and differentiation factor-associated serum protein-1) (Hill *et al.*, 2003) bind to myostatin and inhibit its activity, whereas in skeletal muscle inhibitory myostatin binding proteins are hSGT (human small glutamin-rich tetratricopeptide repeat-containing protein) (Wang *et al.*, 2003), titin cap (Nicholas *et al.*, 2002) and follistatin (Amthor *et al.*, 2004).

Myostatin has direct effects on the proliferation and/or differentiation of numerous muscle cell lines (Rios *et al.*, 2001; Langley *et al.*, 2002). The effect on muscle fibre number is likely to result from the activity of myostatin on myoblast proliferation and/or differentiation during development, while the effects on fibre size appear to be mediated through the action of myostatin on muscle satellite cells (Walsh & Celeste, 2005). Myostatin inhibits proliferation through an up-regulation of p21 and decreases the levels of both Cdk2 and phosphorylated Rb, resulting in cell cycle inhibition (Thomas *et al.*, 2000). The effect on differentiation appears to occur through down-regulation of the myogenic differentiation factors MyoD, Myf-5 and myogenin (Langley *et al.*, 2002).

3.6. Signal transduction

The determination, maintenance and activation of the myogenic program are regulated by many factors. Each of the following molecules has been shown to initiate or to control the expression of at least one MRF: Wnt1, Wnt7, Sonic hedgehog, TGFβ and BMP4 (Rescan, 2001) (Fig. 1.10).

Many distinct mechanisms have been elucidated to explain how growth factors are able to repress or stimulate the myogenic program. Protein kinase C (PKC) activity is increased in response to mitogenic stimulation.

Overexpression of activated PKC represses MRF-mediated transcription of muscle-specific genes and terminal differentiation (Li *et al.*, 1992).

Binding of ligands to cell-surface receptors initiates a cascade of events leading to the activation of $p21^{ras}$. Overexpression of $p21^{ras}$ inhibits MRF-mediated differentiation (Kong *et al.*, 1995). In opposition, inhibition of MEK and rac/rho kinase pathways, which are activated by ras, do not rescue myogenesis suggesting that these pathways are not involved in regulating terminal differentiation (Ramocki *et al.*, 1997). Later stages of differentiation require MKP-1 (mitogen-activated protein kinase phosphatase 1) downregulation for myoblast fusion and myotube formation (Bennett & Tonks, 1997). In summary, an increase in MAPK signalling is required for transmitting growth signals, and a decrease in MAPK activity is required for myogenesis to proceed.

The absence of stimulation by extracellular growth factors leads to apoptosis indicating that the pathways induced by growth factors are essential for cell survival. Expression of activated PI3K is able to induce differentiation, suggesting a direct role of PI3K in myogenesis (Jiang et al., 1998). PKB is a downstream target of PI3K and has been shown to be upregulated during differentiation and its activity to be important for myocyte survival (Fujio et al., 1999). However, overexpression of activated PKB is unable to force differentiation under growth factor conditions suggesting the involvement of mediators that are specifically expressed at the onset of myogenic differentiation (Rommel et al., 1999).

It has been hypothesized that calcium activated signal transduction pathways are important for fibre-type specification. Calcineurin is a calcium-activated protein phosphatase that activates the NFAT (nuclear factor of activated T-cells) transcription factors. The latter are thought to alter fibre-type specific gene expression by interaction with MEF2 (Youn *et al.*, 1999).

In addition to MAPK, PI3K and calcineurin pathways, the signalling induced by growth factors during development could involve miRNA (Martello *et al.*, 2007). The fact that many miRNAs are organ-specific indicates that they are central to cell differentiation and tissue development. It has been suggested that miRNAs may act by inhibiting the genes that should not be expressed in a particular cell type and therefore function to reinforce or fine-tune gene expression (Williams, 2007). miR-1 and miR-133 have distinct roles in modulating skeletal muscle proliferation and differentiation both in vitro and in vivo. miR-1 promotes myogenesis by targeting histone deacetylase 4, a transcriptional repressor of muscle gene expression. In contrast, miR-133 enhances myoblast proliferation by repressing serum response factor (Chen *et al.*, 2006).

In conclusion, MRFs are key transcription factors for the determination, proliferation and terminal differentiation of myogenic cells. Although, several growth factors and transduction pathways regulating MRF expression and activity have been described, the integration of these events still remains to be explored. Coordination of cell cycle and terminal differentiation is a complex process.

Since the general mechanisms of protein synthesis and protein breakdown have been described, we will now focus on their regulation by exercise and nutrients. Exercise and nutrients are generally known to be anabolic agents in skeletal muscle resulting in muscle hypertrophy. However, the mechanisms leading to muscle cell differentiation and growth are only beginning to be elucidated.

4. Factors regulating muscle cell differentiation and growth

4.1. Nutrients

4.1.1. Creatine

Creatine metabolism

Creatine is a nonessential dietary element found in high abundance in meat and fish. Normal daily intake of creatine from an omnivorous diet approximates 1g. However, creatine is also synthesized within the body, primarily in the liver, from two amino acids by a two-step reaction at a rate of about 1g per day (Fig. 1.11) (Wyss & Kaddurah-Daouk, 2000).

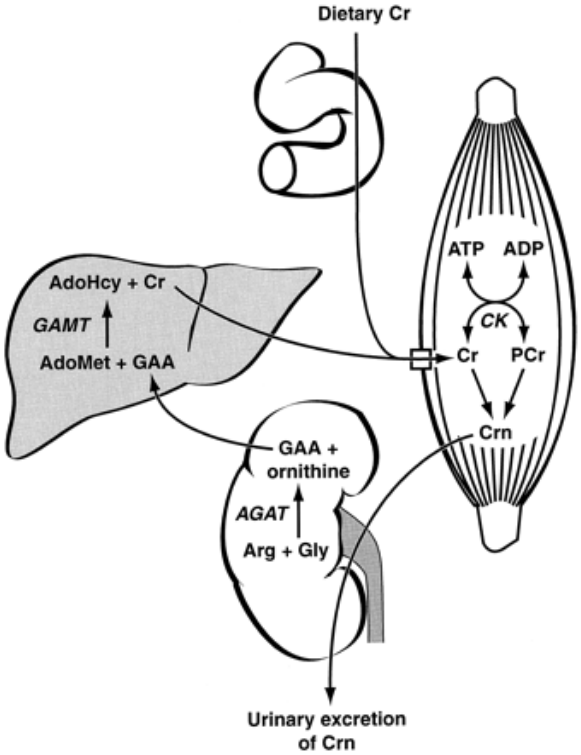

Fig. 1.11. *Creatine metabolism* (Wyss & Kaddurah-Daouk, 2000).

In the first reaction, guanidinoacetate is formed from arginine and glycine in a reaction catalyzed by arginine:glycine amidinotransferase. In the second step, a methyl group of S-adenosylmethionine is transferred to

guanidinoacetate and creatine is formed. Muscle does not synthesize creatine but is dependent on creatine uptake from the circulation by a sodium dependent transporter in the muscle membrane. Once in the muscle, creatine becomes phosphorylated by the activity of creatine kinase. The sole end product of creatine metabolism is creatinine, formed by the nonenzymatic conversion from phosphorylcreatine and creatine. Creatinine is then excreted by the kidneys.

Approximately 120g of creatine is found in a 70kg male, 95% in skeletal muscle. Total creatine exists in muscles as both free creatine (40%) and phosphorylcreatine (60%) (Wyss & Kaddurah-Daouk, 2000). Creatine is an important source of chemical energy for muscle contraction because it can undergo phosphorylation that is rapid, with the formation of phosphorylcreatine, and reversible, with donation of the phosphate group to ADP to form ATP (Fig. 1.12). This reaction is catalyzed by the enzyme creatine kinase and is a rapid source of high-energy phosphate for high-intensity, short-duration physical activity (Greenhaff *et al.*, 1994).

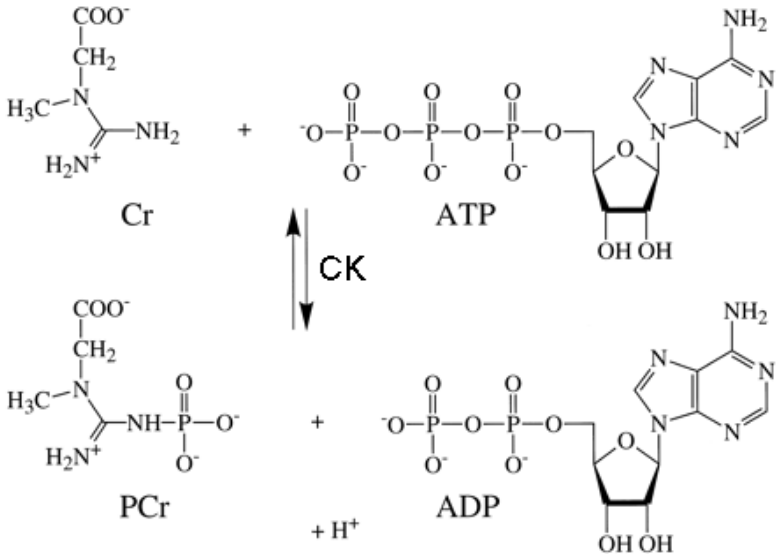

Fig. 1.12. *Creatine kinase reaction*. Cr, creatine; CK, creatine kinase; PCr, phosphorylcreatine (Wyss & Kaddurah-Daouk, 2000).

Creatine supplementation

Various supplementation strategies have been used to increase skeletal muscle creatine concentrations. The most commonly used protocol is to ingest a daily total of 20 to 30g of creatine, usually creatine monohydrate, in 4 doses of 5 to 7g dissolved in fluids, for 5 or 7 days, although some studies used lower doses or supplemented for fewer days (Williams & Branch, 1998). Normal muscle creatine content approximates 125mmol/kg dry matter. Subjects respond very differently to creatine supplementation. Those with a very low basal level generally accumulate larger amounts of creatine but human muscle appears to have an upper limit of creatine storage of 150-160mmol/kg dry matter (Greenhaff, 1995). In average, creatine supplementation increases total creatine concentration in skeletal muscle by 20% (chapters 3 and 5).

Interest for creatine supplementation

Creatine supplementation has become one of the most popular ergogenic aids among athletes and one of the most advantageous for the industry of supplements. A relatively large number of scientific studies have been conducted over the past decade documenting the physiologic and performance effects of creatine supplementation in diverse populations. Creatine has been studied in relation to acute performance (Volek *et al.*, 1997; Casey & Greenhaff, 2000), training adaptations (Dempsey *et al.*, 2002; Kreider, 2003; Rawson & Volek, 2003), factors affecting muscle creatine uptake (Snow & Murphy, 2003), pharmacokinetics (Persky *et al.*, 2003), therapeutic potential in neuromuscular and other metabolic diseases (Wyss & Schulze, 2002), use in adolescents (Unnithan *et al.*, 2001) and the elderly (Tarnopolsky, 2000) and potential adverse effects of creatine supplementation (Poortmans & Francaux, 2000).

Mechanisms of action of creatine

Whereas most studies have focused on the ergogenic effects of creatine supplementation in combination with resistance exercise, only a few studies have tried to determine the molecular mode of action of creatine. Several hypotheses have been proposed to explain the increased muscle strength and improved weight-lifting performance after creatine supplementation in conjunction with resistance training (Volek & Rawson, 2004).

Creatine could act directly on muscle growth and strength. In this case, part of the ergogenic effect of creatine supplementation in training studies is independent of the resistance-training stimulus and is due to the acute effects of creatine loading and subsequent increases in muscle creatine levels.

Creatine has also been proposed to act via an increase in training volume. The increase in the ability to perform more repetitions with the same weight after creatine supplementation could translate into greater gains in lean body mass and strength after several weeks of training.

Nevertheless, these two theories require that creatine interact with known or postulated mechanisms of muscle fibre hypertrophy to induce the enhancement of strength and lean body mass. Creatine supplementation has been shown to result in larger increases in type I, IIA, IIAB muscle fibre cross-sectional area compared with placebo after 12 weeks of heavy resistance training (Volek *et al.*, 1997) and after 2 weeks of leg immobilization followed by 10 weeks of rehabilitation (Hespel *et al.*, 2001). Creatine supplementation also leads to greater increases in type I and II MHC (myosin heavy chain) mRNA abundance and protein content after 12 weeks of resistance training, suggesting that an increase in MHC synthesis may account in part for the greater increase in muscle size with creatine supplementation (Willoughby & Rosene, 2001).

The creatine-induced increase in MHC gene expression has been postulated to be mediated by MRFs. Indeed, MRF4 level was increased after creatine supplementation in conjunction with resistance training (Willoughby & Rosene, 2003) or with rehabilitation following muscle disuse (Hespel *et al.*, 2001) whereas opposite results were obtained for myogenin expression. The increases in MRF4 and myogenin protein content were strongly correlated to muscle creatine kinase mRNA expression (Willoughby & Rosene, 2003).

In summary, creatine supplementation and resistance training influence expression of certain MRFs that possibly upregulate expression of skeletal muscle MHC and creatine kinase, which in turn could explain greater increases in muscle size and strength. It still remains unclear whether creatine has a direct effect on expression of MRFs and MHC or whether the effect is indirectly mediated through a greater training volume.

In the same line, creatine supplementation has recently been shown to amplify the increase in satellite cell number and myonuclei concentration in human skeletal muscle fibres during 4 to 16 weeks of resistance training (Olsen *et al.*, 2006). This could explain why previous studies observed an increase in certain MRFs and could represent the first step in the increase in muscle mass after creatine supplementation.

It has been hypothesized that creatine per se could induce muscle hypertrophy due to water retention since cellular water content may influence protein metabolism (Haussinger *et al.*, 1993). Indeed, creatine has been shown to increase total body water including intracellular water. However, the relative volume of the body water compartments remains constant after creatine supplementation. Therefore the gain in body mass cannot be attributed to water retention alone but to an increase in dry matter accompanied by a normal water volume (Francaux & Poortmans, 1999).

The increase in lean body mass after creatine supplementation could be the result of a larger protein synthesis or a decrease in protein breakdown or both together. Five days of creatine supplementation had no effect on muscle protein synthesis but decreased whole body protein breakdown (Parise *et al.*, 2001). Two other studies have indicated that creatine had no effect on skeletal muscle protein synthesis and breakdown, whether at rest or after resistance exercise (Louis *et al.*, 2003a; Louis *et al.*, 2003b). Collectively, these studies indicate that short-term creatine loading does not have a significant effect on measures of skeletal muscle protein balance. It is possible that creatine only affects protein metabolism under very specific experimental conditions: more intense resistance exercise, longer supplementation periods, older or atrophied subjects,…

It has become apparent that accumulation of creatine in skeletal muscle affects a variety of cellular processes that could account for its ergogenic and therapeutic potential. Recent work has started to provide a better picture of the physiological mechanisms by which creatine supplementation affects skeletal muscle protein metabolism but the molecular signalling is far to be elucidated and is one major purpose of the present work (chapters 3-5).

4.1.2. Amino acids

Regulation of protein metabolism and cell differentiation

Following amino acid administration, muscle protein synthesis increases proportionally to the amount of amino acids administered, up to 3 times the basal postabsorptive value (Biolo & De Cicco, 2004). This effect can be observed independently of any hormones, although insulin may enhance the process. Modulation of protein synthesis via availability of amino acids appears to show a sigmoidal relationship; rises and falls in amino acid availability cause rapid changes in muscle protein synthesis in the basal to postprandial range (Rennie *et al.*, 2002). In humans, the upper limit of amino

acid concentrations at which protein synthesis appears to be saturated is about 50% greater than the blood amino acid concentrations normally achieved after a meal (Rennie *et al.*, 2002).

Amino acids are more efficiently utilized when given in divided doses (bolus) rather than with continuous administration (Biolo & De Cicco, 2004). Moreover, administration of boluses of single essential amino acids (threonine, valine, phenylalanine and leucine) but not non-essential amino acids (proline, glycine, serine and alanine) markedly stimulated the incorporation of labelled amino acids into muscle protein (Smith *et al.*, 1998). It is important to note that after feeding, skeletal muscle in diverse anatomical locations shows increases in protein synthesis by similar amounts (Mittendorfer *et al.*, 2005), indicating that this stimulation is a systemic rather than a muscle-specific effect.

These last years, it has become evident that amino acids not only act as metabolic precursor and substrate for protein synthesis but additionally activate and modulate signal transduction pathways involved in the regulation of proliferation, apoptosis, gene expression and metabolism. One purpose of the present work will thus be to identify potential mechanisms by which two major amino acids in skeletal muscle, namely leucine and glutamine, affect protein metabolism and myogenic cell differentiation (chapter 7).

Leucine and glutamine

Leucine is an essential branched-chain amino acid (BCAA), also called (S)-2-amino-4-methyl-pentanoic acid (Fig. 1.13). It is one of the three amino acids with a branched hydrocarbon side chain together with valine and isoleucine. It has one additional methylene group in its side chain compared with valine. Like valine, leucine is hydrophobic and generally buried in folded proteins.

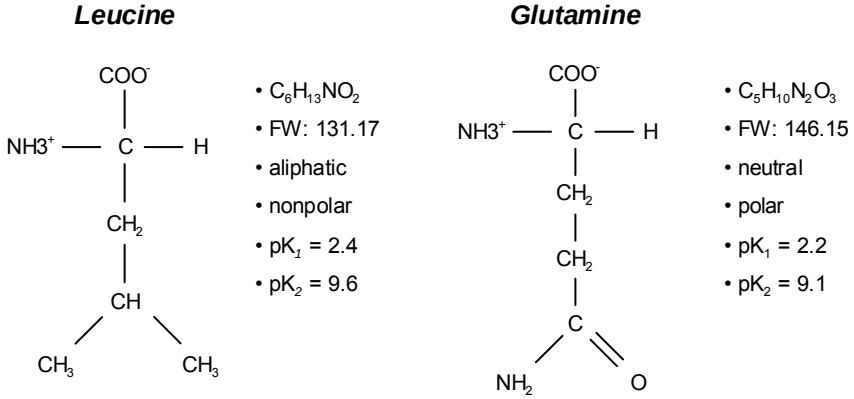

Fig. 1.13. *Leucine and glutamine structures.*

Leucine is transported into skeletal muscle cells by the sodium-independent system L, which mediates uniport of amino acids with bulky side chain such as BCAA (Palacin *et al.*, 1998). The BCAA serve as an essential substrate and important regulator in the synthesis of body proteins and represent the major nitrogen source for glutamine and alanine synthesis in muscle (Fig. 1.14). The first step in BCAA catabolism is reversible transamination leading to the production of corresponding branched-chain keto acids. The skeletal muscle is considered the initial site of BCAA catabolism because of a high activity of BCAA aminotransferase. The amino group released during transaminase reaction is used primarily for synthesis of alanine and glutamine. The branched-chain keto acids formed during transamination undergo oxidative decarboxylation catalyzed by branched-chain keto acid dehydrogenase and/or released into the bloodstream and then taken up by different tissues where they can be oxidized or used for resynthesis of BCAA (Harper *et al.*, 1984).

In summary, BCAA are released from skeletal muscle in lower amounts than would be expected from their content in muscle protein, probably reflecting their active catabolism in muscle. The opposite situation occurs for glutamine and alanine as a consequence of de novo synthesis in muscle (Ruderman & Berger, 1974).

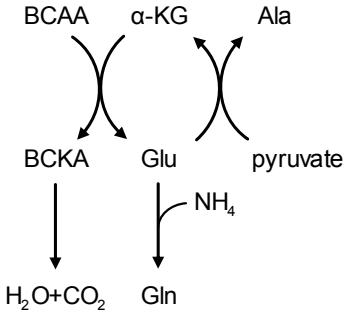

Fig. 1.14. *Relation between glutamine and alanine production and BCAA catabolism in muscle.* α-KG, alpha-ketoglutarate; Ala, alanine; BCAA, branched-chain amino acid; BCKA, branched-chain keto acid; Gln, glutamine; Glu, glutamate (Adapted from Holecek, 2002).

Numerous reports established that, in skeletal muscle, leucine is unique in its ability to initiate signal transduction pathways that modulate translation initiation. Oral administration of leucine stimulates protein synthesis in association with hyperphosphorylation of 4E-BP1 and enhanced phosphorylation of $p70^{s6k}$. The stimulatory effects of leucine on translation initiation are mediated in part through mTOR, where both insulin signalling and leucine signalling converge to promote a maximal response. However, signalling through mTOR alone is not sufficient to explain the increased rates of protein synthesis following leucine administration. Further studies are required to determine additional signalling pathways activated by leucine that regulate protein synthesis (Anthony et al., 2001).

Glutamine is a non essential amino acid also called (2S)-2-amino-4-carbamoyl-butanoic acid. It is the amide of glutamic acid and is uncharged under all biological conditions (Fig. 1.13). Glutamine is the most abundant free amino acid in human plasma, skeletal muscle and cerebrospinal fluid. It can be synthesized by most cells and tissues by the glutamine synthetase-catalyzed ATP-dependent reaction of glutamate with ammonia (Haussinger & Schliess, 2007). Glutamine plays a central role in nitrogen metabolism and represents a storage and transport form of glutamate and ammonia. The skeletal muscle serves as the major glutamine supplier whereas the liver represents a major consumer of glutamine (Bode, 2001).

Glutamine can be transported by sodium-dependent (ASC, $B^{0,+}$, y^+L, A et N) and sodium-independent systems (L, $b^{0,+}$ et n). The N, ATA2 and LAT1 systems have been found to be present in myogenic cells. The N system is the most abundant in skeletal muscle and possesses a specific muscle isoform (Nm). In muscle, both influx and efflux of glutamine are mediated by the Nm transporter. The latter has a Km of 9.25mmol/l in rat skeletal muscle (Bode, 2001). Once in the intracellular compartment, glutamine may serves as substrate for protein synthesis or may be catabolised by transamination in the cytosol followed by a deamination by a glutamate dehydrogenase in the mitochondria (Rennie *et al.*, 1992). Glutamine may also be degraded by a glutaminase, but it seems that degradation by transamination-deamination is more frequent in muscle due to a higher activity of glutamine transaminase and glutamate dehydrogenase than that of glutaminase (Rennie *et al.*, 1996).

Glutamine has been shown to modulate signal transduction pathways in several tissues. For instance, it induces intestinal cell proliferation by stimulating ERK- and JNK (c-Jun NH_2 terminal)-dependent DNA binding activity of activator protein AP-1 (Rhoads *et al.*, 1997). In isolated rat hepatocytes, glutamine increases cell volume and induces an anabolic response characterized by an activation of acetyl-CoA carboxylase, glycogen synthase and $p70^{s6k}$, the key enzymes in fatty acid, glycogen and protein synthesis, respectively (Krause *et al.*, 2002). The latter study and others (Haussinger & Schliess, 1999; Haussinger *et al.*, 2001) have underlined the fact that some of the proliferative and metabolic glutamine signals depend on cellular registration of cell volume-sensitive signal transduction pathways. In cardiomyocytes, glutamine increased abundance of the mRNAs encoding the contractile proteins, α-MHC and cardiac α-actin, and the metabolic enzymes, muscle carnitine palmitoyl transferase I and muscle adenylosuccinate synthetase (Adss1). The induction was mediated through the PKA (protein

kinase A) and mTOR signalling pathways and required a cyclic AMP response element associated with the promoter region of the Adss1 gene (Xia *et al.*, 2003). The effects of glutamine in skeletal myogenic cells remain to be identified and will be addressed in chapter 7.

4.2. Exercise

Protein turnover

Most studies on the response to exercise of human muscle protein turnover have focused on post-exercise changes following resistance exercise, likely to result in muscle hypertrophy. After a single bout of heavy resistance training, mixed muscle protein synthesis is increased for up to 48h (Phillips *et al.*, 1997). Similarly, after a strenuous bout of exercise, myofibrillar protein synthesis peaked at 24h and remained elevated at 72h (Miller *et al.*, 2005). Contrary to the systemic response of feeding on all skeletal muscles, physical activity only stimulates a response in the exercised muscle (Miller, 2007). Interestingly, over the same time period after exercise, the muscle extracellular matrix and tendon increased protein synthesis in a similar pattern (Miller *et al.*, 2005).

The extent of the anabolic response (+80-100%) of protein synthesis to a single bout of exercise is surprising, because the rate of net accretion of muscle protein is very much slower than this. It may take 20 weeks of intense resistance exercise to increase muscle mass by 20%. This is, of course, due to a concomitant elevated protein breakdown after acute exercise if no food is provided (Phillips *et al.*, 1997).

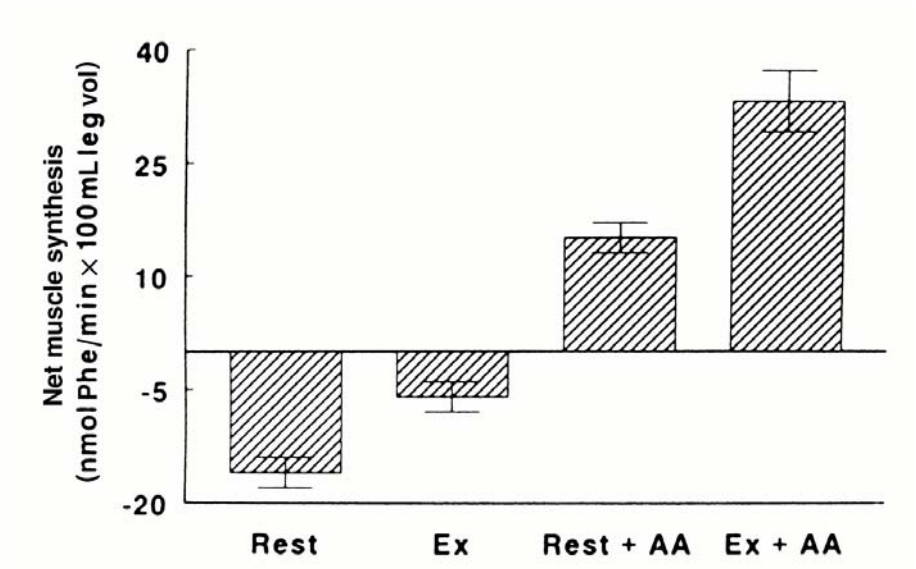

Fig. 1.15. *Net protein balance at rest, after amino acid infusion (AA) and/or exercise (ex)* (Wolfe, 2000).

Protein nutrition after a bout of exercise increases skeletal muscle protein synthesis and net balance to a greater extent than exercise in the fasted state (Tipton *et al.*, 1999). Measurements of protein synthesis and breakdown after a 12h fast indicate that breakdown exceeds synthesis, so net protein balance is negative (Wolfe, 2000) (Fig. 1.15). In the recovery period after exercise without nutrient provision, protein synthesis and protein breakdown are increased compared with the 12h fasted state, although net balance does not improve to a positive balance. When receiving an infusion of mixed amino acids after a fasted period, protein synthesis increases, whereas protein breakdown remains the same or decreases slightly and net protein balance becomes positive. When exercise is combined with amino acids, protein synthesis increases more than after exercise or amino acid feeding alone, and protein breakdown remains similar to exercise without feeding. Net protein balance is enhanced in comparison with amino acid feeding alone.

The increase in protein synthesis after feeding is a transient storage phenomenon, whereas physical activity stimulates a longer term adaptive response. Providing nutrition after physical activity takes advantage of the anabolic signalling pathways that physical activity has initiated by providing amino acid building blocks and energy for protein synthesis (Miller, 2007).

Satellite cells

Exercise training has been shown to increase the number of satellite cells after several days (Crameri *et al.*, 2004), weeks (Olsen *et al.*, 2006) or years (Kadi *et al.*, 2004a). This increase can be maintained as long as the muscles are subjected to training. The cessation of training is associated with the termination of satellite cell activation (Kadi *et al.*, 2004b). The activation of satellite cells can be attributed to exercise *per se*, exercise-induced localised ultrastructural damage, exercise-induced segmental fibre damage, exercise-induced release of inflammatory substances or/and exercise-induced release of growth factors (Kadi *et al.*, 2005). It seems that the amount of muscle fibre damage is not correlated to the changes in satellite cells following training. The trigger for satellite cell activation could rather be the exercise-induced ultrastructural muscle damage since fibres with more ultrastructural damage also contained higher proportions of active satellite cells (Roth *et al.*, 2001).

The molecular mechanisms underlying satellite cells activation by exercise are presently unknown, but it has been proposed that HGF (hepatocyte growth factor) activates satellite cells and that IGF-I and FGF (fibroblast growth factor) increase the proliferation of satellite cells once they are activated. The discovery of two IGF-I isoforms in skeletal muscle, MGF and IGF-IEa, has suggested that MGF initiates satellite cell activation and proliferation, while IGF-IEa promotes differentiation of proliferating satellite cells (Yang & Goldspink, 2002).

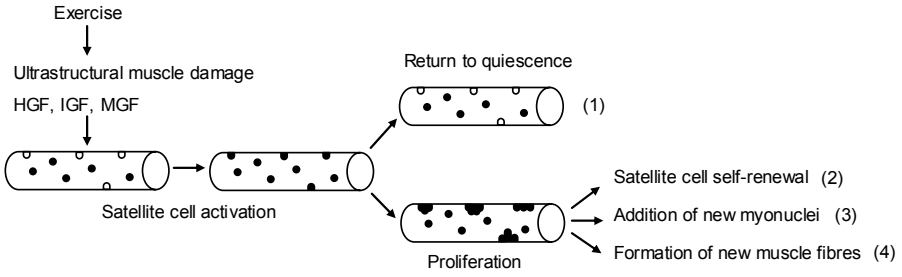

Fig. 1.16. *Activation of satellite cells by exercise* (Adapted from Kadi *et al.*, 2005).

Based on the current knowledge (Fig. 1.16), it can be hypothesised that exercise can induce activation of satellite cells without proliferation (1), proliferation and withdrawal from differentiation (2), proliferation and differentiation to provide more myonuclei (3), and proliferation and differentiation to generate new fibres or to repair segmental fibre injuries (4) (Kadi *et al.*, 2005).

Although the studies mentioned above tend to favour the idea that satellite cell addition participates to muscle hypertrophy following exercise, it seems that skeletal muscle is capable of hypertrophy via a mere increase in protein synthesis with no additional satellite cell incorporation (McCarthy & Esser, 2007). In accordance with the latter view, some studies have shown that contraction-induced skeletal muscle growth occurs with no change in total DNA per muscle (Wong & Booth, 1990b, a), and that inhibition of satellite cell proliferation does not prevent muscle growth (Lowe & Alway, 1999).

The requirement of satellite cells activation for skeletal muscle hypertrophy could be linked to the type of growth stimulus, the magnitude of the growth response, the age of the subjects and the time of sampling after the applied stimulus. Muscle growth consists of multiple phases, including accelerated transcriptional and translational responses followed by possible satellite cell addition during the later stages of hypertrophy. It is likely that satellite cell activation is necessary only if a certain threshold myofibre size is reached (O'Connor *et al.*, 2007).

4.3. Signalling leading to muscle hypertrophy

The main cellular events leading to muscle hypertrophy are summarized in Fig. 1.17 (Rennie *et al.*, 2004). Via receptor binding and cellular signals, certain cytokines (others are obviously involved in muscle degradation) and other growth factors are sensed and activate a network of signal transduction pathways that result in the nuclear translocation or activation of transcription factors. Active transcription factors change the expression of the major muscle growth regulators, IGFI-Ea, MGF and myostatin, or other muscle-specific genes. IGFI-Ea, MGF and insulin activate PI3K-Akt/PKB-mTOR pathway, which enhances protein synthesis via increased translational initiation and the synthesis of ribosomal proteins for ribosome biogenesis. Availability of amino acids will activate mTOR signalling, whereas an increased energy demand sensed by AMPK will inhibit mTOR.

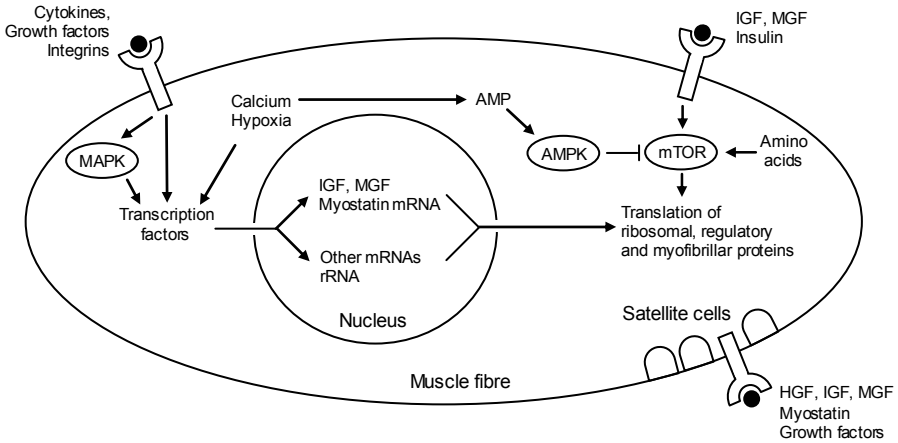

Fig. 1.17. *Main events during signal transduction and gene regulation leading to muscle hypertrophy* (Adapted from Rennie *et al.*, 2004).

In addition to stimulate muscle fibre hypertrophy, IGFI-Ea, MGF, myostatin and various other factors also regulate an increased proliferation and differentiation of satellite cells. Hypertrophy is thus produced through binding of growth factors on the skeletal muscle fibres but also through binding to receptors on activated satellite cells. Although IGF-I protein is one major mediator of these hypertrophic effects, it has been suggested that

the isoform from which IGF-I is produced can affect its potency. MGF has been proposed to be more potent for promoting rat skeletal muscle hypertrophy (Goldspink, 2005).

However, this proposal has been countered in another study in young growing rats, where the ability of MGF to promote muscle hypertophy was not greater than IGF-IEa, with a barely nonexistent effect in adult muscle. It is thus possible that MGF can promote hypertrophy to a similar extent than that achieved by IGF-IEa only when there is an active satellite cell pool, as observed in growing animals. These results suggest that the bioavailability of MGF or its receptor affinity may diminish with age (Barton, 2006).

The signalling pathways leading to transcriptional and translational changes in skeletal muscle in response to resistance exercise are still not fully understood. Four potential stimuli that may regulate these processes have been proposed: mechanical load or stress, intracellular calcium, hypoxia and redox state (Baar *et al.*, 1999). These are thought to be first messengers in a signalling cascade in which various transcription factors, hormones, and other regulatory proteins are activated. Signal transduction pathways shown to be activated in response to various forms of contraction include those involving AMPK (Winder & Hardie, 1999), calcineurin (Meissner *et al.*, 2000), ERK1/2 and p38 (Widegren *et al.*, 2000), JNK (Aronson *et al.*, 1998), NF-κB (nuclear factor kappa B) (Hollander *et al.*, 2001), PI3K-Akt/PKB-mTOR (Turinsky & Damrau-Abney, 1999) and PKC (Richter *et al.*, 2001).

Calcineurin is a calcium-calmodulin-activated protein phosphatase that dephosphorylates the transcription factor NFAT, enabling its nuclear translocation and DNA binding. The calcineurin pathway has been linked to the regulation of skeletal muscle growth but also to the conversion of fast-to-slow phenotype (Olson & Williams, 2000) although currently the link with muscle growth is questioned.

Various sensors of mechanical strain seem to possess the ability to translate strain into chemical signals that induce the activation of skeletal muscle gene promoters (Carson & Wei, 2000). A possible candidate sensor of the increase in mechanical strain is focal adhesion kinase, a protein localized to the sarcolemma (Fluck *et al.*, 1999). The serum response factor, which is a transcription factor, is a substrate of focal adhesion kinase, thereby providing a transcriptional link between membrane, the genome, and subsequent expression of muscle protein. The putative link between focal adhesion kinase and serum response factor is the β1 integrin-RhoA signalling (Wei *et al.*, 2001).

Different modes of exercise affect ERK1/2 and p38 MAPK likely in an intensity-dependent manner (Widegren *et al.*, 2000; Nader & Esser, 2001). However, only those stimuli likely to result in hypertrophy, such as high-frequency electrical stimulation, increased $p70^{s6k}$ and PKB phosphorylation (Nader & Esser, 2001).

IGFI-Ea, MGF and myostatin are not directly regulated by stretch, overload, or muscle contraction, but by the signal transduction pathways that sense these stimuli and, consequently, regulate the availability of these muscle growth factors for receptor binding. The major step controlling the availability of IGFI-Ea, MGF and myostatin in response growth-inducing stimuli appears to be transcriptional regulation (Rennie *et al.*, 2004).

In summary, muscle growth stimuli lead to the activation of a signal transduction network and to a changed availability of the major muscle growth factors IGFI-Ea, MGF and myostatin. The activated signal transduction pathways and changed growth factor availability will then regulate the activity of muscle growth executors, which are the translational or protein synthesis machinery and possibly satellite cells.

It must be stated here that a very recent study (Spangenburg *et al.*, 2007) has seriously questioned the current view implying a major role of IGF-I in skeletal muscle hypertrophy induced by mechanical loading through the activation/proliferation of satellite cells and/or the increase in protein synthesis. The authors demonstrated that IGF-IR was not necessary for the induction of skeletal muscle growth in response to mechanical loading, whereas components of the Akt/PKB pathway were activated. These results suggest that IGF-I is not the key factor necessary for initiation of growth response to mechanical load and that other, undetermined factors are probably responsible for the activation of the Akt/PKB pathway (Spangenburg *et al.*, 2007). Additional work is needed to identify these factors and to determine the requirement of each player in skeletal muscle hypertrophy induced by mechanical loading.

5. Aim of the thesis

Protein loss or gain may result from different combinations of changes in rates of protein synthesis and degradation. Whereas nutrients and/or exercise are anabolic factors leading to muscle hypertrophy, the molecular mechanisms by which they increase muscle mass are not clearly determined. Moreover, the role of nutrients and exercise in muscle cell differentiation is poorly defined. These mechanisms will be studied in two widely used models, C_2C_{12} myogenic cells and human muscle biopsy specimens.

The current knowledge on the myogenic differentiation process and the regulation of protein synthesis and protein degradation in human skeletal muscle has been summarised in this first chapter. The second chapter will be more acutely oriented towards the regulation of the mTOR pathway by amino acids and exercise. Whereas the mTOR pathway was generally presented separately as playing a key role in the regulation of protein synthesis by amino acids and as being activated by exercise, this was the first review to integrate the control of this cascade by both stimuli.

Creatine is a potent anabolic agent, but its mechanisms of action still remain largely unknown. In myogenic cells, creatine increases IGF-I and MRF mRNA expression (Louis *et al.*, 2004). The third chapter of the present work will present a study that was designed to test if the findings obtained on cell cultures could be corroborated in human, and to analyse the possible activation of downstream effectors of IGF-I involved in the regulation of protein synthesis.

The fourth chapter will focus on the question whether creatine affects protein synthesis and cell differentiation in myogenic C_2C_{12} cells. Moreover, the role of IGF-I as a primary mediator in the signalling induced by creatine will be

tested as well as cascades downstream of IGF-IR, namely the Akt/PKB and the MAPK pathways. Chapter 5 will present a follow-up of the previous study. The protocol was designed to test the involvement of the Akt/PKB and MAPK pathways after exercise with or without creatine supplementation in human and the regulation of gene expression by these two cascades.

Resistance exercise is another well-described anabolic stimulus and both the MAPK and the Akt/PKB signalling pathways have been involved in this hypertrophic effect. The sixth chapter will investigate a possible interaction between the MAPK pathway and the Akt/PKB pathways during resistance exercise and the subsequent implication of this crosstalk during the recovery phase when nutrients are provided.

The regulation of protein synthesis, as well as the regulation of muscle specific-gene expression, by leucine and glutamine will be reported in chapter 7, with a specific attention on the mTOR pathway.

In chapter 8, a general discussion will be presented about the new mechanisms highlighted from the present work and by which nutrients and exercise improve myogenic cell differentiation and growth. In addition, perspectives beyond the present work will be developed.

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Chapter 2

Regulation of mTOR by amino acids and resistance exercise in skeletal muscle

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Abstract

Resistance exercise disturbs skeletal muscle homeostasis leading to activation of catabolic and anabolic processes within the muscle cell. A current challenge of exercise biology is to describe the molecular mechanisms of regulation by which contractile activity stimulates net protein breakdown during exercise and net protein synthesis during recovery. Muscle growth is optimized by combining exercise and appropriate nutritional strategies, such as amino acids (AA) and carbohydrate ingestion. The effects are integrated at the level of one central regulatory protein, mTOR (mammalian target of rapamycin). mTOR is a complex protein integrating signals of the energetic status of the cell and environmental stimuli to control protein synthesis, protein breakdown and therefore cell growth. mTOR is known to be activated by insulin, and the mechanisms involved are well documented. The ways by which exercise and AA lead to mTOR activation remain partially unclear. Exercise and AA use different signalling pathways upstream of mTOR. Exercise seems to recruit partially the same pathway as insulin, whereas AA could act more directly on mTOR. During resistance exercise, the activity of mTOR could be acutely blunted by AMPK, thus inhibiting protein synthesis and enhancing AA availability for energy metabolism. During recovery, the inhibition of mTOR by AMPK is suppressed, and its activation is maximized by the presence of AA. There appears to be a requirement for a minimal concentration of plasma insulin to stimulate muscle protein synthesis in response to resistance exercise and AA ingestion.

Keywords

Protein synthesis, $p70^{s6k}$, 4E-BP1, PKB/Akt, AMPK

Introduction

Resistance exercise leads to changes in protein turnover in skeletal muscle up to 48h after the completion of a training session (Phillips *et al.*, 1997). At rest in the fasted state, the net protein balance is negative, because protein breakdown largely exceeds protein synthesis. Following exercise in the fasted state, protein synthesis as well as protein breakdown are enhanced, although the latter is less so, resulting in a less negative net balance (Biolo *et al.*, 1995; Phillips *et al.*, 1997; Tipton & Wolfe, 1998).

Most athletes involved in resistance training are looking for increased muscle mass. Ingesting a mixture containing carbohydrate (CHO) and amino acids (AA), preferentially before (Tipton *et al.*, 2001) or immediately after (Biolo *et al.*, 1997) completion of the training session, counteracts the catabolic state by increasing AA transport and availability into the muscles (Biolo *et al.*, 1995). In this situation, protein synthesis (Tipton & Wolfe, 1998; Louis *et al.*, 2003b), but not protein breakdown (Biolo *et al.*, 1995; Phillips *et al.*, 1997), is enhanced, and the net protein balance becomes positive. The effect of AA is enhanced by an elevated insulin concentration subsequent to CHO supplementation (Rasmussen *et al.*, 2000). Nutritional strategies centred around the ingestion of AA are essential to maximize the effect of resistance training and therefore to accumulate muscle mass (Wolfe, 2000).

The mechanisms of activation of protein synthesis by AA and exercise are somewhat different. Both use distinct signalling pathways converging at the level of one central protein involved in cell growth called mTOR (mammalian target of rapamycin). mTOR is a 289 kDa serine/threonine kinase composed of 2459 AA. This large protein has also been called FRAP (FK506-binding protein 12 [FKBP-12]-and rapamycin associated protein), RAFT1 (rapamycin and FKBP-12 target-1), RAPT1 (rapamycin target-1) or

SEP (sirolimus effector protein). mTOR stimulates protein synthesis mainly via three key regulatory proteins $p70^{s6k}$ (p70 ribosomal S6 kinase), 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1) (Brunn *et al.*, 1997; Burnett *et al.*, 1998) and eIF4G (eukaryotic initiation factor 4G) (Raught *et al.*, 2001). The purpose of this review is to describe the roles and the mechanisms of regulation of the mTOR-mediated signalling by AA and exercise. The first part will describe the roles and the regulation mechanisms of mTOR and its downstream effectors. Next the role of AA in mTOR activation will be discussed. Finally the effect of exercise and AA on mTOR signalling will be analyzed. Optimal stimulation of muscle growth is highly dependent on the fine-tuned integration of these two pathways.

Roles of mTOR

Some authors present mTOR as an energetic sensor although mTOR has a relatively high Km for ATP (~1mM) (Dennis *et al.*, 2001). Therefore, a large drop in ATP is needed to induce a substantial effect on mTOR activity, as opposed to AMPK which is a more sensitive energetic sensor to detect small variations in AMP concentration. A major role of mTOR is to integrate environmental stimuli (nutrient and growth factor levels, mitochondrial signals and exercise) in order to control cellular growth (Fig. 2.1) (Raught *et al.*, 2001; Proud, 2002).

Protein synthesis is one of the most highly regulated processes. Cell growth is well organized and occurs at specific moments and in well-defined cellular compartments. To develop, the cell must integrate mitogenic and nutritional signals. One of the most powerful anabolic signals is insulin, which acts, among others, through mTOR-mediated signalling. Once bound to its receptor, insulin activates the intrinsic kinase activity of the receptor leading to its autophosphorylation (Kasuga *et al.*, 1982) and phosphorylation of

several substrates, including members of the insulin receptor substrate (IRS) family (White *et al.*, 1985). Phosphorylation of IRS-1 recruits another signalling molecule, the phosphatidylinositol 3 kinase (PI3K) (Backer *et al.*, 1993). One downstream target of PI3K is the serine/threonine protein kinase B (PKB) which activates mTOR directly and indirectly (see below) and its downstream effectors involved in the control of protein translation, $p70^{s6k}$, 4E-BP1 and eIF4G (Fig. 2.2).

mTOR could control other processes such as autophagy (Blommaart *et al.*, 1995; Noda & Ohsumi, 1998), cell proliferation (Coolican *et al.*, 1997; Shah *et al.*, 2001; Gao *et al.*, 2004) and cytoskeletal organization (Berven & Crouch, 2000; Berven *et al.*, 2004; Qian *et al.*, 2004) (Fig. 2.1). Although the main focus of this review is on protein translation, it should be noted that mTOR also regulates gene transcription (Mayer *et al.*, 2004), as evidenced by studies using rapamycin, an inhibitor of mTOR.

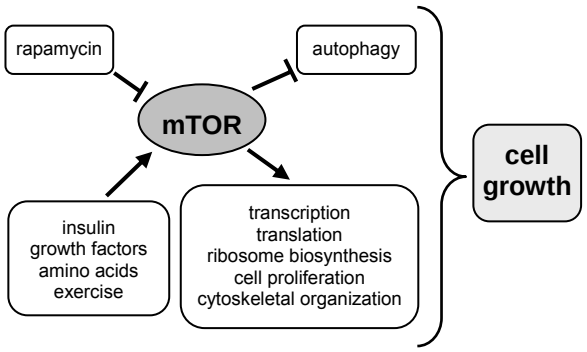

Fig. 2.1. *Roles of mTOR.* mTOR signalling is activated in the presence of insulin, growth factors, AA and during exercise, whereas it is repressed by rapamycin. When activated, mTOR inhibits autophagy and stimulates transcription, translation of mRNAs coding for components of the translation machinery, ribosomes biosynthesis, cell proliferation and cytoskeletal organization to favour cell growth.

Regulation of mTOR and its downstream targets

In the N-terminal region, mTOR possesses 16 HEAT domains which allow the binding of various regulatory proteins. The activity of mTOR is also influenced by changes in its phosphorylation state. Up to now three phosphorylation sites have been discovered: Ser 2481, an autophosphorylation site, Ser 2448 and Thr 2446, the roles of which are described below. Once phosphorylated, mTOR can in turn phosphorylate downstream targets. The major proteins regulated by mTOR and playing a role in translation are 4E-BP1, eIF4G, and $p70^{s6k}$ (Raught *et al.*, 2001). The mechanism by which mTOR activates its effectors is not fully elucidated. mTOR could stimulate 4E-BP1, eIF4G, and $p70^{s6k}$ either directly by phosphorylation (Raught *et al.*, 2001), indirectly by activating another protein kinase (Raught *et al.*, 2001) or by inhibiting a phosphatase (e.g. protein phosphatase 2A, PP2A) (Li *et al.*, 2004).

4E-BP1 is an inhibitor of the initiation translation factor eIF4E (eukaryotic initiation factor 4E). When 4E-BP1 is phosphorylated, eIF4E is released and can form the eIF4F complex, together with eIF4G, which is also under the control of mTOR (Raught *et al.*, 2001), and eIF4A. The assembly of this complex is necessary for initiation to continue. 4E-BP1 possesses many phosphorylation sites. The phosphorylation of Ser 65 and Thr 70 (and to a lesser extend Thr 37 and 46) is blocked in the presence of rapamycin suggesting an essential role of mTOR in the regulation of 4E-BP1 (Proud, 2002).

$p70^{s6k}$ is a member of the S6K family. The S6Ks are a product of two genes, S6K1 and S6K2, $p70^{s6k}$ being encoded by the first one. Up to now twelve sites of phosphorylation have been described for $p70^{s6k}$ (Shah *et al.*, 2000). The Thr 389 is a direct target of mTOR *in vitro* (Dennis *et al.*, 1999). The phosphorylation of Thr 389 by mTOR allows subsequent phosphorylation of

Thr 229 by the phosphoinositide-dependant kinase 1 (PDK1). It is generally assumed that, when activated, the S6K proteins phosphorylate the ribosomal S6 protein. The latter controls the translation of a class of mRNAs called the 5'TOP (tract of pyrimidine) mRNAs, although, for some authors, this process does not require S6K or S6 phosphorylation (Stolovich *et al.*, 2002). The TOP mRNAs encode for ribosomal proteins and proteins implicated in translation, like eEF2 (eukaryotic elongation factor 2) (Proud, 2002). eEF2 mediates the translocation during the elongation phase. It binds the guanidic nucleotides and is active when bound to GTP. Furthermore, the activity of eEF2 can be inhibited by phosphorylation on Thr 56 via its kinase eEF2K. $p70^{s6k}$ can inhibit eEF2K by phosphorylation, thus increasing the activity of eEF2 (Browne *et al.*, 2004). In summary, $p70^{s6k}$ is involved in the expression as well as in the activity of eEF2. Another potent target of the S6K family is the phosphoprotein eIF4B which stimulates the activity of eIF4A (see above) (Raught *et al.*, 2001).

Rapamycin-FKBP12

Rapamycin regulates the protein kinase activity of mTOR. Once bound to its intracellular receptor FKBP12 (also called FK506-binding protein), rapamycin specifically interacts with mTOR and inhibits the signalling downstream of mTOR *in vitro*. However, the concentrations of rapamycin required to inhibit the activity of mTOR *in vitro* are much higher than *in vivo* (500nM *vs.* 25nM). It is likely that rapamycin does not directly modulate the activity of mTOR, but when bound to it, rapamycin could activate a phosphatase which could act on the effectors downstream of mTOR (Raught *et al.*, 2001).

Raptor, rictor and GβL

The activity of mTOR is also controlled by several binding partners such as the regulatory associated protein to mTOR (raptor), the rapamycin-insensitive companion of mTOR (rictor) and GβL (also called mLST8).

Raptor and rictor independently associate with mTOR and GβL and define two distinct mTOR complexes (Sarbassov *et al.*, 2004). Raptor seems to serve as an mTOR scaffolding protein whose association with mTOR is required for an efficient phosphorylation of 4E-BP1 and $p70^{s6k}$ (Nojima *et al.*, 2003). Binding of raptor to mTOR could influence the sensitivity of mTOR to rapamycin and AA (Kim *et al.*, 2002). However, further investigations are needed to clarify the interaction between mTOR and raptor, and how raptor alters the kinase activity of mTOR.

Raptor not only binds to mTOR but also to $p70^{s6k}$ and 4E-BP1 through their respective TOS (conserved TOR signalling) motifs. The TOS motif is a short conserved sequence required for AA- and mTOR-dependent activation of the downstream effectors of mTOR. A mutation in the TOS motif of $p70^{s6k}$ and 4E-BP1 that prevents both AA- and mTOR-regulation also abolishes their binding to raptor.

Unlike raptor, the recently discovered rictor is insensitive to rapamycin and does not regulate $p70^{s6k}$. The rictor-mTOR complex rather modulates the organization of the actin cytoskeleton and cell morphology. PKCα (protein kinase C α) is a mediator of this function but it is likely that other effectors are involved in controlling the actin cytoskeleton (Sarbassov *et al.*, 2004).

TSC1/TSC2 and Rheb

mTOR can be directly activated by PKB phosphorylating Ser 2448 (Nave *et al.*, 1999). Recently, an additional control mechanism of mTOR via PKB has been suggested, implicating the proteins TSC1 and TSC2 (tuberous sclerosis complex 1 and 2). TSC1 and TSC2, also called hamartin and tuberin respectively, form a complex and repress mTOR. This inhibition on mTOR can be reversed when TSC2 is phosphorylated by PKB, which induces the dissociation of the complex (Fig. 2.2). PKB phosphorylates TSC2 on Ser 939, Ser 1086, Ser 1088 and Thr 1422. According to some authors (Liu *et*

al., 2002; Nellist *et al.*, 2003), the phosphorylation of these sites by PKB is responsible for the binding of the 14-3-3 protein to TSC2. The 14-3-3 protein family has many roles in cell metabolism (Siles-Lucas Mdel & Gottstein, 2003), amongst which the modulation of the TSC2 activity. According to others (Li *et al.*, 2002; Shumway *et al.*, 2003), it is the phosphorylation of TSC2 on Ser 1210 by the p38 mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2) that would be essential for the binding of the 14-3-3 protein.

TSC2 is a GTPase-activating protein (GAP) for the small G protein Rheb (Ras homolog enriched in brain). When Rheb is bound to GTP, mTOR is activated (Fig. 2.2). The GAP domain of TSC2 increases the hydrolysis of the GTP form of Rheb preventing the signal to propagate through mTOR. Up to now no guanine-nucleotide exchange factor (GEF) to counter the GAP activity of TSC2 has been discovered. It has been proposed that Rheb might not require a GEF for its activation because Rheb exists in a highly GTP-bound state and has low intrinsic GTPase activity (Im *et al.*, 2002). Unlike TSC2, TSC1 has no GAP activity. Its role is rather to stabilize TSC2 and thereby to prevent TSC2 from ubiquitinisation and degradation (Li *et al.*, 2004).

Several mechanisms have been proposed to explain how Rheb could activate mTOR (Fig. 2.2). First Rheb could be a component of the complex formed by mTOR and other proteins, thus regulating mTOR directly or via these binding proteins. As up to now no direct association between Rheb and mTOR has been demonstrated, it is likely that Rheb activates a binding protein of mTOR rather than mTOR directly. Secondly mTOR could play a role in the detection of the intracellular AA level. It is then possible that Rheb activates mTOR indirectly by facilitating the transport of AA into the cell (Li *et al.*, 2004). Thirdly Rheb could activate an unknown kinase that phosphorylates mTOR. In HEK293 cells, overexpression of Rheb has been

shown to increase the phosphorylation of mTOR on Ser 2448 (Inoki *et al.*, 2003a). This activation of mTOR by overexpressing Rheb can even occur in the absence of growth factors or AA (Inoki *et al.*, 2003a; Tee *et al.*, 2003). Conversely, overexpression of TSC1 and TSC2 blocks the ability of both amino acids (Gao *et al.*, 2002; Inoki *et al.*, 2002; Tee *et al.*, 2002) and Rheb (Inoki *et al.*, 2003a; Tee *et al.*, 2003) to activate mTOR. In summary, several hypotheses have been proposed to explain the mechanism of activation of mTOR by Rheb, but the effector(s) of Rheb that regulates mTOR directly or indirectly remain(s) unknown.

AMPK

The protein kinase activated by AMP (AMPK) is a heterotrimeric complex including one catalytic subunit α and two non-catalytic subunits β and γ. AMPK is activated when AMP increases and when the catalytic subunit α is phosphorylated by one or several AMPK kinases on Thr 172, located in the "T loop" of the catalytic domain. Once activated AMPK phosphorylates many substrates, the purpose of which is to maintain the intracellular ATP level. When the ATP concentration falls, AMPK prevents the consumption of ATP by inhibiting key enzymes of anabolic pathways. AMPK also increases ATP synthesis by stimulating for example the rate of fatty acid oxidation and the uptake of glucose by the cell (Yonezawa, 2003). It has been reported that, when activated, AMPK could either phosphorylate TSC2 on Thr 1227 and Ser 1345 thereby enhancing its activity (Inoki *et al.*, 2003b), or phosphorylate mTOR on Thr 2446 resulting in its inhibition (Cheng *et al.*, 2004). However, the latter results need to be confirmed, and it is more likely that AMPK does not inhibit mTOR directly but through the phosphorylation of TSC2 (Inoki *et al.*, 2003b).

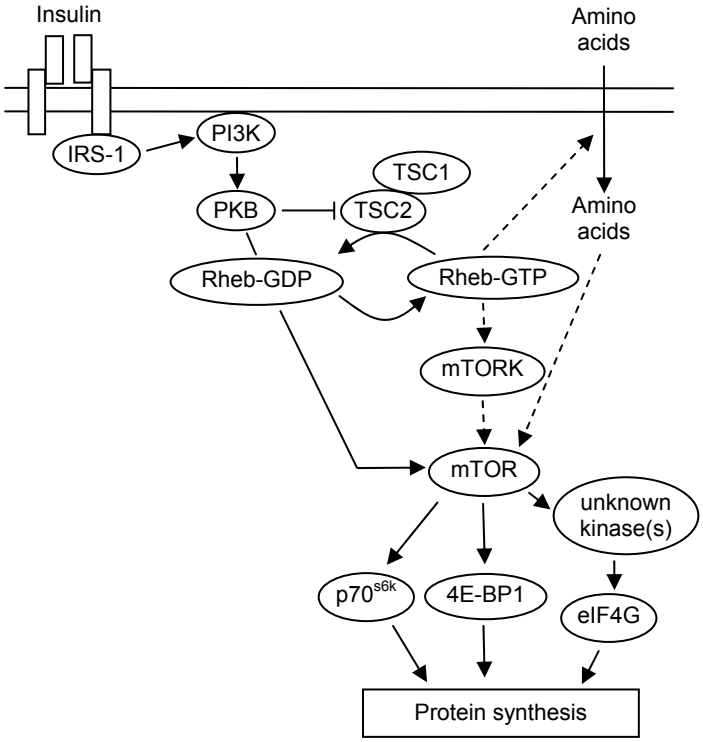

Fig. 2.2. *Inhibition of mTOR by TSC1/TSC2.* TSC2 is a GTPase-activating protein for the small G protein Rheb. The GAP domain of TSC2 increases the hydrolysis of the GTP form of Rheb, inhibiting the signal to propagate through mTOR. Rheb, under active form (Rheb-GTP), could facilitate the transport of AA into the cell which in turn could activate mTOR. IRS-1, insulin receptor substrate 1; PI3K, phosphatidylinositol 3 kinase; PKB, protein kinase B; TSC1/TSC2, tuberous sclerosis complex 1/2; Rheb, Ras homolog enriched in brain; mTORK, mammalian target of rapamycin kinase; mTOR, mammalian target of rapamycin; $p70^{s6k}$, p70 ribosomal S6 kinase; 4E-BP1, eukaryotic initiation factor 4E-binding protein.

mTOR and amino acids

Protein synthesis is one of the most energy consuming processes. If AA are lacking, the cell should conserve its energy and avoid expensive anabolic processes. On the other hand, if AA availability is sufficient, the cell has to increase the rate of protein translation to ensure cell growth. There is evidence for AA playing a role in the translational regulation of protein synthesis. In skeletal muscle of rats in the fasted state, the mTOR-mediated

signalling is reduced compared with the fed state. In a study with fasted animals, 4E-BP1 was highly present in hypophosphorylated forms (Yoshizawa *et al.*, 1997; Yoshizawa *et al.*, 1998), sequestering eIF4E and inhibiting the formation of the initiation complex eIF4F. On the contrary, when the rats received a protein-containing meal, 4E-BP1 became hyperphosphorylated and promoted the formation of the eIF4F complex. This positive effect on muscle protein synthesis can also be observed with oral leucine administration alone (Anthony *et al.*, 2000). In addition to promoting eIF4F assembly, both feeding a complete meal or oral administration of leucine promote phosphorylation of $p70^{s6k}$ (Anthony *et al.*, 2000).

Types of amino acids

AA are known to stimulate protein synthesis but the mechanisms of activation differ according to the AA studied (Meijer & Dubbelhuis, 2004). AA can be classified into two major categories. The first one induces cell swelling because of an increase in the intracellular osmolarity following Na^+-dependent AA transport across the membrane, and because some products of their metabolism accumulate in the cell (e.g., glutamate). Cell swelling *per se* induces protein, glycogen and lipid synthesis. The second category of AA is represented by leucine which does not induce cell swelling and which essentially regulates protein metabolism by stimulating protein synthesis and by inhibiting autophagy.

A group of AA, the branched chain AA (BCAA), including leucine, valine and isoleucine, seem to be particularly effective anabolic agents, especially leucine which has the most potent effect on the phosphorylation state of $p70^{s6k}$ in the skeletal muscle (Anthony *et al.*, 2000) and in various cell lines (Patti *et al.*, 1998; Xu *et al.*, 1998). It is possible that AA activate mTOR through a permissive action of insulin (Jefferson & Kimball, 2003). Even

low levels of insulin could maintain a minimal activation level of the PI3K/PKB pathway by stimulating the PI3K class I and subsequently allowing AA to activate the downstream targets of mTOR in many different cell types (hepatocytes, adipocytes, myocytes and pancreatic cells).

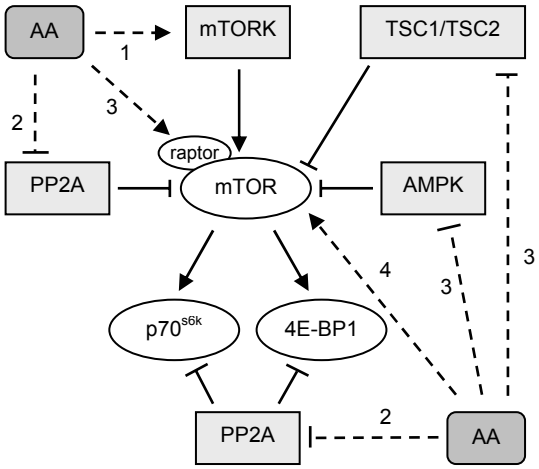

Fig. 2.3. *Hypotheses for activation of mTOR by AA (see text for details).* 1 AA could stimulate a protein kinase (mTORK) acting on mTOR. 2. AA could inhibit a protein phosphatase such as PP2A which could act on mTOR or on the downstream proteins of mTOR. 3. AA could interact with proteins associated with mTOR (raptor) or acting on mTOR (TSC1/TSC2, AMPK). 4. AA could activate mTOR directly. AA, amino acids; mTORK, mammalian target of rapamycin kinase; TSC1/TSC2, tuberous sclerosis complex 1/2; PP2A, protein phosphatase 2A; 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.

Mechanisms involved in amino acid regulation

The way by which AA stimulate mTOR is not fully understood, but they do not act like growth factors or insulin which modulate PI3K and its downstream effector PKB (see above). Indeed, activation of mTOR via AA does not require activation of PKB (Jefferson & Kimball, 2003). Several hypotheses have been proposed to explain the activation of mTOR by AA (Fig. 2.3). AA could stimulate a protein kinase (other than PKB) acting on mTOR or could inhibit a protein phosphatase like PP2A. This protein phosphatase could act on mTOR or on the downstream proteins. AA could

also interact with proteins associated with mTOR (raptor) or acting on mTOR (TSC1/TSC2, AMPK) (Meijer & Dubbelhuis, 2004). Some authors have hypothesized that AA increase the phosphorylation on Ser 2448 by facilitating the dissociation of the complex TSC1/TSC2 from mTOR and allowing PKB to access on Ser 2448 (Kanazawa *et al.*, 2004). Finally a direct activation of mTOR by AA could be possible but remains controversial. In conclusion, the molecular nature of AA sensing and the regulation of TSC1/TSC2, Rheb and mTOR by AA remains poorly understood.

Although the mTOR pathway plays a major role in the regulation of protein synthesis, it should be mentioned that AA can stimulate cell growth independently of mTOR, and that the involvement of mTOR varies greatly among cell lines. In L6 myoblasts, eIF2B (eukaryotic initiation factor 2B), which is not under the control of mTOR, appears to be more involved in the activation of protein synthesis by AA than eIF4E (Kimball *et al.*, 1998). In isolated rat ventricular myocytes, protein synthesis is acutely activated by insulin (Wang *et al.*, 2000) and this effect is blunted by only around 50% by rapamycin. In skeletal muscle, the activation of protein synthesis by leucine is only partially inhibited by rapamycin (Anthony *et al.*, 2000). Thus it is likely that leucine stimulates protein synthesis via a mTOR-dependent and a mTOR-independent pathway(s) (Proud, 2002).

Autophagy

The presence of AA not only stimulates protein synthesis but also represses autophagy. Autophagy is the only proteolytic system that can be regulated by the nutritional state. When the nutritional state is low, eukaryotic cells degrade their proteins to scavenge AA. The first step of this autophagy consists in the sequestration of a portion of the cytoplasm inside a double membrane structure called autophagosome. This structure fuses then with lysosomal membranes where the cytoplasmic components can be degraded

(Raught *et al.*, 2001). AA and insulin are the main physiological regulators of autophagy in mammals, but it seems that they use different ways to control it. The pathway used by insulin to regulate autophagy is partially the same as for protein synthesis. On the contrary, it seems that the signalling used by AA in autophagy is independent of protein synthesis and that AA have their own signal transduction mechanisms (Kanazawa *et al.*, 2004). In a perfused liver, autophagy is controlled by the extracellular concentrations of phenylalanine and leucine. Leu8-MAP, an analog of leucine that can not be transported inside the cell, mimics the effect of leucine. It can be speculated that AA have their own recognition site on the surface of the membrane and their own signal transduction mechanisms up to the formation site of the autophagosome in the cell (Kanazawa *et al.*, 2004).

mTOR could be a central component of the signal transduction of autophagy (Fig. 2.1), whereas downstream effectors of mTOR are not well known. In yeast, autophagy induced by fasting is controlled by TOR via a complex Apg1-Apg13, which plays a role in the initial formation of the phagosome (Kamada *et al.*, 2000). In isolated hepatocytes, inhibition of autophagy by AA occurs via the phosphorylation of the ribosomal protein S6 (Blommaart *et al.*, 1995). As described above, phosphorylation of S6 is activated by $p70^{s6k}$, a direct target of mTOR. However some studies do not support the hypothesis of mTOR involvement in autophagy. In mouse C_2C_{12} myotubes, mTOR signalling pathway was not involved in the regulation of autophagy by leucine (Mordier *et al.*, 2000). Furthermore rapamycin had no effect on the suppression of autophagic proteolysis by AA in isolated hepatocytes (Kanazawa *et al.*, 2004). The discrepancy between these studies and the study of Blommaart et al. (1995) could be explained by methodological considerations which are beyond the scope of this review. Clearly more research is warranted to clarify if and how mTOR regulates autophagy in skeletal muscle.

mTOR and exercise

Acute resistance exercise is known to increase skeletal muscle protein synthesis during the recovery phase. Both concentric and eccentric contractions seem to be effective in promoting protein synthesis (Wong & Booth, 1990b; Phillips *et al.*, 1997; Phillips *et al.*, 1999). Two major pathways leading to protein synthesis have been proposed to induce this increase: the PI3K/PKB/mTOR and the calcineurin/NFAT (nuclear factor of activated T cells) pathways. The PI3K/PKB/mTOR pathway could be involved mainly in skeletal muscle growth (Pallafacchina *et al.*, 2002), whereas the calcineurin/NFAT pathway, and to a lesser extend the Ras/MAPK (mitogen-activated protein kinase) pathway, would rather control muscle fibre type (Fig. 2.4) (Serrano *et al.*, 2001). Indeed, inhibitors of calcineurin, cyclosporin A and FK506, do not blunt muscle hypertrophy, as shown by increases in plantaris muscle mass and fibre size of rats after overload (Bodine *et al.*, 2001). In contrast, specific inhibition of mTOR with rapamycin leads to a 95% blockade of hypertrophy indicating that mTOR and its downstream targets, $p70^{s6k}$ and 4E-BP1, are crucial regulators of muscle hypertrophy (Bodine *et al.*, 2001).

PKB

No information is available to date on the activation of PI3K following skeletal muscle overload. However, a series of studies have demonstrated upregulation of PKB activity. PKB phosphorylation on Ser 473 was almost tripled immediately after high frequency electrical stimulation (Nader & Esser, 2001) and was higher in rat plantaris muscle after a few days overloading compared to control conditions (Bodine *et al.*, 2001). PKB activity increased 2.5 fold from day 3 to day 10 in regenerating innervated soleus muscle after injury. In the same study overexpression of active PKB increased fibre size in regenerating soleus and in adult EDL and soleus muscles (Pallafacchina *et al.*, 2002).

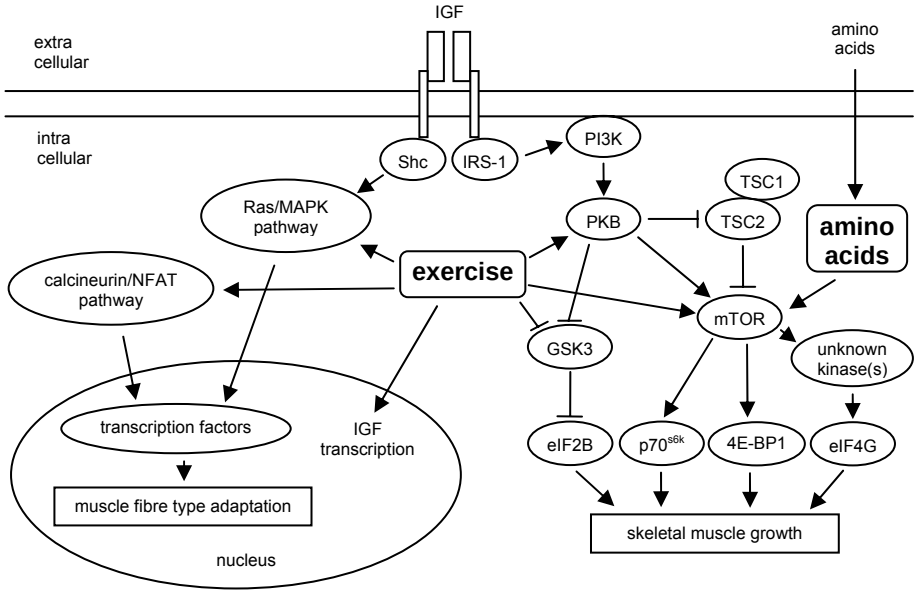

Fig. 2.4. *AA and exercise signalling.* AA stimulate skeletal muscle growth by activating mTOR and its downstream effectors (for details see Fig. 2.3), whereas exercise acts upstream of mTOR via PKB to promote hypertrophy. Exercise also activates the calcineurin/NFAT and Ras/MAPK pathways leading to muscle fibre type adaptation. IGF, insulin-like growth factor; Shc, Src-homology and collagen homology; IRS-1, insulin receptor substrate 1; PI3K, phosphatidylinositol 3 kinase; PKB, protein kinase B; TSC1/TSC2, tuberous sclerosis complex 1/2; mTOR, mammalian target of rapamycin; $p70^{s6k}$, p70 ribosomal S6 kinase; 4E-BP1, eukaryotic initiation factor 4E-binding protein; GSK3, glycogen synthase kinase 3; eIF2B, eukaryotic initiation factor 2B.

Some results suggest that PKB phosphorylation is fibre type specific. Indeed, contraction- or stretch-induced activation of PKB was only observed in isolated fast-twitch EDL but not in isolated slow-twitch soleus (Sakamoto *et al.*, 2003; Sakamoto *et al.*, 2004). These data provide evidence that the different contractile and/or metabolic properties of slow or fast fibres confer distinct signalling properties to mixed skeletal muscles. Because no activation of PKB by exercise has been observed in isolated soleus muscle but well *in vivo*, it is likely that systemic factors and/or oxidative stress mediate the activation of PKB by exercise (Shaw *et al.*, 1998; Cooper *et al.*, 2002).

mTOR

In opposition to the above described results, some experiments did not reveal an enhanced activity or phosphorylation of PKB after exercise (Brozinick & Birnbaum, 1998; Markuns *et al.*, 1999; Sherwood *et al.*, 1999; Sakamoto *et al.*, 2002; Parkington *et al.*, 2003) or sciatic nerve stimulation, despite mTOR activation (Parkington *et al.*, 2003). These observations suggest a PKB-independent activation of mTOR signalling by contractile activity. In the study of Parkington et al. (2003), mTOR phosphorylation on Ser 2448 increased in tibialis anterior muscle after 6h recovery and in plantaris immediately after and 6h after the stimulation. mTOR was not altered in soleus muscle at either time point. This implies that like for PKB, mTOR phosphorylation depends on muscle fibre type. Indeed, mTOR phosphorylation in tibialis anterior is preferentially localized in type IIa fibres, suggesting that this class of fibres may be more responsive to hypertrophy (Parkington *et al.*, 2003).

p70^{s6k}

The first report on the phosphorylation state of p70^{s6k} after exercise in rats was published a few years ago (Baar & Esser, 1999). The phosphorylation of p70^{s6k} was unchanged immediately after tibialis anterior and extensor digitorum longus (EDL) muscles stimulation but tripled 3h and 6h post-exercise. This elevated phosphorylation was maintained until 36h of recovery. The changes in p70^{s6k} phosphorylation after a single bout of resistance exercise could be a good marker for the long-term increases in muscle mass. A later study confirmed that the activity of p70^{s6k} is elevated between 6h and 24h after acute resistance exercise but not immediately after exercise (Hernandez *et al.*, 2000).

GSK3

GSK3 (glycogen synthase kinase 3) is another effector of PKB that can regulate muscle hypertrophy, independently of the mTOR-mediated pathway (Bodine *et al.*, 2001). Although GSK3 is controlled by PKB, other kinases can modulate its activity such as MAP kinase-activated protein (MAPKAP) kinase-1 (also known as RSK2 or $p90^{rsk}$) and $p70^{s6k}$ (Sutherland *et al.*, 1993; Cross *et al.*, 1995). The phosphorylation of GSK3 results in its inhibition and in activation of eIF2B, which stimulates global protein synthesis. Indeed, eIF2B activity has been shown to coincide with increased protein synthesis several hours following resistance exercise (Farrell *et al.*, 1999). Moreover, exercise itself could inhibit GSK3, thus increasing the effect of enhanced protein synthesis (Fig. 2.4). Stretch-induced activation of PKB in EDL muscle of rats was fully prevented by wortmannin, an inhibitor of PI3K, whereas GSK3 phosphorylation was only partially reduced (Sakamoto *et al.*, 2003). These results suggest that exercise also regulates GSK3 by a PKB-independent pathway. In humans, phosphorylation and deactivation of GSK3 by exercise is less clear (Wojtaszewski *et al.*, 2001; Sakamoto *et al.*, 2004).

Time course of activation of the mTOR-mediated pathway

Some authors have investigated immediate responses of the mTOR-mediated signalling after resistance exercise in skeletal muscle of fed rats (Bolster *et al.*, 2003). Their results revealed that the PKB/mTOR pathway was induced after a few minutes following resistance exercise. The phosphorylation state of PKB, mTOR, $p70^{s6k}$, ribosomal protein S6, 4E-BP1 and the association of eIF4E to eIF4G peaked between 5min and 10min of recovery. These early responses may serve as a priming event favouring the growth response by the cell during exercise recovery. It should be noted that most variables measured in this study showed a “bell-shaped” curve. Thus, the components of the PKB/mTOR pathway could be phosphorylated in an oscillatory

fashion during the beginning of recovery. The summation of these transient activations could be critical in inducing muscle hypertrophy with repeated training sessions (Bolster *et al.*, 2003). The oscillatory fashion of activation of the PKB/mTOR pathway after exercise makes its study very difficult, and the time points at which the samples are taken following exercise may lead to opposing results, as illustrated by the above-described studies.

Even if the literature is abundant regarding the study of the PKB/mTOR pathway and exercise, one major question remains unanswered at this time: what is the link between contraction and activation of the PKB/mTOR pathway? Several hypotheses have been proposed to explain the changes in muscle protein synthesis induced by exercise. On short term, calcium release (Ji *et al.*, 2002) and integrin signalling (MacKenna *et al.*, 1998) are important actors in the responses to stretch in muscle. However, up to now there is no evidence linking one of these actors, exercise and the activation of PKB/mTOR signalling. On long term, IGFs and the so-called mechanical growth factor (Goldspink, 1999) could stimulate a late sustained response leading to muscle hypertrophy (Rennie & Wackerhage, 2003). Indeed, resistance exercise increases the expression of IGF-I, which is known to be a potent activator of the PKB/mTOR pathway. However the involvement of other, so far unknown, players can not be excluded. The summation of both short and long term processes lead to increased muscle protein synthesis and hypertrophy via activation of the PKB/mTOR pathway (Rennie & Wackerhage, 2003).

mTOR, exercise and amino acid supplementation

All the studies cited above were carried out on fed animals. Because AA and exercise both use the mTOR pathway, it is difficult to isolate the effect of exercise on this pathway. A recent study in humans examined the effect of exercise on the phosphorylation state of $p70^{s6k}$ and the S6 ribosomal protein after ingestion of a placebo or a drink containing BCAA. The phosphorylation of the S6 ribosomal protein and of $p70^{s6k}$ on Thr 389, which corresponds to its highest level of phosphorylation and its highest activity, were only slightly higher 1h and 2h after exercise in the placebo condition and were significantly increased at the same time points when the subject received the drink containing BCAA (Karlsson *et al.*, 2004). These data suggest that BCAA could exert a permissive action on the activation of the mTOR-mediated pathway by exercise (Karlsson *et al.*, 2004). Furthermore, as previously discussed, there appears to be a requirement for a minimal concentration of plasma insulin to stimulate muscle protein synthesis in response to resistance exercise. However, the concentration of insulin needed is low, values equivalent to or less than fasting values are sufficient to induce the effect (Kimball *et al.*, 2002).

Summary and conclusion

Protein synthesis is enhanced up to 48h after the completion of a resistance exercise concomitantly with a lesser increase in protein breakdown, leading to a positive protein net balance (Tipton & Wolfe, 1998). The opposite situation occurs during exercise, protein breakdown is greater than protein synthesis resulting in a negative net balance. From a biochemical point of view, we could propose the following hypothesis: during acute exercise, AMPK is activated due to an increase in the AMP/ATP ratio (Kemp *et al.*, 1999; Chen *et al.*, 2003; Nielsen *et al.*, 2003), and AMPK inhibits mTOR either directly (Cheng *et al.*, 2004) or via activation of the repressor TSC2

(Inoki *et al.*, 2003b). mTOR is thus less active in promoting protein synthesis and preventing autophagy. Increased breakdown allows for more AA to be available as fuel for exercise. Once exercise is completed, AMPK activity quickly returns to basal values. The inhibition on mTOR is suppressed and the latter becomes able to reduce autophagy and to promote protein synthesis via phosphorylation of $p70^{s6k}$, 4E-BP1 and eIF4G. The role of mTOR could be to preserve AA from being enrolled in protein synthesis during exercise through an inhibition by AMPK and, on the other hand, to favour their utilisation and to promote muscle mass accumulation during recovery.

Many recent investigations focus on exercise and its influence on translational control of protein synthesis. Interpretation of the results is complex since the type, the frequency and the intensity of muscle contractions, but also the type of muscle studied and the training status of the subjects represent factors that may lead to diverse responses. Different models and experiments may induce various degrees of muscle damage, AA availability and hormonal responses, and therefore different adaptations to exercise. The heterogeneity in the protocols could explain some discrepancies in the literature about the exercise signalling. It is, however, very clear that AA ingestion before or immediately after exercise is necessary to fully activate protein synthesis signalling in muscle and therefore to accumulate muscle mass.

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Chapter 3

Increased IGF mRNA in human skeletal muscle after creatine supplementation

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Abstract

Purpose: We hypothesized that creatine supplementation would facilitate muscle anabolism by increasing the expression of growth factors and the phosphorylation of anabolic signaling molecules; we therefore tested the responses of mRNA for IGF-I and IGF-II and the phosphorylation state of components of anabolic signaling pathways $p70^{s6k}$ and 4E-BP1 to a bout of high intensity resistance exercise after 5 days of creatine supplementation. **Methods:** In a double-blind cross-over design muscle biopsies were taken from the m. vastus lateralis at rest and 3 and 24 h post-exercise in subjects who had taken creatine or placebo for 5 days (21 g/day). For the first 3 h post-exercise the subjects were fed with a drink containing maltodextrin (0.3 $g.kg^{-1}$ body $wt.h^{-1}$) and protein (0.08 $g.kg^{-1}$ body $wt.h^{-1}$). **Results:** After creatine supplementation resting muscle expressed more mRNA for IGF-I (+30%, $P<0.05$) and IGF-II (+40%, $P=0.054$). Exercise caused an increase by 3 h post-exercise in IGF-I (+24%, $P<0.05$) and IGF-II (+48%, $P<0.05$) and by 24 h post-exercise in IGF-I (+29%, $P<0.05$), but this effect was not potentiated by creatine supplementation. The phosphorylation states of $p70^{s6k}$ and 4E-BP1 were not affected by creatine at rest; phosphorylation of both increased (150 – 400 %, $P<0.05$) to similar levels under placebo and creatine conditions at 3 h post-exercise plus feeding. However, the phosphorylation state of 4E-BP1 was higher in the creatine vs placebo condition at 24 h post-exercise. **Conclusion:** The increase in lean body mass often reported after creatine supplementation could be mediated by signaling pathway(s) involving IGF and 4E-BP1.

Keywords

Resistance exercise; protein synthesis; $p70^{s6k}$; 4E-BP1; real time RT-PCR

Introduction

Over the last 15 years, the use of creatine as an ergogenic aid has increased markedly, especially among strength and power athletes. Surprisingly, creatine supplementation has been found not only to increase the short term power output during high intensity exercises but to result in significant increases in lean body, particularly muscle mass (Balsom *et al.*, 1993). There is evidence that this increase is mainly intracellular, suggesting that the cell dry mass is increased (Francaux & Poortmans, 1999). Additionally, a number of workers have found increases in muscle fiber area (Volek *et al.*, 1999; Tarnopolsky *et al.*, 2001; Willoughby & Rosene, 2001). Taken together, these observations suggest that creatine could enhance muscle growth by stimulating protein synthesis.

In previous studies (Louis *et al.*, 2003a; Louis *et al.*, 2003b), we set out to discover if 5 days of oral creatine supplementation could potentiate protein synthesis in human muscle. We could not confirm this hypothesis either at rest in the post-absorptive or fed state, or at rest after an acute bout of resistance exercise. These results could be interpreted in several ways. First, it is possible that creatine has no anabolic effect on muscle, which would, however, not be in line with previous observations (see above). Secondly, creatine might have an effect, but beyond the time frame of our previous experiments (<3.5 h). Thirdly, the effect of creatine could be small and below the detection limits of our previously used methods.

Insulin-like growth factor I (IGF-I) is known to increase muscle protein synthesis and to stimulate satellite cell proliferation and differentiation *in vitro* (Allen & Boxhorn, 1989). Recently we showed that myogenic C_2C_{12} cells incubated with creatine differentiated more rapidly than in control conditions; this observation was associated with an increase in IGF-I mRNA expression (Louis *et al.*, 2004).

IGF-II, another member of the IGF family, is also produced by skeletal muscle and is involved in muscle differentiation and regeneration (Kaliman *et al.*, 1999). IGFs activate several cellular pathways regulating muscle atrophy and hypertrophy, amongst which the PI3K-Akt/PKB-mTOR signaling pathway (Sartorelli & Fulco, 2004).

The PI3K-Akt/PKB-mTOR pathway is involved in the regulation of skeletal muscle fiber size and in the stimulation of translation initiation by activating mTOR and two of its downstream effectors: $p70^{s6k}$ and 4E-BP1 (Eukaryotic Initiation Factor-4E Binding Protein-1) also called PHAS-I (Phosphorylated Heat- and Acid-Stable protein regulated by Insulin). $p70^{s6k}$ and 4E-BP1 are two key regulators of protein synthesis. $p70^{s6k}$ phosphorylates the ribosomal phosphoprotein S6 on the 40S subunit (Proud, 1996). S6 is involved in the translation of a class of mRNA called TOP mRNAs (due to their Terminal region rich in OligoPyrimidines). This class of mRNA encodes for components of the translational machinery and elongation factors (Jefferies *et al.*, 1997). The phosphorylation state of $p70^{s6k}$ and consequently its activity are increased by amino acids, insulin and exercise (Sakamoto & Goodyear, 2002).

4E-BP1, a translational repressor (Brunn *et al.*, 1997), can bind to the initiation factor eIF4E to prevent the formation of the eIF4F scaffolding complex, which is necessary for efficient binding of the 40S ribosomal subunit to mRNA. When 4E-BP1 is hyperphosphorylated, it dissociates from eIF4E allowing it to participate in translation initiation. In human skeletal muscle, 4E-BP1 is activated by branched chain amino acid infusion (Liu *et al.*, 2001) and exercise (Rennie, 2001).

Since we previously could not demonstrate an acute increase in protein synthesis following creatine ingestion (Louis *et al.*, 2003a; Louis *et al.*, 2003b), we hypothesized that creatine has a measurable effect on

downstream regulatory factors from the PI3K-Akt/PKB-mTOR pathway, and that it could act upstream, in a longer time frame, via the IGFs. Therefore, the aim of the present study was to test the prediction that creatine supplementation alone would enhance IGF-I and IGF-II gene expression and increase the phosphorylation state of $p70^{s6k}$ and 4E-BP1. Furthermore, we speculated that high intensity resistance exercises would potentiate these effects.

Material and Methods

Subjects and study design

Six healthy young men (age 23 ± 0.6 y, body mass 77.1 ± 5.1 kg, height 186 ± 1.0 cm) physically active but not specifically trained for resistance exercises participated in this double-blind, cross-over study. All subjects were given an oral and written account of the study before signing an informed consent form. This study was approved by the Ethic Committee of the Université Catholique de Louvain and all the procedures used were in accordance with the Declaration of Helsinki. The subjects were randomly divided into two groups: one group (N=3) received 21 g oral creatine monohydrate per day for 5 days while the second group (N=3) received a placebo (maltodextrin 21 g). After a wash-out period of one month, the treatments were crossed-over.

Protocol

During an initial familiarization session one week prior to the main study, each subject completed a leg press test with one leg to determine the one-repetition maximum (1-RM). The subjects were free to choose their preferred leg for exercise, but they were asked to use the same leg subsequently for both the creatine and placebo trials.

The day before and the day of the experiment, the subjects were asked not to participate in any sport activities and to maintain their usual diet. On the day of testing, subjects reported to the laboratory at 8.00 a.m. after an overnight fast and a muscle biopsy was taken at rest from the vastus lateralis. Subsequently, subjects received a standardized meal (2 croissants (2 x 60 g) and 1 glass of orange juice (200 ml)) after which they began the exercise session. They first warmed-up with 3 sets of 10 repetitions on the leg press. The subsequent exercise test consisted of 10 sets of 10 leg press repetitions (every 5 s) at 70% of each individual 1-RM. The sets were interspersed with

2-min rest periods and the subjects received verbal encouragement throughout the exercise session. During the first 3 h post-exercise, the subjects ingested a drink containing 0.3 $g.kg^{-1}$ body $wt.h^{-1}$ maltodextrin (Nestlé; Caloreen, Brussels, Belgium) and 0.08 $g.kg^{-1}$ body $wt.h^{-1}$ skimmed milk protein powder (Nutricia; Protifar Plus, Bornem, Belgium) dissolved in water. A second muscle biopsy was taken 3 h post-exercise from the exercised leg (with the subjects in the fed state) and a third biopsy was taken the following day, 24 h post-exercise, in the fasted state. All the biopsies were taken from the mid portion of the vastus lateralis muscle with a 4-mm Bergström biopsy needle after local anesthesia with 1% lidocaine. Blood, macroscopically visible fat and connective tissue were quickly removed, and the sample was immediately frozen in liquid nitrogen and stored at -80 °C for later analysis.

RNA extraction and quantitative Real-Time PCR

Total RNA was prepared from frozen tissue samples (90 mg) using TRIZOL® (Gibco Life Technologies, Paisley, UK). RNA was quantified by spectrophotometry (λ = 260 nm) and its concentration adjusted to 1 µg/µl using RNase-free water. Reverse transcription (RT) was performed using the GeneAmp PCR system 2400 (Applied Biosystems, Foster City, CA, USA) with 1 µg of total RNA in a reaction volume of 20 µl, containing 7.5 µM random hexamers, RT buffer 1X, 9 mM dithiothreitol, 220 µM of each deoxyribonucleotide triphosphate (dNTP), 20 U ribonuclease inhibitor (Applied Biosystems) and 50 U of reverse transcriptase (Superscript®, Gibco, BRL). The final RT product was adjusted to 40 µl using RNase free water. Real time RT-PCR (reverse transcription-polymerase chain reaction) primers were designed (Table 3.1, Primer Express Software, Applied Biosystems) for human IGF-I, IGF-II and β-2-microglobulin. The latter was used as the “house keeping” gene, because preliminary experiments as well as a previous study (Murphy *et al.*, 2003) had revealed that it was not

affected by either exercise or creatine supplementation. In the current study, the intra-subject coefficient of variation (CV) of the Ct values varied between 0.9 and 3.9%. Sybr Green® real time RT-PCR analyses were carried out on the GeneAmp 5700 (Applied Biosystems) using the following cycle conditions: 10 min at 95 °C, followed by 40 cycles of 1 min at 60 °C and 15 s at 95 °C. For each gene, real time RT-PCR was conducted in duplicate with 25 µl reaction volume of 5 ng of cDNA, 2.5 µl Sybr Green® buffer, 250 µM dNTP, 3 mM $MgCl_2$, 400 nM of each primer and 0.625 U AmpliTaq Gold® DNA Polymerase (Applied Biosystems). To ensure the quality of the measurements, each plate included, for each gene, a negative control (sample replaced by RNase free water) and a positive control consisting of an aliquot from a mixed pool of cDNA of positive samples. The threshold cycle (Ct) from a positive sample was used to calculate the inter-assay CV. For each gene, the CV was calculated as standard deviation/mean of the 2 Ct determined on five different plates and with different mixes. Typically the CV obtained was ~ 5%. The mRNA results are presented as ΔCt: $Ct_{\text{gene of interest}} - Ct_{\text{house keeping gene}}$.

Table 3.1. *Sequences of primers used for mRNA quantification by real time RT-PCR.*

Primer name	**Sequence (5' to 3')**	**Accession n°**
β-2-µglobulin Fw	gga ggc tat cca gcg tac tcc	NM_004048
β-2-µglobulin Rv	cgg atg gat gaa acc cag ac	
IGF-I Fw	atc agc agt ctt cca acc caa	NM_000618
IGF-I Rv	cag cgc cag gta gaa gag atg	
IGF-II Fw	agc ttg ttg aca cgc ttc agt	NM_000612
IGF-II Rv	tgc cct ctc tca act ctt tga	

Western immunoblotting techniques

$p70^{S6k}$ About 20 mg of frozen muscle were ground in a mortar and homogenized in ice-cold buffer [20 mM Tris, pH 7.0, 0.27 M 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 (DTT) and a protease inhibitor cocktail containing 1mM EDTA (Roche Applied Science, Mannheim, Germany)] (Sneddon *et al.*, 2001). The homogenate was immediately centrifuged at 10,000 × g for 10 min at 4 °C. The supernatants were stored in liquid nitrogen. Protein concentration was determined using the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Cell lysates (40 µg of protein) were combined with Laemmli sample buffer and separated by SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) using an 8% gel. After electrophoretic separation at 40 mA, the proteins were transferred at 80 V for 90 min to a PVDF membrane (Amersham Biosciences, Uppsala, Sweden) for Western analysis. All membranes were incubated for the same time, and in the same conditions. Membranes were incubated for 2 h in a 5% Blotto solution (5% powdered milk in Tris buffer saline with 0.01% Tween-20, TBST) containing 50 mM NaF to inhibit phosphatases. The blocking solution was then poured off. The phospho-specific $p70^{s6k}$ antibody (1:1,000) [(Thr389), Santa Cruz Biotechnology, Santa Cruz, CA] was added and incubated overnight at 4 °C in Blotto containing 50 mM NaF. Membranes were washed (3 × 10 min) in TBST and then incubated for 1 h at room temperature in secondary antibody conjugated to horseradish peroxidase (1:10,000) (Calbiochem, San Diego, CA) in a 5% Blotto solution. After an additional 3 washes in TBST, chemiluminescent detection was carried out using an ECL Western blotting kit (Amersham Biosciences). As a loading control, membranes were then stripped and reprobed with an anti-$p70^{s6k}$ (1:1000) (Santa Cruz Biotechnology) that recognizes both phosphorylated and non-phosphorylated forms. Total $p70^{s6k}$

was found to be unchanged by creatine and exercise. The films were scanned on an ImageScanner using the Labscan Software and quantified with the Image Master 1D Image Analysis Software (Amersham Biosciences).

4E-BP1 Muscle samples were treated as described by Xu et al. (1998). About 20 mg of muscle were homogenized in ice-cold buffer (50 mM sodium β-glycerophosphate, pH 7.3, 100 mM sodium fluoride, 5 mM EDTA, 2 mM EGTA, 0.1 mM sodium orthovanadate, 1 mM benzamidine, 10 µg/ml leupeptin, 0.2 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 10,000 × g for 20 min at 4° C. The supernatant was heated at 100 °C for 10 min and was then centrifuged at 10,000 × g for 30 min at 4 °C. The supernatants were stored in liquid nitrogen for further analysis. The proteins were combined with Laemmli sample buffer and separated on a 15% gel by SDS-PAGE. Proteins were transferred to a PVDF membrane. After incubation in a 5% Blotto solution, PHAS-I antibody (1:1,000) (Calbiochem) was added and incubated overnight at 4 °C. Membranes were then treated as described above.

Muscle creatine

A fraction of the initial muscle biopsy (~30 mg) was used for spectrophotometric determination of total creatine (i.e. creatine phosphate + free creatine). Ground muscle was extracted in 0.25 N $HClO_4$ and neutralized with 1 N KOH. To hydrolyze creatine phosphate, 2 N HCl was added to the supernatant which was then heated for 15 min at 60 °C. The reaction was stopped on ice and the supernatant neutralized with 2 N NaOH. Total creatine was immediately determined enzymatically using a spectrophotometric method described by Guder et al. (1986) (Creatinine PAP, Boerhinger Mannheim, Germany from which the creatininase was omitted).

Statistics

Results are expressed as means ± SEM. A repeated measures ANOVA design was used to assess the statistical significance of differences between mean values over time and between conditions. Dunnett's pairwise multiple comparison t test was used as post-hoc test to compare post-exercise with control resting conditions. The threshold of significance was set at 0.05.

Results

Muscle total creatine

Muscle total creatine concentration increased from 113 ± 7 μmol.g^{-1} dry wt to 141 ± 10 μmol.g^{-1} dry wt (P<0.05) as a result of the creatine supplementation.

IGF-I and IGF-II mRNA

Real time RT-PCR. Resting human skeletal muscle expressed IGF-I and IGF-II mRNA. Based on ΔCt values, IGF-I mRNA was less abundant, about one half, than IGF-II (data not shown).

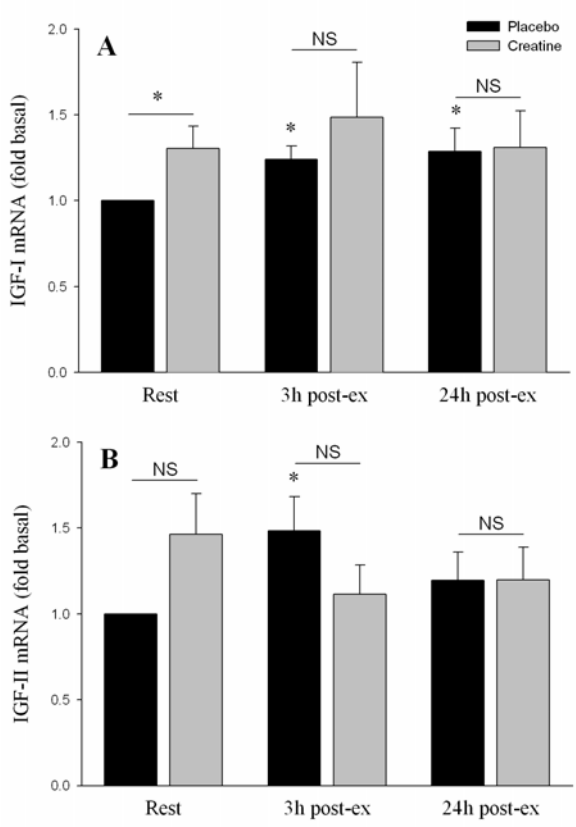

Fig. 3.1. *mRNA level of IGF-I (A) and IGF-II (B) in placebo and creatine conditions at rest and after exercise.* Symbols above histograms depict significant differences with rest under the same treatment. *P<0.05.

IGF-I. In the placebo condition, heavy resistance exercise increased IGF-I mRNA by 3 h post-exercise (24%, P<0.05), which was maintained 24 h post-exercise (29%, P<0.05, Fig. 3.1A). After 5 days of creatine

supplementation, IGF-I mRNA expression at rest was increased by 30% (P<0.05), but exercise had no further effect on this. The mRNA expression measured after exercise was similar for the creatine supplemented and the placebo conditions.

IGF-II. Under placebo, IGF-II mRNA level was increased by 48% 3 h post-exercise (P<0.05), and returned to values similar to rest 24 h post-exercise (Fig. 3.1B). Creatine supplementation up-regulated the expression of IGF-II mRNA at rest (46%), although the statistical threshold was not reached (P=0.054) due to the variability induced by one of the six subjects. In creatine-supplemented subjects, IGF-II mRNA expression dropped 20% 3h and 24h after exercise but these values were not statistically different from rest condition.

Phosphorylation state of $p70^{s6k}$ and 4E-BP1

$p70^{s6k}$. Three hours post-exercise plus feeding, the phosphorylation state of $p70^{s6k}$ increased by about 400% both in the placebo (P<0.001) and creatine conditions (P<0.05, Fig. 3.2A). The phosphorylation state of $p70^{s6k}$ returned to basal values 24 h post-exercise. Creatine had no additional effect on the phosphorylation state of $p70^{s6k}$.

4E-BP1. In the placebo condition, the fraction of 4E-BP1 in the gamma form was approximately doubled 3 h post-exercise (P<0.05, Fig. 3.2B). It also increased 1.5 fold in the creatine condition, but the significance threshold was not reached (P=0.06). Twenty-four hours post-exercise, the phosphorylation state of 4E-BP1 displayed a slight decrease towards basal values. It was not affected by creatine at rest and at 3 h post-exercise, but remained more elevated, and thus different from placebo, at 24 h post-exercise (P<0.05).

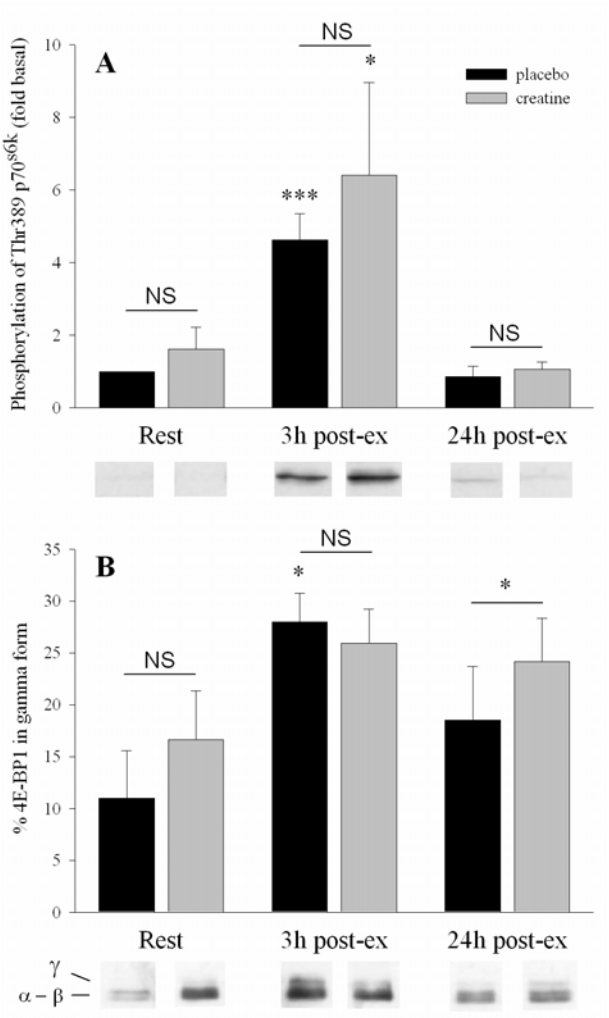

Fig. 3.2. *Changes in the phosphorylation state of $p70^{s6k}$ (A) and 4E-BP1 (B) in placebo and creatine conditions at rest and after exercise.* Symbols above histograms depict significant differences with rest under the same treatment. **P*<0.05., ****P*<0.001.

Discussion

Recently, we observed that creatine added to the culture medium of myogenic cells (C_2C_{12}) improved cell differentiation and increased expression of the IGF-I mRNA (Louis *et al.*, 2004). In the present study, we hypothesized that expression of IGF would also be increased in human skeletal muscle after 5 days of creatine supplementation. Indeed, the amount of IGF-I was higher in resting muscle after creatine intake and IGF-II mRNA showed an increasing trend (Fig. 3.1). 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 (see below) (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 (Fig. 3.1A and B). This indicates that both exercise and creatine increase the expression of IGF, but that these effects are not cumulative.

Even though the design of the present study does not enable us to distinguish the confounding effects of exercise and feeding at 3 h post-exercise, a 20% increase in IGF mRNA was observed 3 h and 24 h after a session of resistance exercise. Bamman et al. already reported a 62% increase in IGF-I mRNA after a bout of eccentric exercises at 110% of the 1-RM and a trend to up-regulation after concentric exercises at 85% of the 1-RM (Bamman *et al.*, 2001). The results of the present study (+24%) compare well with the data reported by those authors since the leg press exercise was performed against a load equal to 70% of the 1-RM. On the other hand, the effect of creatine in the resting condition reached similar range of increase (+30%). The up-regulation of IGF-I mRNA could be a mechanism by which exercise and creatine exert their effect on muscle hypertrophy.

Whereas evidence linking muscle activity and local IGF-I and IGF-II availability is growing, the mechanism by which muscle overload modulates

IGF-I and IGF-II expression is unclear. In mammals, the IGF-I gene consists of six exons with several regulating promoters (Hall *et al.*, 1992). In skeletal muscle, IGF-I transcription appears to be mostly regulated by the exon 1 promoter, which is activated by MyoD and the calcium signaling molecule calcineurin (McCall *et al.*, 2003).

Once bound to their common receptor, IGFs can stimulate several signaling pathways, amongst others the PI3K-Akt/PKB-mTOR pathway (Coolican *et al.*, 1997) which plays an important role in the regulation of muscle hypertrophy. The PI3K-Akt/PKB-mTOR pathway and its downstream targets $p70^{s6k}$ and 4E-BP1 can also respond to other signals such as exercise and feeding. In rats, the activation of this pathway following exercise is rapid and large, more than double in a few minutes (Bolster *et al.*, 2003). Thus, in contrast to the delayed-response regulation by IGFs, exercise and feeding induce an early response at the level of $p70^{s6k}$ and 4E-BP1, comparable to an on/off switch (Rennie & Wackerhage, 2003), as confirmed by the present results. Phosphorylation of $p70^{s6k}$ was measured using an antibody recognizing phosphorylated Thr 389, which corresponds to the highest level of phosphorylation of $p70^{s6k}$ and its highest activity (Weng *et al.*, 1998). The phosphorylation state of $p70^{s6k}$ increased 4 fold 3 h post-exercise (Fig. 3.2A) in accordance with previous studies on rats (Baar & Esser, 1999). Six hours after high-resistance lengthening contractions the activity of $p70^{s6k}$ in rats was tripled and the phosphorylation of $p70^{s6k}$ correlated with increased skeletal muscle mass after 6 weeks of training (Baar & Esser, 1999). Creatine ingestion did not potentiate the phosphorylation of $p70^{s6k}$ in the present study.

The effect of exercise on 4E-BP1 has been less extensively investigated. In our study, exercise doubled the gamma form of 4E-BP1 which is the most phosphorylated form of the protein (Mothe-Satney *et al.*, 2000) (Fig. 3.2B). No supplementary effect of creatine was found 3 h post-exercise. However,

the gamma form of 4E-BP1 remained more elevated than in the placebo condition 24 h post-exercise. The inhibition of 4E-BP1 by phosphorylation results in its release from eIF4E and allows the latter to participate in the initiation of protein synthesis by enabling the formation of the eIF4F complex.

The dissimilar effects of creatine on 4E-BP1 and $p70^{s6k}$ are surprising since both proteins are downstream targets of the same pathway. Nevertheless, other involved factors, like the protein phosphatase 2A and/or a crosstalk between the MEK/MAPK pathway and $p70^{s6k}$, can not be excluded and may explain the difference. Creatine could have a more direct effect on 4E-BP1 and maybe not on $p70^{s6k}$. Clearly, further research is warranted to clarify these issues.

In summary, creatine supplementation had no potentiating effect on the activation of $p70^{s6k}$ and 4E-BP1 three hours after acute exercise. As already pointed out, the increase in the phosphorylation state of $p70^{s6k}$ and 4E-BP1 after 3 h was not due to exercise alone, but was induced by both acute exercise (Bolster *et al.*, 2003) and feeding (Liu *et al.*, 2001). These observations are in accordance with our previous finding that in comparable conditions, creatine did not enhance protein synthesis within a short timeframe (Louis *et al.*, 2003a; Louis *et al.*, 2003b). Creatine increased the expression of IGF-I only at rest with no additional effect of exercise. The phosphorylation state of 4E-BP1 was also found to be more elevated 24 h post-exercise. Our results indicate 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.

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Chapter 4

Creatine enhances differentiation of myogenic C_2C_{12} cells by activating both p38 and Akt/PKB pathways

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Abstract

In myogenic C_2C_{12} cells, 5mM creatine increased the incorporation of labelled [^{35}S] methionine into sarcoplasmic (+20%, $P<0.05$) and myofibrillar (+50%, $P<0.01$) proteins. Creatine also promoted the fusion of myoblasts assessed by an increased number of nuclei incorporated within myotubes (+40%, $P<0.001$). Expression of myosin heavy chain type II (MHC II, +1300%, $P<0.001$), troponin T (+65%, $P<0.01$) and titin (+40%, $P<0.05$) was enhanced by creatine. Neither mannitol, taurine, nor beta-alanine mimicked the effect of creatine, ruling out an osmolarity-dependent mechanism. The addition of rapamycin, the inhibitor of mammalian target of rapamycin/70kDa ribosomal S6 protein kinase (mTOR/p70^{s6k}) pathway, and SB202190, the inhibitor of p38, completely blocked differentiation in control cells, and creatine did not reverse this inhibition, suggesting that the mTOR/p70^{s6k} and the p38 pathways could be potentially involved in the effect induced by creatine on the differentiation. Creatine upregulated phosphorylation of protein kinase B (Akt/PKB, +60%, $P<0.001$), glycogen synthase kinase-3 (GSK-3, +70%, $P<0.001$) and p70^{s6k} (+50%, $P<0.001$). Creatine also affected the phosphorylation state of p38 (-50% at 24h and +70% at 96h, $P<0.05$) as well as the nuclear content of its downstream targets myocyte enhancer factor-2 (MEF-2, -55% at 48h and +170% at 96h, $P<0.05$) and MyoD (+60%, $P<0.01$). In conclusion, this study points out the involvement of the p38 and the Akt/PKB-p70^{s6k} pathways in the enhanced differentiation induced by creatine in C_2C_{12} cells.

Keywords

Protein synthesis, IGF, MAPK, ERK1/2, p70^{s6k}

Introduction

Over the last decade, the interest for oral creatine supplementation has grown. Creatine has been used in different contexts, not only among athletes as an ergogenic aid for improving muscle power output during high-intensity exercise (Greenhaff *et al.*, 1993; Balsom *et al.*, 1995), but also as a potential therapeutic agent for patients suffering from muscle wasting and myopathies (Tarnopolsky & Martin, 1999; Vorgerd *et al.*, 2000; Walter *et al.*, 2002; Louis *et al.*, 2003). Creatine supplementation leads to an increase in lean body mass (Hultman *et al.*, 1996) and in muscle cross section area when the supplementation is associated with resistance exercise training (Volek *et al.*, 1999; Tarnopolsky *et al.*, 2001) or in the case of rehabilitation after muscle atrophy (Hespel *et al.*, 2001). Evidence accumulated over the past 10 years suggests that creatine exerts an anabolic effect by enhancing the regeneration process subsequent to a degeneration phase induced by exercise, disease or immobilisation.

Recently, creatine supplementation has been shown to amplify the increase in satellite cell number and myonuclei concentration in human skeletal muscle fibres during 4 to 16 weeks of resistance training. These creatine-induced modifications were associated with an enhanced muscle fibre growth in response to strength training (Olsen *et al.*, 2006). Nevertheless, the precise mechanism by which creatine affects growth and differentiation of myogenic cells still remains unknown.

Creatine improves growth and differentiation of various other cell types in culture. It stimulates metabolic activity, differentiation and mineralization of osteoblast-like cells (Gerber *et al.*, 2005). In cultured striatal tissue, creatine increases the density of GABA-ergic neurons without affecting total cell number (Andres *et al.*, 2005). In 1972, Ingwall et al. had already reported

that creatine increases the expression of myosin heavy chain (MHC) and stimulates muscle-specific protein synthesis in both skeletal and cardiac chicken myotubes in culture (Ingwall *et al.*, 1972). Previous work of our group on C_2C_{12} myogenic cells has shown that creatine stimulates growth and protein accretion, associated with an increase in insulin-like growth factor I (IGF-I) mRNA and a coordinated up-regulation of myogenic regulatory factors mRNA (Louis *et al.*, 2004).

While some targets have been identified, it is not clear whether IGF or other signalling pathways are involved in creatine action. Therefore, the purpose of the present study was to identify the signalling of creatine by using a myogenic cellular model. Since the p38 and the ERK1/2 mitogen activated-protein kinase (MAPK) as well as the phosphatidylinositol 3-kinase (PI3K) pathways are known to be key signalling cascades in the differentiation process of myoblasts (Gredinger *et al.*, 1998; Zetser *et al.*, 1999; Li *et al.*, 2000; Li & Johnson, 2006), we tested their possible involvement in creatine action.

Material and Methods

Cell culture

C_2C_{12} murine skeletal muscle myoblasts (ATCC, Manassas, VA, USA) were seeded in 100mm-diameter culture dishes and were grown in Dulbeccos's Modified Eagle Medium (DMEM, Life technologies) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (5000U/5000µg/ml) and 100µM non-essential amino acids. For immunohistochemistry, coverslips were placed on the bottom of the plates at the beginning of the proliferation phase. When cells were 70% confluent, the proliferation medium was replaced by a differentiation medium containing 1% horse serum, 1% penicillin/streptomycin (5000U/5000µg/ml) and 100µM non-essential amino acids.

At the beginning of the differentiation phase, creatine (Flamma) was added to reach a final concentration of 5mM to half of the plates, the other plates serving as controls. This concentration of creatine was chosen because it had the largest effect on the growth of C_2C_{12} cells (Louis *et al.*, 2004). To test a potential activation of cell signalling by disturbance of extracellular or intracellular osmolarity, we used 5mM mannitol, taurine or beta-alanine (Sigma). Rapamycin (100nM, inhibitor of mammalian target of rapamycin, mTOR; Alexis Biochemicals), SB202190 (10µM, inhibitor of p38; Sigma) or picropodophyllin (1-10nM, inhibitor of IGF-I receptor, Calbiochem) were added to the culture medium at the beginning of the differentiation phase in control and creatine conditions.

Protein extraction

Sarcoplasmic proteins. Cells were rinsed with PBS (phosphate buffer saline) and harvested in a lysis buffer containing 20mM Tris, pH 7.0, 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 (DTT) and a protease inhibitor cocktail (Roche Applied Science). The lysate was immediately centrifuged at 10 000g for 10min at 4°C. The supernatants were stored at -80°C and protein concentration was determined using the DC protein assay kit (Bio-Rad Laboratories) with bovine serum albumin (BSA) as a standard.

Nuclear proteins. Plates were rinsed with PBS and washed with cold hypotonic buffer (20mM Hepes, 5mM sodium fluoride, 1mM sodium molybdate, 0.1mM EDTA). Cells were then scraped in a lysis buffer (20mM Hepes, 0.5% NP-40, 5mM sodium fluoride, 1mM sodium molybdate, 0.1mM EDTA) and immediately centrifuged for 30s at 10 000g. The pellet was re-suspended in 100µl of a buffer containing 20mM Hepes, 5mM sodium fluoride, 1mM sodium molybdate, 0.1mM EDTA, 20% glycerol and 150µl of a saline buffer (20mM Hepes, 5mM sodium fluoride, 1mM sodium molybdate, 0.1mM EDTA, 20% glycerol, 0.8M NaCl) was added. The lysate was mixed for 30min at 4°C and centrifuged for 10min at 10 000g. The supernatant was stored at -80°C and protein concentration was determined as described above.

Myofibrillar proteins. Myofibrillar proteins were extracted according to the method described by Artaza (2002). Briefly, plates were rinsed with PBS and scraped in a homogenisation buffer (250mM sucrose, 100mM potassium chloride, 5mM EDTA, 20mM Tris, pH 6.8). Cells were then centrifuged at 10 000g for 10min at 4°C. The pellet was washed (175mM potassium chloride, 2mM EDTA, 0.5% Triton, 20mM Tris, pH 6.8), centrifuged and re-suspended in a buffer containing 150mM potassium chloride and 20mM Tris (pH 7.0). Protein content was measured as described above.

Fusion index

The fusion index corresponds to the proportion of nuclei present within myotubes that contain 3 or more nuclei (Jacquemin *et al.*, 2004). To determine clearly the boundaries of each myotube, the cells were incubated with a specific antibody directed against the membrane protein desmin (see immunohistochemistry section), while the nuclei were stained with 2-(4-amidinophenyl)-6-indolecarbamidine 300nM (DAPI, Sigma) for 5min (Fig. 4.1A and B). The fusion index was determined after 4 days of differentiation by counting at least 1000 nuclei per culture in 3 independent cultures.

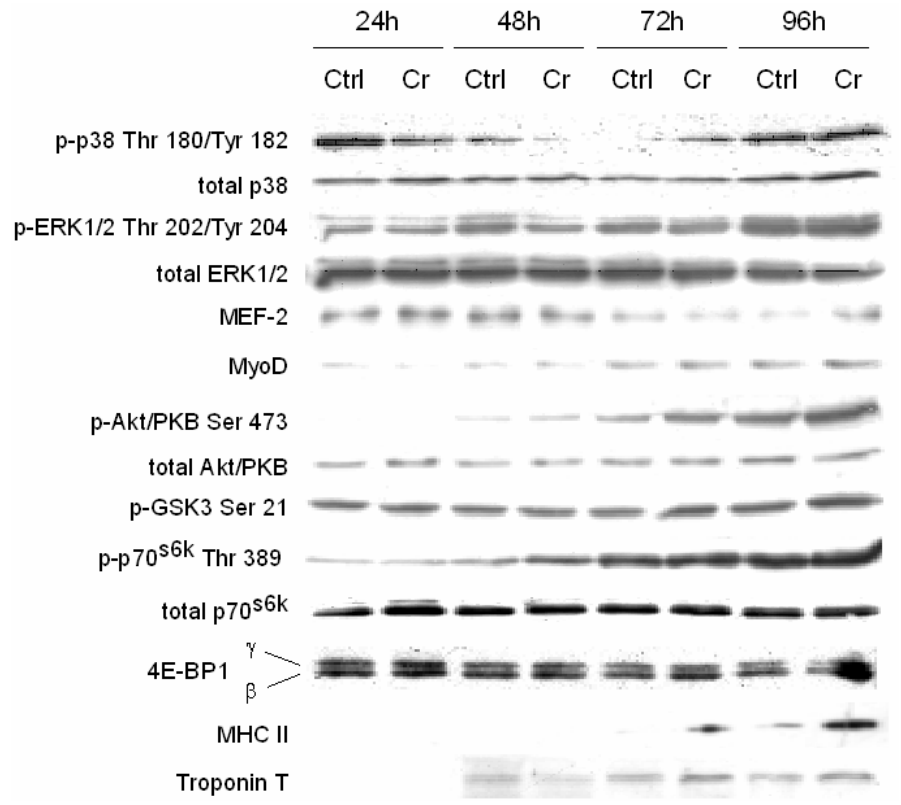
Incorporation of labelled [^{35}S] methionine

Cells were cultured in control and creatine conditions for 4 days of differentiation and then incubated for 24h in the presence of 1mM [^{35}S] methionine (100µCi/ml). Sarcoplasmic and myofibrillar proteins were extracted and the incorporation of labelled methionine was measured separately. Proteins were precipitated with 10% trichloroacetic acid (TCA), subsequently dissolved in 0.1N NaOH and precipitated again. Proteins were washed twice with 5% trichloroacetic acid. The final pellet was re-suspended in 1ml of formic acid. Five ml of scintillating liquid (Ultimagold, Perkin Elmer) was then added and [^{35}S] incorporation was counted in a scintillation counter (Beckman).

SDS/PAGE and immunoblotting

Cell lysates were combined with Laemmli sample buffer and separated by SDS/PAGE (8%-15%). After electrophoretic separation, the proteins were transferred to a PVDF membrane for a western blot analysis except for titin, the expression of which was assessed by a 3% SDS/PAGE strengthened with agarose (Tatsumi & Hattori, 1995). Membranes were incubated in a 5% Blotto solution. After the blocking step, they were incubated with the following antibodies overnight at 4°C (Supplementary fig. 4.1): anti-

phospho Akt/PKB Ser 473 (1:2000, Upstate Biotechnology), total Akt/PKB (1:1000, Upstate Biotechnology), anti-phospho $p70^{s6k}$ Thr 389 (1:1000, Santa Cruz), total $p70^{s6k}$ (1:1000, Santa Cruz), 4E-BP1/PHAS-I recognizing all phosphorylated forms between 19 and 25kDa (1:1000, Calbiochem), anti-phospho p38 Thr 180/Tyr 182 (1:1000, Cell Signaling), total p38 (1:1000, Cell Signaling), anti-phospho ERK1/2 Thr 202/Tyr 204 (1:1000, Cell Signaling), total ERK1/2 (1:1000, Cell Signaling), anti-phospho GSK-3 Ser 21 (1:1000, Upstate Biotechnology), MyoD (1:500, Santa Cruz), MEF-2 (1:1000, Santa Cruz), MHC II (1:100, Alexis Biochemicals), Troponin T fast isoform (1:500, Santa Cruz).



Supplementary fig. 4.1. *Typical western blot bands.* All bands in one row have been obtained from one western blot. Ctrl, control; Cr, creatine; p38, protein 38; ERK1/2, extracellular signal regulated-kinase 1 and 2; MEF-2, myocyte enhancer factor 2; Akt/PKB, protein kinase B; $p70^{s6k}$, 70kDa ribosomal S6 kinase; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; GSK-3, glycogen synthase kinase-3; MHC II, myosin heavy chain type II.

Membranes were washed in TBST (Tris buffer saline, 0.1% Tween-20) and incubated for 1h at room temperature with a secondary antibody conjugated to horseradish peroxidase (1:10 000, Calbiochem). After an additional 3 washes, chemiluminescent detection was carried out using an ECL Western blotting kit (ECL Plus, Amersham Biosciences). Then, the steady expressions of the analyzed proteins through the differentiation were verified with a total antibody. The films were scanned with an ImageScanner using the Labscan software and quantified with the Image Master 1D Image Analysis Software (Amersham Biosciences). The results represent the phosphorylated form of the protein.

Akt/PKB activity

Akt/PKB activity was measured by the phosphorylation of a synthetic peptide after immunoprecipitation (100µg of protein extract) with a total Akt/PKB antibody recognizing the PH-domain (Upstate Cell Signaling) as previously described (Bertrand *et al.*, 1999). In short, 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). After 20min at 30°C, the reaction mixture was quickly centrifuged and 20µl samples were spotted on P81 phosphocellulose paper, followed by washes in cold 75mM phosphoric acid. [^{32}P] incorporation was counted in a scintillation counter (Beckman).

Immunohistochemistry

Cells were fixed on the coverslips with 4% paraformaldehyde for 20min, washed twice with PBS and permeabilised for 15min with 0.2% Triton. After 2 washes, the blocking step was performed with goat serum for 30min followed by a 1h incubation with an antibody against MHC II (1:10, Alexis

Biochemicals) or with an antibody against desmin (1:100, DAKO) diluted in PBS containing 0.5% BSA. Coverslips were washed 3 times and a FITC (fluorescein isothiocyanate) secondary anti-mouse antibody (1:100, Sigma) was applied for 1h. After several washes, the coverslips were mounted with Vectashield (Vector Laboratories) on glass slides. The slides were examined with an Axiovert 40 fluorescent microscope (Zeiss).

Adenosine triphosphate, phosphorylcreatine, free creatine and total creatine contents

Cells were rinsed with cold PBS and scraped with 3N perchloric acid (PCA) on ice. Cell lysates were centrifuged at 10 000g for 3min at 4°C and the supernatants were neutralized with 2N $KHCO_3$. Adenosine triphosphate (ATP), phosphorylcreatine and free creatine were analyzed enzymatically with standard fluorometric assays (Lowry & Passonneau, 1972). Muscle total creatine content was calculated as the sum of free creatine and phosphorylcreatine.

Statistical analysis

The difference between control and creatine conditions was tested for significance using either one-way or two-way analyses of variance (ANOVA). When appropriate, ANOVA for repeated measures were applied. When the significance threshold, set to $P<0.05$, was reached, Bonferroni post hoc tests were used. The results are presented as the means ± SEM.

Results

Myogenic effects of creatine

Phosphorylcreatine (PCr), free creatine (Cr) and total creatine (total Cr) contents were increased 3- to 6-fold both after 3 and 4 days of differentiation (P<0.001, Table 4.1), whereas ATP content was not modified in the presence of creatine (Table 4.1).

Table 4.1. *ATP, PCr, Cr and Total Cr content.*

	72h		96h	
	Control	**Creatine**	**Control**	**Creatine**
ATP	45 ± 3.2	37 ± 3.8	52 ± 6.8	47 ± 0.4
PCr	73 ± 8.9	287 ± 3.5***	80 ± 5.3	418 ± 1.8***
Cr	106 ± 12.0	424 ± 3.5***	63 ± 7.0	427 ± 22.6***
Total Cr	179 ± 20.5	712 ± 0.9***	143 ± 11.4	845 ± 21.2***

Adenosine triphosphate (ATP), phosphorylcreatine (PCr), free creatine (Cr) and total creatine (Total Cr) content in control and in creatine-treated cells after 72h and 96h of differentiation. Results are expressed as the means ± SEM (nmol/mg protein) (n=3). *** P<0.001 creatine vs control.

To quantify the effect of creatine on the differentiation of myogenic C_2C_{12} cells, we assessed the index of fusion by measuring the number of nuclei within myotubes. After 4 days of differentiation, creatine increased the number of nuclei within myotubes by about 40% (P<0.001, Fig. 4.1A-C). The increase in fusion of myoblasts was accompanied by an increase in the incorporation of labelled [^{35}S] methionine into both sarcoplasmic (P<0.05) and myofibrillar proteins (P<0.01) after 5 days of differentiation (Fig. 4.1D). After 4 days of differentiation, troponin T and titin expressions were increased in the plates treated with creatine (P<0.01 and P<0.05 respectively, Fig. 4.1E and F).

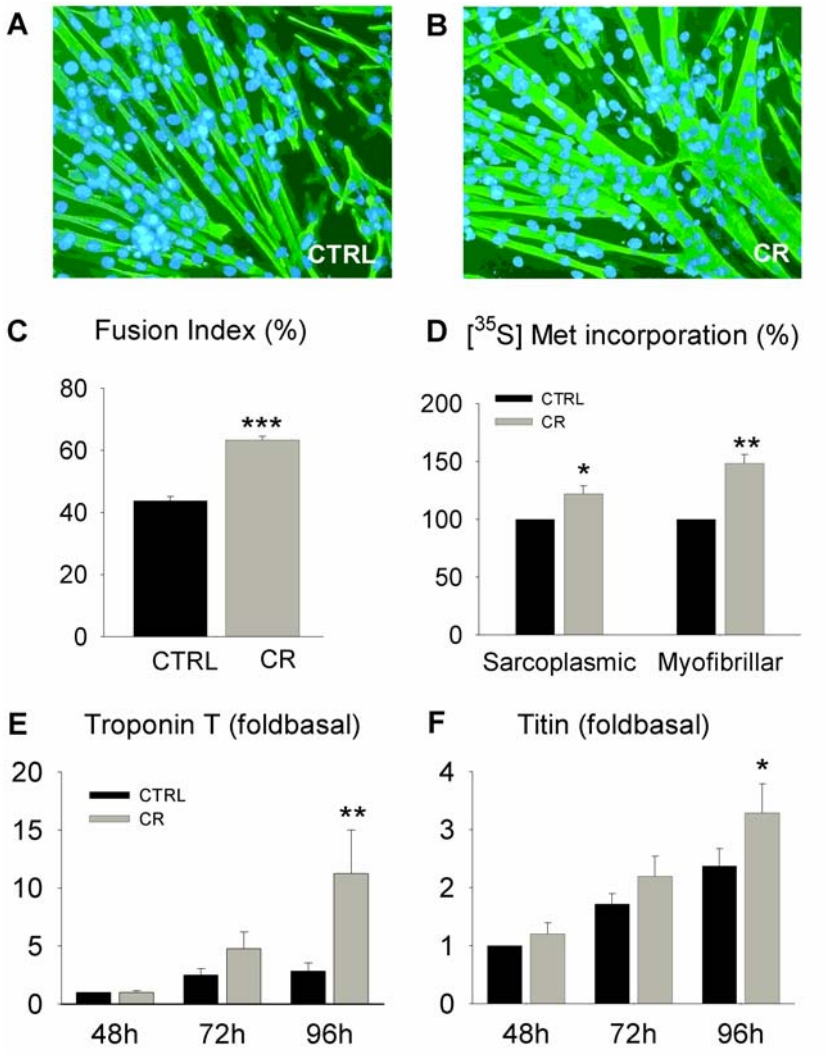


Fig. 4.1. *Effect of creatine on differentiation, incorporation of labelled methionine and expression of troponin T and titin in C_2C_{12} cells.* Control (A) and creatine-treated (B) myotubes after 4 days of differentiation. To determine the boundaries of each myotube, the cells were incubated with a specific antibody directed against the membrane protein desmin, while the nuclei were stained with DAPI. (C) Creatine induces an increase in the fusion index. The fusion index was defined as the percentage of nuclei located within myotubes (at least 3 nuclei) with respect to the total number of nuclei. (D) Effects of creatine on [^{35}S] methionine (Met) incorporation into C_2C_{12} myotubes for 24h. Results are expressed as the percentage of the control condition after 5 days of differentiation (n=3). (E-F) Creatine increases the expression of troponin T (E) and titin (F) (n=4). Results are expressed as the means ± SEM. *P<0.05, **P<0.01, ***P<0.001 creatine vs ctrl same time.

At the same time, the expression of MHC II in the myotubes was markedly enhanced (P<0.001, Fig. 4.2A-C). Since this increase has already been observed by Ingwall (Ingwall *et al.*, 1972), and since MHC is the most abundant and one of the most important myofibrillar proteins in the muscle, MHC II was used as a marker of the creatine effect in the experiments described below. On the other hand, creatine did not influence the activity of citrate synthase and lactate dehydrogenase (data not shown).

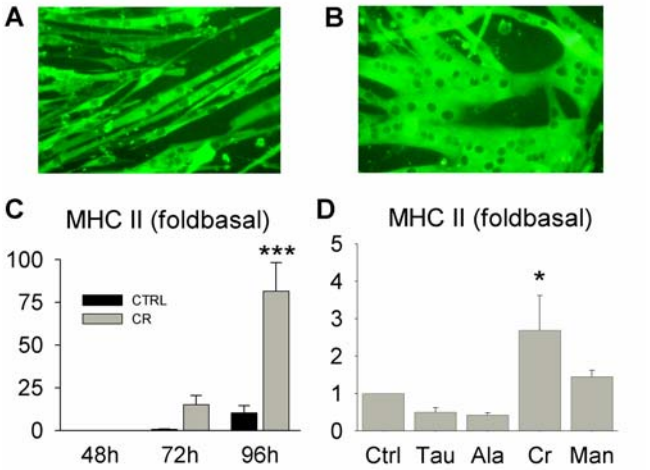


Fig. 4.2. *Effect of creatine and disturbance of osmolarity on the expression of MHC II.* Control (A) and creatine-treated (B) myotubes immunostained with an antibody against MHC II (myosin heavy chain type II) after 96h of differentiation. (C) Creatine increases the expression of MHC II after 96h of differentiation. (D) Effect of disturbance in intracellular (taurine 5mM or beta-alanine 5mM) or extracellular osmolarity (mannitol 5mM) on the expression of MHC II after 96h of differentiation. Results are expressed as the means ± SEM (n=5). *P<0.05, ***P<0.001 vs ctrl.

To test if the enhancement of differentiation by creatine was caused by a disturbed osmolarity, we added mannitol, taurine or beta-alanine to the medium. Increased extracellular osmolarity by 5mM mannitol, which is not taken up by the cell, did not change MHC II expression (Fig. 4.2D). Taurine or beta-alanine (5mM) increased their intracellular concentration by ~16-fold (not shown), but in contrast with creatine, MHC II expression tended to be decreased (Fig. 4.2D). These data demonstrate that changes in osmolarity were not able to mimic the action of creatine.

Signalling of the myogenic effects of creatine

To test the involvement of IGF-I in creatine action, we used picropodophyllin, a specific inhibitor of IGF-I receptor (Vasilcanu *et al.*, 2004). We had previously tested the effectiveness of this inhibitor in our experimental conditions and confirmed that it prevented the increase in p38 phosphorylation induced by IGF-I (data not shown). Incubation of the cells with picropodophyllin (1nM-10nM) at the beginning of the differentiation phase did not prevent the enhancement of MHC II induced by creatine (Fig. 4.3). This suggests that IGF-I is not initiating the signalling leading to the enhancement of MHC II expression.

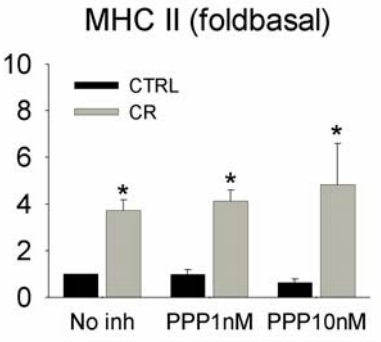


Fig. 4.3. *Effect of picropodophyllin on the expression of MHC II.* Effect of increasing doses of picropodophyllin (PPP, 1-10nM), a specific inhibitor of the IGF-I receptor, on the expression of MHC II (myosin heavy chain II) in control (Ctrl) and creatine (Cr) conditions after 96h of differentiation. No inh, no inhibitor. Results are expressed as the means ± SEM (n=3). **P*<0.05 creatine vs ctrl.

The time course of p38 phosphorylation was bi-phasic under control conditions (Fig. 4.4A): it decreased during the first 48h of differentiation and then it increased again after 72h. In the presence of creatine, the time-course was accelerated, with an earlier decrease and a larger re-increase ($P<0.05$). The potential implication of p38 in the differentiation process of myogenic cells was also suggested by the use of SB202190, an inhibitor of p38, which abolished the expression of MHC II in control conditions (Supplementary fig. 4.2B). Creatine did not reverse this effect suggesting that the p38 pathway is likely involved in the enhanced differentiation induced by creatine. In contrast to p38 which was affected throughout the differentiation by creatine, ERK1/2 phosphorylation only increased after 4 days of differentiation in creatine conditions (Fig. 4.4B, $P<0.01$).

The nuclear expression of myocyte enhancer factor-2 (MEF-2), a downstream target of p38, followed the pattern of p38 phosphorylation with a 24h delay and was affected by creatine ($P<0.05$, Fig. 4.4C). Another putative target of p38, the myogenic regulatory factor MyoD, was also modified by creatine. In control conditions, the expression of MyoD in the nucleus increased throughout the differentiation (Fig. 4.4D). At the end of the differentiation, this increase was larger in the myotubes treated with creatine ($P<0.01$).

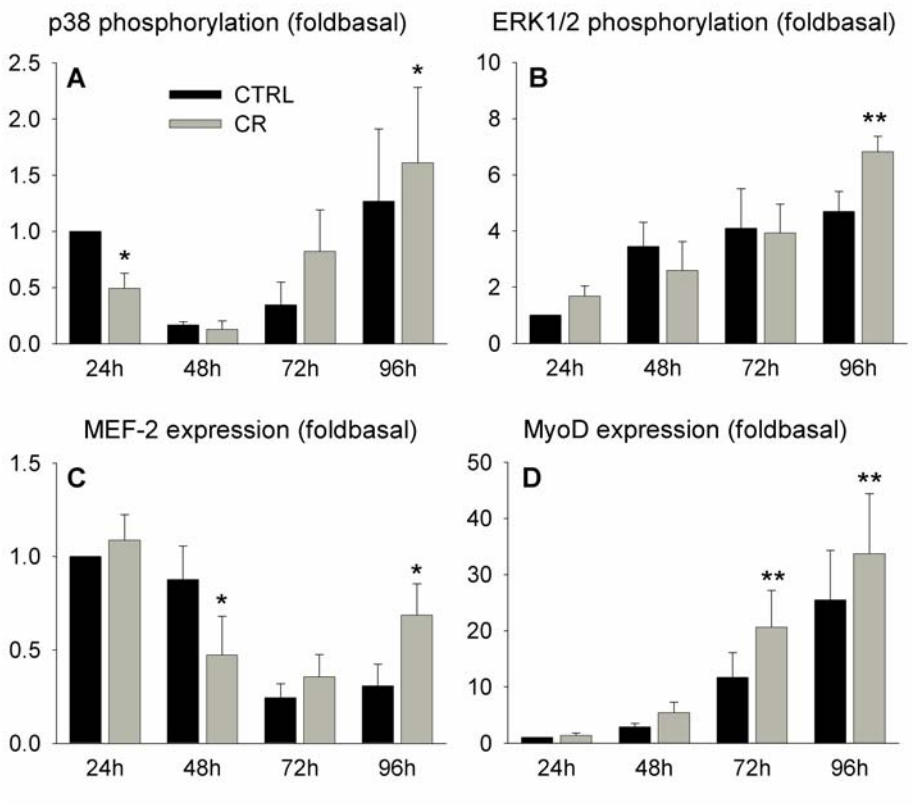


Fig. 4.4. *Time-course of p38 and ERK1/2 phosphorylation and time-course of MEF-2 and MyoD expressions in C_2C_{12} cells incubated with creatine.* (A) p38 phosphorylation on Thr 180/ Tyr182 (n=4) and (B) ERK1/2 phosphorylation on Thr 202/Tyr 204 (B, n=4) as well as (C) MEF-2 expression (n=3) and MyoD expression (D, n=6) in the nucleus during the differentiation phase. Results are expressed as the means ± SEM. *$P<0.05$, **$P<0.01$ creatine vs ctrl same time.

Incubation with creatine led to an increased activity (2.5-fold) of Akt/PKB after 72h of treatment ($P<0.01$, Fig. 4.5A) and to an increased Ser 473 phosphorylation at 72h ($P<0.01$) and 96h of differentiation ($P<0.001$) (Fig. 4.5B). Creatine also increased the phosphorylation state of glycogen synthase kinase-3 (GSK-3), a downstream effector of Akt/PKB, at 72h ($P<0.01$) and 96h ($P<0.001$, Fig. 4.5C). Downstream of the cascade are 70kDa ribosomal S6 kinase ($p70^{s6k}$) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). The percentage of 4E-BP1 in gamma form, which represents the most phosphorylated form of the protein, was not affected by creatine (Fig. 4.5D), but creatine increased the phosphorylation state of $p70^{s6k}$ on Thr 389 from the second day of differentiation until the end of the period studied ($P<0.05$ at 48h and 72h and $P<0.001$ at 96h, Fig. 4.5E). The phosphorylation state of $p70^{s6k}$ was only barely detectable after 24h of differentiation in two cultures out of four. Therefore the basal state has been referred to 48h rather than to 24h (Fig. 4.5E).

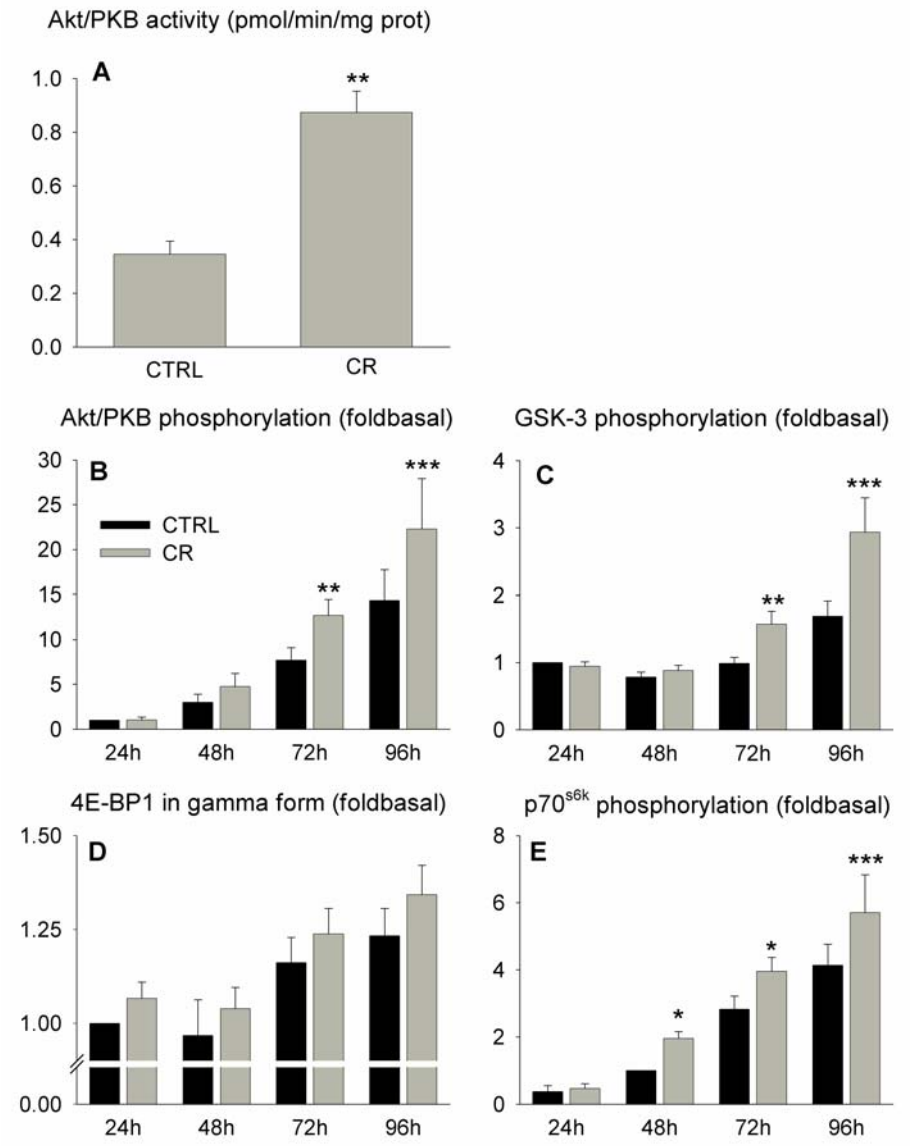
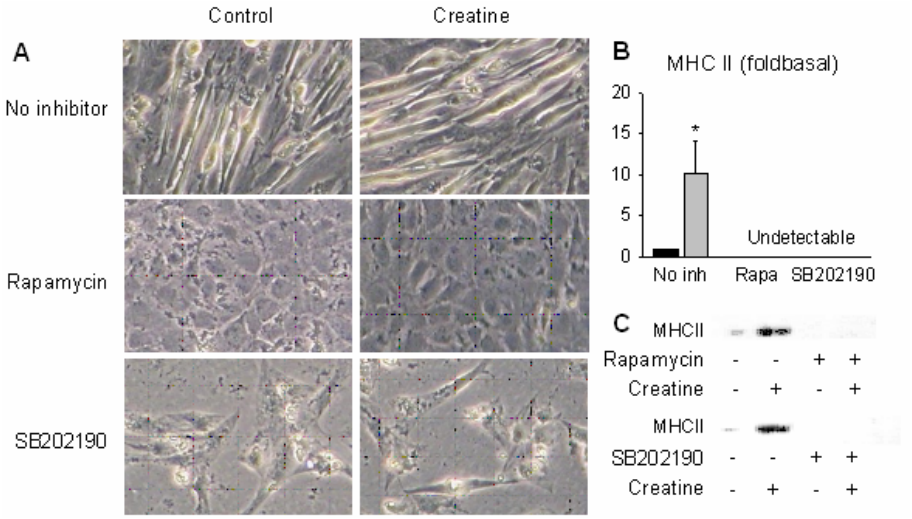


Fig. 4.5. *Time-course of Akt/PKB, GSK-3, 4E-BP1 and p70^{s6k} phosphorylation in C_2C_{12} cells incubated with creatine.* (A) Akt/PKB activity after 72h of differentiation (n=3). (B) Akt/PKB phosphorylation on Ser 473, (C) GSK-3 phosphorylation on Ser 21, (D) 4E-BP1 in gamma form and (E) p70^{s6k} phosphorylation on Thr 389 (n=4). Results are expressed as the means ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001 creatine vs ctrl same time.

As observed for the p38 pathway, the Akt/PKB-mTOR-p70^{s6k} cascade is required for the occurrence of the differentiation process. Indeed, the addition of rapamycin, an inhibitor of mTOR/p70^{s6k} pathway, completely repressed differentiation and the expression of MHC II in control conditions. Creatine did not reverse this effect indicating that the Akt/PKB-mTOR-p70^{s6k} pathway is also needed for the enhanced differentiation induced by creatine (Supplementary fig. 4.2B).



Supplementary fig. 4.2. *Effect of rapamycin and SB202190 on the expression of MHC II.* (A) Pictures of C_2C_{12} cells after 96h of differentiation in control and creatine conditions with or without 100nM rapamycin (inhibitor of mTOR) or 10µM SB202190 (inhibitor of p38). (B) Effect of 100nM rapamycin (Rapa) and 10µM SB202190 on the expression of MHC II in control and creatine conditions after 96h of differentiation. The level of MHC II was undetectable when cells were incubated with rapamycin or SB202190 both in control and creatine conditions. No inh, no inhibitor. Results are expressed as the means ± SEM (n=4). *P<0.05 vs ctrl same condition. (C) Typical western blot bands obtained after addition of rapamycin or SB202190 in control and creatine conditions for 96h of differentiation.

An interaction between p38 and Akt/PKB in myogenic cells has previously been described in the literature (Cabane *et al.*, 2004). To confirm that p38 and the Akt/PKB-mTOR-p70^{s6k} pathways could crosstalk in our model, we studied the effects of SB202190 (inhibitor of p38) on the phosphorylation state of Akt/PKB and p70^{s6k} (Fig. 4.6). SB202190 reduced Akt/PKB and p70^{s6k} phosphorylation in both control and creatine conditions.

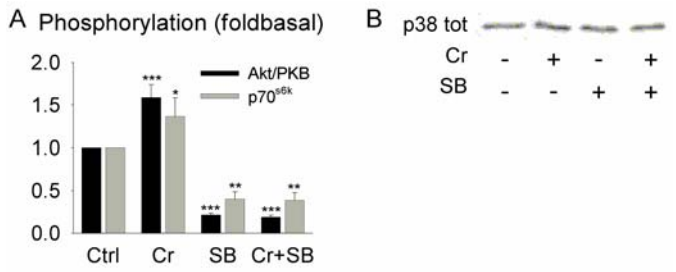


Fig. 4.6. *Crosstalk between p38 and Akt/PKB-p70^{s6k}*. (A) Phosphorylation state of Akt/PKB on Ser 473 and p70^{s6k} on Thr 389 after addition of 10µM SB202190 (SB, inhibitor of p38) in control (Ctrl) and creatine (Cr) conditions at the beginning of the differentiation phase for 48h. (B) The total form of p38 was not affected by the inhibitor SB202190. **P*<0.05, ***P*<0.01, ****P*<0.001 vs ctrl.

Discussion

The present study shows that, in the myogenic C_2C_{12} cell line, as in skeletal and cardiac chicken myotubes in culture (Ingwall *et al.*, 1972; Ingwall *et al.*, 1974; Ingwall, 1976; Louis *et al.*, 2004), creatine promotes differentiation. It also shows that creatine enhances protein synthesis (Fig. 4.1D) and fusion of myoblasts (Fig. 4.1C). The effect of creatine was rather specific since the expression of troponin T, titin and MHC II were clearly increased after 4 days of differentiation (Fig. 4.1E-F, Fig. 4.2C) whereas the activities of citrate synthase and lactate dehydrogenase were unchanged by creatine (data not shown).

The enhancement of differentiation by creatine was independent of a change in intracellular ATP content and could be mediated by an increase in free creatine as well as phosphorylcreatine levels (Table 4.1). The present data confirm previous data on endothelial (Nomura *et al.*, 2003), myogenic L6 (Ceddia & Sweeney, 2004) and C_2C_{12} cells (Alfieri *et al.*, 2006) that ATP levels are not affected by creatine whereas free creatine and phosphorylcreatine are increased. It seems that intracellular free creatine concentration rapidly (<24h) increases and reaches a maximal level after addition of creatine into the cell culture medium. This maximal content is, to a certain extent, independent of the dose of creatine added and the duration of incubation period. Indeed, the addition of 0.5mM creatine for 24h-48h (Ceddia & Sweeney, 2004), 5mM in the present study for 72h-96h or 20mM for 16h (Alfieri *et al.*, 2006) leads to an intracellular free creatine concentration of about 400nmol/mg protein. This is not the case for phosphorylcreatine which increases through the differentiation phase, from 175nmol/mg protein (Alfieri *et al.*, 2006) to 400nmol/mg protein (Table 4.1), implying an increase in the PCr/Cr ratio throughout the differentiation phase. This could be partially explained by muscle creatine kinase which

only reaches maximal expression and activity after 4-5 days of differentiation in C_2C_{12} myotubes (Ritchie *et al.*, 1991; Trask *et al.*, 1992) allowing the cell to accumulate more phosphorylcreatine throughout the differentiation phase.

Since the experiments with mannitol, taurine and beta-alanine ruled out an osmolarity-dependent action of creatine, we further focused our work on the signalling enhancing growth and differentiation. The p38 pathway is potentially one major signalling cascade activated by creatine since p38 is known to regulate the expression of muscle-specific genes and fusion of myoblasts into myotubes (Cuenda & Cohen, 1999). In the present study, creatine essentially affected the fusion of myoblasts (Fig. 4.1C) and the expression of muscle-specific proteins (Fig. 4.1E-F and Fig. 4.2C). Moreover, SB202190, the inhibitor of p38, abolished the expression of MHC II, and this repression was not influenced by the addition of creatine (Supplementary fig. 4.2B).

The phosphorylation state of p38 during the differentiation phase of C_2C_{12} cells is known to follow a U-curve (Li *et al.*, 2000) similar to the one presented in figure 4.3A in control conditions. The addition of creatine to the culture medium anticipated the changes in the phosphorylation state of p38. Creatine also affected the nuclear expression of MEF-2 and MyoD (Fig. 4.4C and D), two muscle-specific transcription factors, their expression and activation known to be potentially controlled by p38 (Gredinger *et al.*, 1998; Wu *et al.*, 2000). MyoD is a member of the myogenic bHLH proteins which forms heterodimers with a ubiquitous class of bHLH transcription factors called E proteins to bind a consensus DNA sequence referred as an E-box in the control regions of muscle-specific genes (Black & Olson, 1998). All myogenic regulatory factors (MyoD, Myf5, MRF4 and myogenin) bind to an E-box, and we have previously shown that creatine potentiated the

transcription of all the members of this family (Louis *et al.*, 2004). MEF-2 is an essential coactivator of MyoD to initiate and to control skeletal myogenesis. Through binding to MyoD, MEF-2 increases the myogenic activity of the latter (Black *et al.*, 1998). Nearly all skeletal muscle genes possess a MEF-2 binding site in their control regions (Black & Olson, 1998). These genes encode a wide variety of proteins including GLUT-4 (Liu *et al.*, 1994), troponin T (Wang *et al.*, 1994) and MyoD itself (Wong *et al.*, 1994), as well as other myogenic regulary factors (Black *et al.*, 1995).

The ERK pathway has been shown to be involved in the hypertrophy of terminally differentiated myotubes (Wu *et al.*, 2000) and creatine increased the phosphorylation state of ERK1/2 after 4 days of differentiation (Fig. 4.4B). Our results suggest that creatine accelerates the differentiation program via p38, and that ERK1/2 could potentially act at the very end of the differentiation to induce myotube hypertrophy.

Rapamycin completely inhibited cell differentiation, and creatine did not reverse this inhibition, indicating that mTOR activity is required for the effect of creatine. Therefore, involvement of the Akt/PKB-mTOR-$p70^{s6k}$ pathway in creatine signalling was tested. Akt/PKB is an upstream kinase of mTOR-$p70^{s6k}$ which plays a central role in the signal transduction pathways stimulated by growth factors and insulin. It mediates a wide range of cellular functions including nutrient metabolism (Cross *et al.*, 1995), cell growth and apoptosis (Bodine *et al.*, 2001; Lai *et al.*, 2004; Nader, 2005). The phosphorylation state of Akt/PKB increases throughout the differentiation and is potentiated by creatine (Fig. 4.5B). The first identified substrate for Akt/PKB is GSK-3. Creatine increases the phosphorylation state of GSK-3 at the end of the differentiation (Fig. 4.5C).

Akt/PKB is known to activate $p70^{s6k}$ and to inhibit 4E-BP1 through mTOR (Nave *et al.*, 1999). In the present study creatine potentiated the increase in Akt/PKB activity at the end of the differentiation phase (Fig. 4.5A and B) and a similar activation pattern was observed on $p70^{s6k}$ (Fig. 4.5E). A possible crosstalk with the p38 pathway could contribute to the higher phosphorylation state of Akt/PKB and $p70^{s6k}$ at the end of the differentiation. Indeed SB202190, the inhibitor of p38, diminished the phosphorylation state of Akt/PKB and $p70^{s6k}$ (Fig. 4.6), as already previously reported (Cuenda & Cohen, 1999). Contrary to Akt/PKB and $p70^{s6k}$, the phosphorylation state of 4E-BP1 remained unchanged by creatine (Fig. 4.5D). Recent work has revealed that 4E-BP1 and $p70^{s6k}$ are regulated by distinct signalling events downstream of mTOR (Wang *et al.*, 2005) which may be regulated differently by creatine.

The involvement of IGF-I in creatine signalling, reported in a previous study (Louis *et al.*, 2004), is probably minimal, because inhibition of IGF-I receptor by picropodophyllin had no effect (Fig. 4.3A), indicating that creatine recruits additional mediators to enhance differentiation and to activate the p38 and the Akt/PKB cascades. Since IGF-I transcription is regulated by MyoD (McCall *et al.*, 2003), the expression of which is enhanced by creatine (Fig. 4.4D), the increase in IGF-I expression is probably a consequence of a nuclear accumulation in MyoD. By its autocrine action, IGF-I would then amplify creatine signalling through the MAPK and the Akt/PKB pathway.

In summary, we identified two major signalling cascades by which creatine promotes differentiation of C_2C_{12} cells (Fig. 4.7). The first is the p38 MAPK pathway which would in turn activate MEF-2, MyoD and probably other unidentified transcription factors. Through this cascade, creatine would promote 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 MAPK which is known to promote myotube growth (Wu *et al.*, 2000). Through activation of the Akt/PKB pathway, creatine would increase protein synthesis. More specifically, the activation of $p70^{s6k}$ could prime the machinery required to accelerate the translation of specific mRNA increased by creatine.

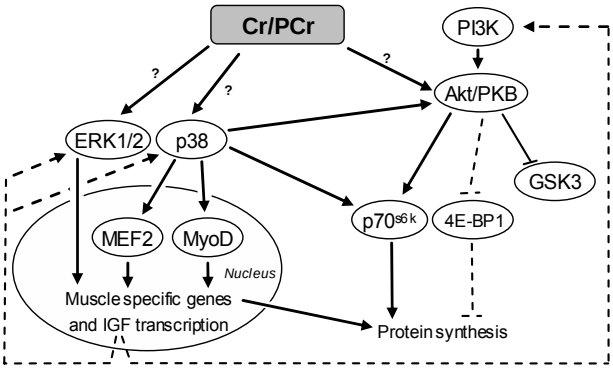


Fig. 4.7. *Hypothetical model for creatine signalling.* Cr/PCr, creatine/ phosphorylcreatine; p38, protein 38; ERK1/2, extracellular signal regulated-kinase 1 and 2; MEF2, myocyte enhancer factor 2; IGF, insulin-like growth factor; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; $p70^{s6k}$, 70kDa ribosomal S6 kinase; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; GSK3, glycogen synthase kinase-3. Bold lines, effect of creatine; dashed lines, no effect of creatine or untested.

Creatine has often been studied for its beneficial effect on human muscle performance or as a treatment for patients, but only a few studies have tried to understand the mechanisms by which creatine leads to muscle hypertrophy or stimulates muscle regeneration. Putting in perspective the results of Olsen et al. (Olsen *et al.*, 2006) and the present findings, future research should be directed towards the highlighting of the mechanisms by which creatine stimulates satellite cells in vivo and the possible involvement of the p38 and Akt/PKB pathways.

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Chapter 5

Effects of resistance exercise with and without creatine supplementation on gene expression and cell signalling in human skeletal muscle

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Abstract

To test the hypothesis that creatine supplementation would enhance the anabolic responses of muscle cell signalling and gene expression to exercise, we studied nine subjects who received either creatine or a placebo (maltodextrin) for 5d in a double-blind fashion before undergoing muscle biopsies: at rest; immediately after exercise (10×10 repetitions of one leg-extension at 80% 1-RM); and 24h and 72h later (all in the morning after fasting overnight). Creatine supplementation decreased the phosphorylation state of protein kinase B (PKB) on Thr308 at rest by 60% (P<0.05) and that of eukaryotic initiation factor 4E-binding protein on Thr37/46 (4E-BP1) by 30% 24h post-exercise (P<0.05). Creatine increased mRNA for collagen 1(α1), glucose transporter-4 (GLUT-4) and myosin heavy chain I at rest by 250%, 45% and 80% respectively, and myosin heavy chain IIA (MHCIIA) mRNA immediately after exercise by 70% (all P<0.05). Immediately after exercise, and independent of creatine, mRNA for muscle atrophy F-box (MAFbx), MHCIIA, peroxisome proliferator-activated receptor γ coactivator-1α and interleukin-6 were up-regulated (by 60 to 350%, P<0.05); the phosphorylation state of p38 both in the sarcoplasm and nucleus were increased (12 and 25 fold respectively, both P<0.05). Concurrently, the phosphorylation states of PKB (Thr308) and 4E-BP1 (Thr37/46) were decreased by 50% and 75% respectively (P<0.05). Twenty-four hours post-exercise, MAFbx, myostatin and GLUT-4 mRNA expression decreased below pre-exercise values (-35 to -50 %, P<0.05); calpain 1 mRNA increased 70% 72h post-exercise (P<0.05) and at no other time. In conclusion, 5d of creatine supplementation does not enhance anabolic signalling but does increase the expression of certain targeted genes.

Keywords

MAPK, PKB, protein synthesis

Introduction

Creatine supplementation has been used in various contexts, not only among athletes as an ergogenic aid for improving muscle power output during high-intensity exercise (Greenhaff *et al.*, 1993), but also as a potential therapeutic agent for patients suffering from general muscle wasting and myopathies (Tarnopolsky & Martin, 1999; Vorgerd *et al.*, 2000; Louis *et al.*, 2003a). Creatine supplementation leads to an increase in muscle fiber area when associated with resistance exercise training (Volek *et al.*, 1999) or in the case of muscle regeneration after atrophy (Hespel *et al.*, 2001). More recently, creatine supplementation has been shown to amplify the increase in satellite cell number and myonuclei concentration in human skeletal muscle fibers during 4 to 16 weeks of resistance training. These creatine-induced adaptations were associated with an enhanced muscle fiber growth in response to strength training (Olsen *et al.*, 2006).

Evidence accumulated over the past 15 years suggests that the primary mechanism by which creatine exerts its anabolic effect in healthy subjects who are weight training is by allowing them to work at a higher proportion of their maximal voluntary contraction force and thus increase the training stimulus (Greenhaff *et al.*, 1993). However this mechanism may not explain the higher force production observed in patients suffering from myopathy (Tarnopolsky & Martin, 1999; Louis *et al.*, 2003a). Therefore the search for alternative mechanisms for the beneficial effects of creatine has been under scrutiny. Several studies suggest that creatine could be more effective in muscle submitted to degeneration/regeneration process induced by exercise (Olsen *et al.*, 2006), immobilization (Hespel *et al.*, 2001) or disease (Tarnopolsky & Martin, 1999; Louis *et al.*, 2003a). Therefore we also hypothesised that creatine would slow down the degeneration phase and accelerate the regeneration phase after high-intensity exercise, which usually occur 1 and 3 days post-exercise respectively (Faulkner *et al.*, 1993).

In two previous studies, we were unable to detect any difference in the myofibrillar or sarcoplasmic protein synthetic rates or the breakdown rate of human muscle after creatine supplementation, whether at rest (Louis *et al.*, 2003c) or post-exercise (Louis *et al.*, 2003b). This lack of effect of creatine after exercise might have been explained by the timing of the measurements made. For example, we measured the protein turnover rates immediately after exercise following 5 days of supplementation, and it might have been that the creatine-plus-exercise effects were present at a later time point. Indeed, exercise-induced increases in protein synthesis can last for up to 72 h (Miller *et al.*, 2005).

Furthermore, there may be intricate changes in translational signalling attributable to creatine, which are undetectable by direct measures of muscle protein synthesis. Indeed, many recent studies in human muscle have implicated changes in such candidate pathways with resistance exercise, including the PKB (protein kinase B)-mTOR and the MAPK (mitogen-activated protein kinase) pathways, key cascades in the regulation of skeletal muscle protein synthesis and remodelling by resistance exercise (Williamson *et al.*, 2003; Karlsson *et al.*, 2004; Cuthbertson *et al.*, 2006; Dreyer *et al.*, 2006; Eliasson *et al.*, 2006).

Furthermore, in the past studies we did not examine the possibility of a 'priming' of muscle gene expression that could be important for forthcoming synthetic responses to resistance exercise or indeed other adaptive processes. In support of the notion for the modulation of gene expression by creatine, there has been evidence of increased MHC (myosin heavy chain) and IGF-I and II (insulin-like growth factor I and II) in human subjects after creatine ingestion (Willoughby & Rosene, 2001; Deldicque *et al.*, 2005). Furthermore, such gene expression changes, either in the satellite cells or in the myofiber after each exercise session, could be one mechanism by which

the induction of satellite cell nuclei into the myofiber occurs after several weeks of resistance training, when coupled with creatine supplementation (Olsen *et al.*, 2006).

The aims of the present study were to test if 5 days of creatine supplementation induces short-term changes in gene expression and cellular signalling at rest and after a single bout of exercise. We hypothesised that short-term creatine supplementation would increase expression of genes associated with the control of muscle mass, phenotype and metabolism and augment anabolic signal transduction activity through the MAPK and the PKB pathways.

Material and Methods

Subjects

Nine healthy young men (21.7 ± 0.55 y, BMI 24 ± 0.9 kg/m^2) who did not partake in any formal resistance exercise regime, were recruited for this double-blind cross-over study. All subjects were given an oral and written account of the study before signing a consent form. This study was approved by the Ethic Committee of the Université catholique de Louvain and the investigation was performed according to the principles outlined in the Declaration of Helsinki.

Experimental protocol

Before the experiment, subjects participated in a pre-test to determine one repetition maximum (1-RM) for each leg on a leg extension apparatus. The exercise consisted of a one-leg knee extension movement from an angle of 90° to 160°. After a warm-up comprising 3 sets of 10 repetitions at 5 kg, the load was progressively increased until the subject could not perform more than one single repetition. Subjects were allowed 2 min rest between each set and reached 1-RM within 5-6 trials. Subjects were instructed to refrain from vigorous physical activity 2 days prior to and during the experimental phase. Food intake on the evening preceding each muscle biopsy was controlled by administering a standardised dinner (22 % protein, 48 % carbohydrate and 30 % fat). Subjects were randomly divided into two groups: one group (n=5) received 21 g (3×7 g) oral creatine monohydrate per day for 5 days before the beginning of the experiment and during the 3 days of the experiment while the second group (n=4) received a placebo (maltodextrin 3×7 g per day) during the same period (the protocol is summarized in Fig. 5.1). During the experimental days, the subjects were asked to ingest creatine during the breakfast after biopsy sampling. After a wash-out period of 6 weeks, the treatments were crossed-over for the second trial.

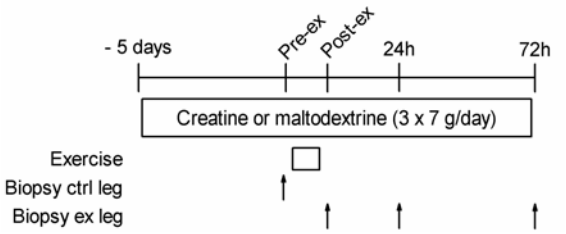

Fig. 5.1. *Experimental protocol.*

On the first morning of the study, participants reported to the laboratory after a 10 h overnight fast and a first biopsy was taken at rest in the control leg chosen at random. The procedure involved the administration of local anaesthesia (1 % lidocaine) and sample extraction from the mid portion of the vastus lateralis muscle with a 4-mm Bergström biopsy needle. Blood, macroscopically-visible fat and connective tissue were quickly removed, and the sample was immediately frozen in liquid nitrogen and stored at -80 °C. The exercise was then performed with the other leg after a warm-up of 3 sets of 10 repetitions at 5 kg. The main exercise session consisted of 10 sets of 10 repetitions at 80 % of the 1-RM of the exercising leg which corresponds to a mean value of positive work of 19471 ± 1403.5 J for the placebo trial and 19902 ± 1504.1 J for the creatine one. The positive work of each repetition was calculated by multiplying the moved mass by "g" (9.81 m.s-2) and by the distance (height to which the mass was raised). In this calculation, we neglected the friction due to the pulleys. The 1-RM was not exactly the same for both legs in each subject resulting in different mean work between placebo and creatine trials. These values were not statistically different. All subjects performed the same number of repetitions during both trials. A second biopsy was taken from the exercising leg within 30 s following the completion of the last repetition. A standardised breakfast was given after the exercise session (475kcal; 7 % protein, 74 % carbohydrate and 19 % fat). Each participant received a standardised dinner in the evening. Additional biopsies were taken 24 h and 72 h later from the exercising leg, each after a 10 h overnight fast.

Protein extraction and cell fractionation

About 20-30 mg of frozen muscle were ground in a mortar and homogenized in ice-cold hypotonic buffer (20 mM Hepes, 5 mM sodium fluoride, 1 mM sodium molybdate, 0.1 mM EDTA, 0.5 % NP-40, protease inhibitor cocktail (Roche Applied Science) for 5 min on ice. The homogenates were then centrifuged for 30 s at 10,000 g. The supernatant, containing the sarcoplasmic proteins, was stored at –80 °C. The pellet was re-suspended in a buffer containing 20 mM Hepes, 5 mM sodium fluoride, 1 mM sodium molybdate, 0.1 mM EDTA, 20 % glycerol, a protease inhibitor cocktail and the same volume of a saline buffer containing 20 mM Hepes, 5 mM sodium fluoride, 1 mM sodium molybdate, 0.1 mM EDTA, 20 % glycerol, 0.8 M NaCl and a protease inhibitor cocktail. The solution was then homogenized on a rotary mixer for 30 min at 4 °C and centrifuged for 10 min at 10,000 g. The supernatant, containing the nuclear proteins, was stored at -80 °C. Sarcoplasmic and nuclear protein concentrations were determined using a protein assay kit (Bio-Rad Laboratories) with BSA as a standard. Fraction purity was verified and confirmed by immunoblotting for nuclear histone 1 (anti-histone 1, 1:1000, Santa Cruz).

SDS/PAGE and immunoblotting

Cell lysates (70 µg for sarcoplasmic proteins and 30 µg for nuclear proteins) were combined with Laemmli sample buffer and separated by SDS/PAGE. After electrophoretic separation at 40 mA, the proteins were transferred to a PVDF membrane at 80 V for 4 h for a western blot analysis. Membranes were then incubated in a 5 % Blotto solution. Subsequently, membranes were incubated with the following antibodies (1:500) overnight at 4 °C: phospho-PKB Ser 473 (Cell Signaling), phospho-PKB Thr 308 (Cell Signaling), total PKB (Cell Signaling), phospho-$p70^{s6k}$ Thr 389 (Santa Cruz), total $p70^{s6k}$ (Santa Cruz), phospho-p38 Thr 180/Tyr 182 (Cell Signaling), total p38 (Cell Signaling), phospho-ERK1/2 Thr 202/Tyr 204

(Cell Signaling), total ERK (Cell Signaling), phospho-4E-BP1 Thr 37/46 (Cell Signaling), total 4E-BP1 (Cell Signaling) and MEF-2 (Santa Cruz). Antibodies from Cell Signaling were diluted in TBST containing 1 % BSA and antibodies from Santa Cruz were diluted in a 5 % Blotto solution. PKB phosphorylated on Thr 308 and Ser 473 was analyzed because the phosphorylation of both sites is required to achieve a high level of kinase activity (Alessi *et al.*, 1996). Membranes were washed in TBST and incubated for 1 h at room temperature in a secondary antibody conjugated to horseradish peroxidase (1:10,000, Cell Signaling). After an additional 3 washes, chemiluminescence detection was carried out using an Enhanced Chemiluminescent Western blotting kit (ECL Plus, Amersham Biosciences) and hyperfilms (Hyperfilm ECL, Amersham Biosciences). Then, the membranes were stripped and re-probed with a total antibody to verify the relative amount of the analyzed proteins through the whole experiment. The films were scanned with an ImageScanner using the Labscan software and quantified with the Image Master 1D Image Analysis Software (Amersham Biosciences). The results represent the phosphorylated form of the protein. A value of 1 was arbitrarily assigned to the control conditions (placebo pre-exercise) to which the post-exercise and the creatine values were reported. The choice of this baseline enables to depict the effects of creatine supplementation in addition to the effects of acute exercise.

RNA extraction and quantitative Real-Time PCR

Frozen tissue samples (~30 mg) were homogenized in TRIZOL® using a Polytron. Total RNA was extracted according to the instructions provided by the manufacturer. RNA was quantified by spectrophotometry (260 nm) and its concentration adjusted to 1 µg/µl using RNase-free water. A RNA agarose gel was run to verify the integrity of the RNA. Reverse transcription (RT) was performed using the iScript synthesis kit (Bio-Rad) on a iQ5 Real-Time PCR Detection System (Bio-Rad) with 1 µg of total RNA in a reaction

volume of 20 µl (4 µl iScript reaction mix 5X, 1 µl iScript reverse transcriptase, 1 µl RNA template, 14 µl RNase-free water). The final RT product was adjusted to 140 µl using RNase free water.

Real time RT-PCR (reverse transcription-polymerase chain reaction) primers were designed (Table 5.1) for human calpain 1, collagen 1(α1), C2 subunit of proteasome, GLUT-4 (glucose transporter 4), IL-6 (interleukin 6), MAFbx (muscle atrophy F-box), MHC I (myosin heavy chain type I), MHC IIA (myosin heavy chain type IIA), MyoD, myostatin, PCNA (proliferating cell nuclear antigen), PGC-1α (peroxisome proliferator-activated receptor gamma coactivator-1 α) and β-2-microglobulin. The latter was used as the "house keeping" gene, because preliminary experiments as well as a previous study (Murphy *et al.*, 2003) had revealed that it was not affected by either exercise or creatine supplementation.

Table 5.1. *Primer sequences.*

	Forward	**Reverse**
Calpain 1	GCC AAG CAG GTG AAC TAC	TGA AGT CTC GGA ATG ACA TC
Collagen 1(α1)	GTG CTA AAG GTG CCA ATG GT	CTC CTC GCT TTC CTT CCT CT
C2	CAT TGA AAA GGG CGC AAT C	GCC ATA TCG TTG TGT TGG TA
GLUT-4	CAG TAT GTT GCG GAG GCT AT	CCT CGA GTT TCA GGT ACT CTT
IL-6	TGG ATT CAA TGA GGA GAC TTG	GAT TCT TTG CCT TTT TCT GC
MAFbx	CGA CCT CAG CAG TTA CTG CAA C	TTT GCT ATC AGC TCC AAC AGC C
MHC I	ACA AGC TGC AGC TAA AGG TC	TCA AGA TGT GGC AAA GCT AC
MHC IIA	AGG CTT CAA GAT TTG GTA GA	TTC CTT TGC AAC AGG GTA GA
MyoD	CCG CCT GAG CAA AGT AAA TG	GCC CTC GAT ATA GCG GAT G
Myostatin	CTA CAA CGG AAA CAA TCA TTA CCA	GTT TCA GAG ATC GGA TTC CAG TAT
PCNA	AGG AGG AAG CTG TTA CCA TAG AG	AAG TGT CCC ATA TCC GCA AT
PGC-1α	GAT GAT GGA GAC AGC TAT GGT	GAG TCA TAC TTG CTC TTG GTG
β-2-microglobulin	ATG AGT ATG CCT GCC GTG TGA	GGC ATC TTC AAA CCT CCA TG

Sybr Green® real time RT-PCR analyses were carried out on the iQ5 Real-Time PCR Detection System (Bio-Rad) using the following cycle conditions: 10 min at 95 °C, followed by 40 cycles of 1 min at 60 °C and 15 s at 95 °C. For each gene, real time RT-PCR was conducted in duplicate with 25 µl reaction volume containing 12.5 µl Platinium Sybr Green qPCR SuperMix UDG (Invitrogen), 0.75 µl of each primer (10 pmol/µl), 9 µl RNAse-free water and 2 µl of 1:5 diluted cDNA. A melt analysis was run to verify the amplified DNA product. A value of 1 was arbitrarily assigned to the control conditions (placebo pre-exercise) to which the post-exercise and the creatine values were reported.

Muscle creatine concentration

Muscle creatine concentration was measured on the pre-exercise biopsy as previously described (Deldicque *et al.*, 2005). Briefly, a fraction of the muscle biopsy (~15 mg) was used for spectrophotometric determination of total creatine (i.e. creatine phosphate + free creatine). Ground muscle was extracted in 0.25 M HClO4 and neutralized with 1 M KOH. A fraction of the supernatant was used to determine free creatine, the remaining serving to determine total creatine content. To hydrolyze creatine phosphate, 2 M HCl was added to the supernatant which was then heated for 15 min at 60 °C. The reaction was stopped on ice and the supernatant neutralized with 2 M NaOH. Total creatine was immediately determined enzymatically using a spectrophotometric method described by Guder et al. (1986) (Creatinine PAP, Boerhinger Mannheim, Germany from which the creatininase was omitted). Phosphorylcreatine concentration was calculated by subtracting free creatine from total creatine concentrations.

Statistical analysis

The difference in muscle creatine content between placebo and creatine conditions was tested for significance using a paired t-test. Treatment by time interactions were evaluated using a two-way analysis of variance for repeated measures (ANOVA). When appropriate, Student-Newman-Keuls post hoc tests were applied. The significance threshold was set to $P < 0.05$. The results are presented as the means ± SEM.

Results

Muscle creatine concentration (Table 5.2)

After 5 days of supplementation muscle total creatine concentration increased by ~20 % (P<0.01).

Table 5.2. *Creatine, phosphorylcreatine and total creatine concentrations in muscle.*

	Placebo	Creatine
Cr	9.6 ± 0.64	12.6 ± 0.67*
PCr	16.3 ± 1.17	17.6 ± 0.63
Total Cr	25.8 ± 1.18	30.2 ± 0.71**

Free creatine (Cr), phosphorylcreatine (PCr), and total creatine (total Cr) levels after creatine supplementation for 5 days (3 × 7 g/day). Results are expressed as the means ± SEM (mmol/kg wet weight). *P<0.05, **P<0.01.

Transcriptional regulation by exercise and creatine (Figure 5.2 A-L)

Immediately after exercise, increases in PCNA mRNA (+150 %, P<0.05), MAFbx (+70 %, P<0.05), MHC IIA (+60 %, P<0.05), PGC-1α (+80 %, P<0.05) and IL-6 (+350 %, P<0.05) were observed. The expression of MHC IIA mRNA, PGC-1α and IL-6 all returned to pre-exercise values by 24 h or 72 h post-exercise (P<0.05). At 24h post-exercise, PCNA mRNA nearly returned to pre-exercise values before increasing again at 72h post-exercise (+130 %, P<0.05). MAFbx mRNA decreased below pre-exercise values at 24 h post-exercise (-50 %, P<0.05) but had returned to basal values two days later. As for MAFbx, mRNA for myostatin (-35 %, P<0.01) and GLUT-4 (-45 %, P<0.001) was also decreased at 24 h post-exercise, in comparison with pre-exercise values, but had returned to baseline by 72 h post-exercise. Calpain 1 mRNA was increased 72 h post-exercise in comparison with pre-exercise (+70 %), post-exercise (+20 %) and 24 h post-exercise (+40 %) (P<0.05).

Creatine increased the expression of collagen 1(α1) (+250 %, $P<0.05$), GLUT-4 (+45 %, $P<0.05$) and MHC I mRNA (+80 %, $P<0.05$) at rest and the expression of MHC IIA mRNA immediately after exercise (+70 %, $P<0.05$).

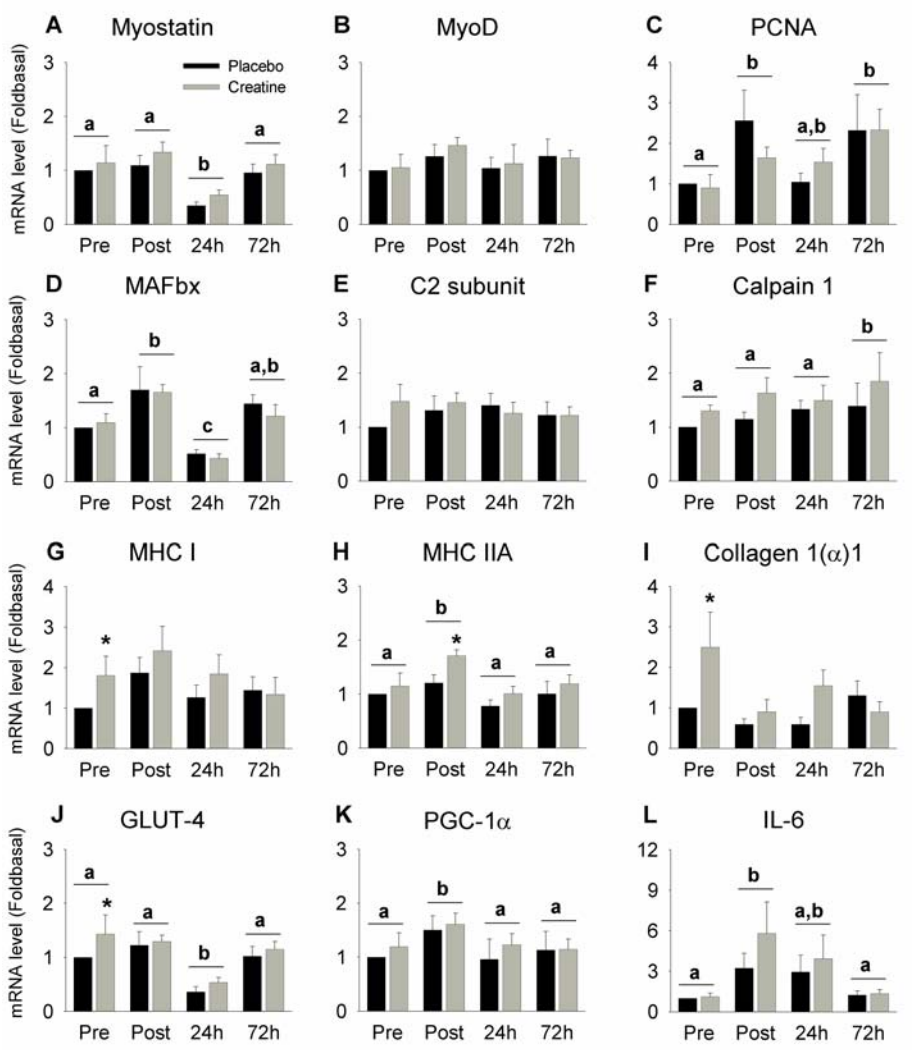

Figure 5.2. *Effect of creatine supplementation on the mRNA for myostatin (A), MyoD (B), PCNA (C), MAFbx (D), C2 subunit of proteasome (E), calpain 1 (F), MHC I (G), MHC IIA (H), collagen 1(α1) (I), GLUT-4 (J), PGC-1α (K) and IL-6 (L).* Results are expressed as the means ± SEM (n=9) and relative to the placebo condition before exercise. * indicates a significant difference ($P<0.05$) of creatine vs. placebo same time. Two different letters above the histograms indicate a significant difference ($P<0.05$) between the different time points.

Activation of the MAPK pathway by exercise (Figures 5.3 A-E and 5.5)

Immediately after exercise, the phosphorylation state of p38 was increased more than 10-fold in the sarcoplasm ($P<0.05$) and more than 20-fold in the nucleus ($P<0.01$). It returned to pre-exercise values 24 h and 72 h post-exercise in both fractions. The phosphorylation of ERK1/2 in the nucleus tended to be increased by exercise but did not reach the statistical threshold ($P=0.065$). Immediately post-exercise, the expression of MEF-2 was doubled in the nucleus ($P<0.05$). Creatine had no effect on the phosphorylation state of p38 and ERK1/2 or on the nuclear expression of MEF-2.

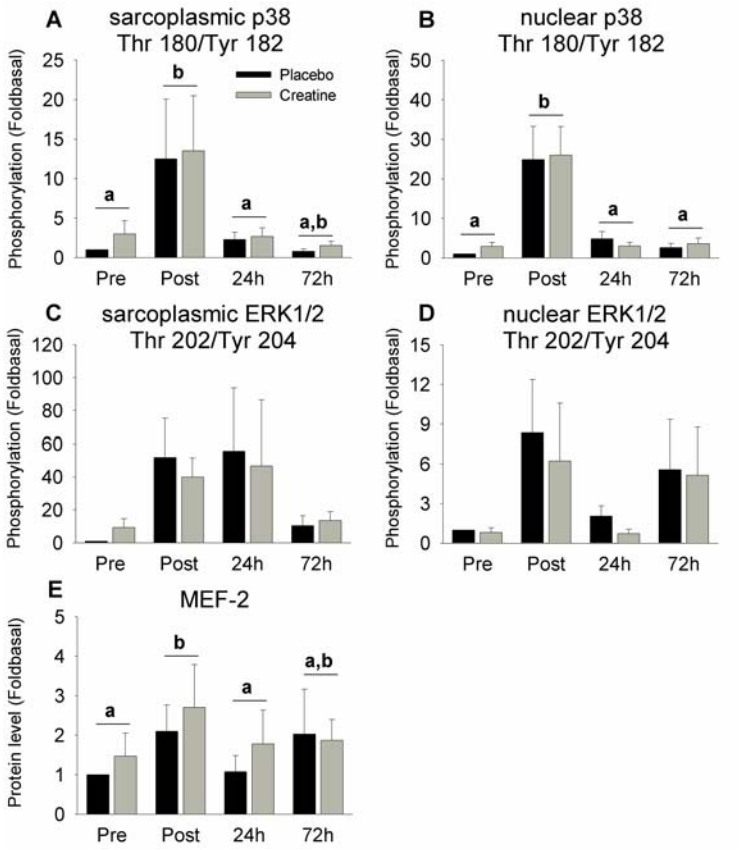

Fig. 5.3. *Effect of creatine supplementation on the phosphorylation state of sarcoplasmic p38 on Thr 180/Tyr 182 (A), nuclear p38 on Thr 180/Tyr 182 (B), sarcoplasmic ERK1/2 on Thr 202/Tyr 204 (C), nuclear ERK1/2 on Thr 202/Tyr 204 (D) and on the expression of MEF-2 in the nucleus (E).* Results are expressed as the means ± SEM (n=9) and relative to the placebo condition before exercise. Two different letters above the histograms indicate a significant difference ($P<0.05$) between the different time points.

Alteration of the PKB pathway by exercise (Figures 5.4 A-D and 5.5)

Immediately after exercise, the phosphorylation state of PKB on Thr 308 (P<0.05) and 4E-BP1 on Thr 37/46 (P<0.01) decreased by 50 % and 75 % respectively and returned to pre-exercise values at 24 h and 72 h post-exercise. The same trend to inhibition was observed immediately post-exercise on both PKB on Ser 473 and on $p70^{s6k}$ on Thr 389 but the statistical significance was not reached. Twenty-four hours post-exercise, two of the nine subjects showed a markedly elevated phosphorylation state of $p70^{s6k}$ on Thr 389 whereas it remained unchanged in the other subjects. Creatine decreased the phosphorylation state of PKB on Thr 308 at rest by 60 % (P<0.05) and the phosphorylation state of 4E-BP1 on Thr 37/46 24 h post-exercise by 30 % (P<0.05).

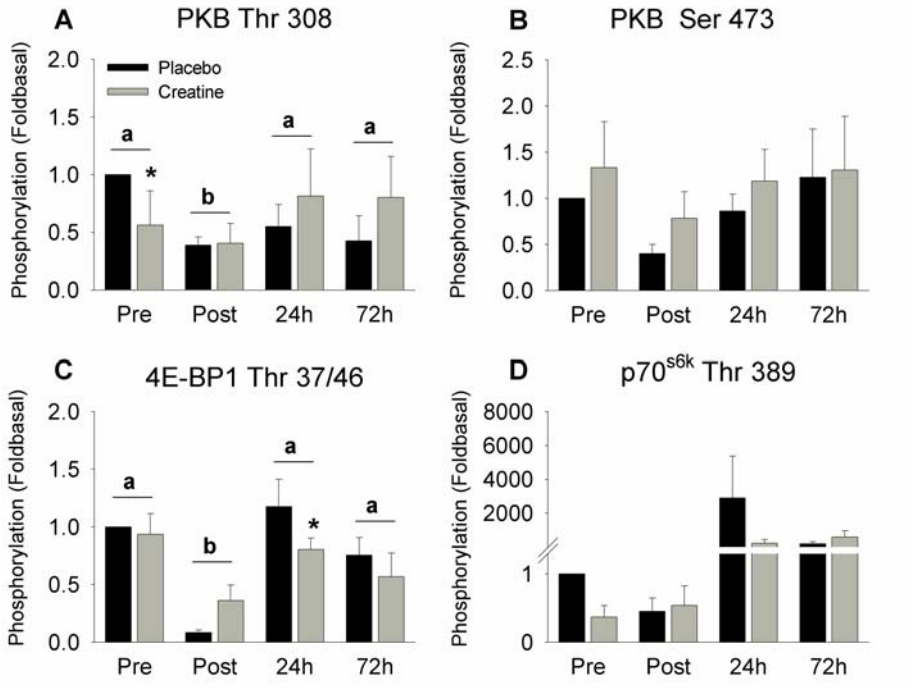

Fig. 5.4. *Effect of creatine supplementation on the phosphorylation state of PKB on Thr 308 (A), PKB on Ser 473 (B), 4E-BP1 on Thr 37/46 (C) and $p70^{s6k}$ on Thr 389 (D).* Results are expressed as the means ± SEM (n=9) and relative to the placebo condition before exercise. * indicates a significant difference (P<0.05) of creatine vs. placebo same time. Two different letters above the histograms indicate a significant difference (P<0.05) between the different time points.

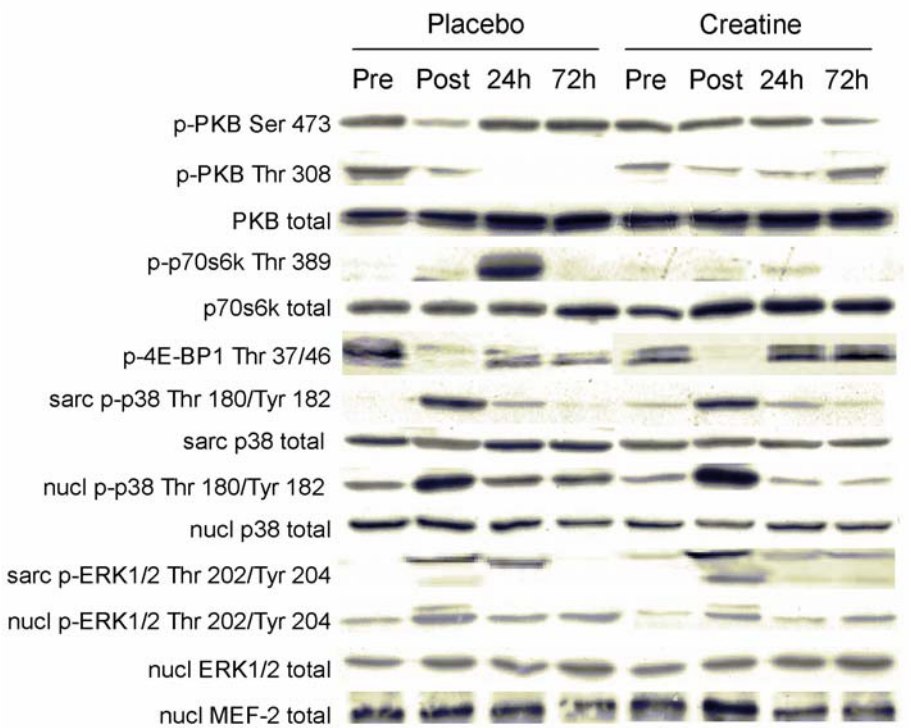

Fig. 5.5. *Typical Western blot bands.* All bands in one row have been obtained from one Western blot. PKB, protein kinase B; $p70^{s6k}$, p70 ribosomal protein S6 kinase; 4E-BP1, eukaryotic initiation factor 4E-binding protein; ERK1/2, extracellular signal-regulated kinase 1 and 2; MEF-2, myocyte enhancer factor 2; sarc, sarcoplasmic; nucl, nuclear; pre, pre-exercise; post, post-exercise; 24 h, 24 h post-exercise; 72 h, 72 h post-exercise.

Discussion

We designed the studies described above to determine principally whether or not the combination of acute exercise with 5 days of creatine supplementation would cause a greater than normal increase in the expression of genes and in signal transduction likely to be involved in adaptation of muscle size or biochemical properties. Indeed, exercise is known to activate multiple signal transduction pathways and to modulate transcriptional and translational processes.

The only significant gene alterations we observed in response to creatine supplementation were increases in the expression of GLUT-4, collagen 1(α1) and MHC I mRNA at rest and increased expression of MHC IIA mRNA immediately post exercise. Most of the changes in mRNA levels observed in the present study and elsewhere (Deldicque *et al.*, 2005) occurred at rest before the resistance exercise session. This suggests that contrary to our hypotheses creatine per se is able to modify the expression of several genes and that exercise training and/or a process of muscle degeneration/ regeneration are not essential prerequisite. In addition to the modulation of gene expression, creatine supplementation has been shown to augment the increase in satellite cells and myonuclei number induced by several weeks of strength training (Olsen *et al.*, 2006). Therefore the activation, proliferation and differentiation of satellite cells are other mechanisms by which creatine might increase muscle mass after several weeks of supplementation combined with training. In the current study, we found that although exercise did acutely increase a marker of satellite cell proliferation (PCNA, a protein involved in DNA replication maximally expressed in S phase) there was no prolonged effect over the next 24 h, but a rebound at 72 h and no additional effect of creatine. MyoD is another key regulator of muscle remodelling associated with cell proliferation. We did not observe any change in MyoD mRNA by creatine nor by exercise whereas others found a doubling of

MyoD expression immediately post-exercise (Psilander *et al.*, 2003) or a peak expression at 8 h to 12 h post-exercise (Yang *et al.*, 2005).

Although there is good evidence that the MAPK and the PKB pathways are involved in the regulation of skeletal muscle protein synthesis and remodelling by resistance exercise (Baar & Esser, 1999; Nader & Esser, 2001; Widegren *et al.*, 2001; Cuthbertson *et al.*, 2006), short-term creatine supplementation does not seem to act via these two cascades to increase muscle mass, contrary to our hypothesis. Creatine supplementation had no additional effect on the phosphorylation of p38 and ERK1/2 or on MEF-2 protein expression nor did creatine increase signalling through the PKB pathway. The results on the MAPK and the PKB pathways are in agreement with our previous studies showing no effect upon protein synthesis (Louis *et al.*, 2003b; Louis *et al.*, 2003c).

Taken together, our results indicate that, although the most remarkable effects of creatine are seen after several weeks of supplementation, some of them could be initiated at the transcription level as soon as after 5 days of supplementation. Nevertheless the extent of these increases is rather small and we have no evidence to relate them to activation of satellite cells and to muscle mass accumulation observed when creatine is combined with resistance training (Olsen *et al.*, 2006).

This study also produced novel results in the context of resistance exercise. These can be summarized as follows: (i) an acute rise in the expression of MAFbx mRNA seen immediately at the end of exercise followed by a subsequent fall at 24 h before the return to basal values by 72 h; (ii) a fall in myostatin mRNA at 24 h; (iii) PCNA, a marker of cell proliferation, was activated in a biphasic way, increasing immediately post-exercise and after 3 days recovery, (iv) the increases in both the sarcoplasmic and nuclear complements of p38 and ERK1/2; (v) the immediate rise after exercise of

MEF-2 protein. Therefore, acute resistance exercise induced very rapid, pronounced alterations in gene transcription and cellular signalling, indicative by the early modifications of some specific mRNAs seen at the end of exercise. Additional effects of creatine were much less pronounced than the effects of exercise alone. However, since no control group undertook muscle biopsies without resistance exercise and considering the potential influence of biopsies sampling on gene expression (Vissing *et al.*, 2005), the effects of exercise should be analysed with care.

A large number of studies have shown modulation of multiple gene clusters in response to resistance exercise stimulus (Psilander *et al.*, 2003; Bickel *et al.*, 2005; Yang *et al.*, 2005). Indeed a recent microarray study highlights the degree of such regulation (Kostek *et al.*, 2007). Furthermore, some gene changes may be associated with the known effects of resistance exercise upon protein metabolism. It has been suggested that during contractile activity, protein synthesis is depressed and protein breakdown is stimulated. The observed changes in the component of the ubiquitin/proteasome pathway, MAFbx, fits in well with this scenario although we did not assess MAFbx protein level. The current results are the first to show an acute upregulation of MAFbx mRNA in human muscle immediately after exercise, likely to reflect increased protein breakdown at this time. At 24 h post exercise, MAFbx mRNA expression was reversed and depressed, suggesting a reduction in protein degradation.

Myostatin is a negative regulator of muscle mass and reportedly regulates the expression of MAFbx to modulate ubiquitin-dependent proteolysis (McFarlane *et al.*, 2006). Similar to MAFbx, 24 h post-exercise, mRNA for myostatin was depressed. The decrease in MAFbx and myostatin mRNA observed 24 h after exercise suggests that the ubiquitin/proteasome pathway is suppressed at this time after exercise.

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 stimulates the expression of MHC IIA observed after resistance training (Adams *et al.*, 1993).

We observed changes in metabolic genes after resistance exercise. Immediately after exercise, IL-6 mRNA more than tripled confirming that skeletal muscle is a major site of IL-6 production (Pedersen *et al.*, 1998). GLUT-4 mRNA abundance decreased by 24 h after exercise. GLUT-4 mRNA seems to be regulated by the type of exercise. Endurance exercise increased GLUT-4 mRNA (Kraniou *et al.*, 2000) while the opposite effect was observed after resistance exercise (Churchley *et al.*, 2007). PGC-1α mRNA has been reported to increase after resistance exercise and the maximal expression level is reached about 3 h after exercise (Pilegaard *et al.*, 2003; Vissing *et al.*, 2005; Coffey *et al.*, 2006a). 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). An increase in the expression of MEF-2 has already been reported after endurance exercise (McGee *et al.*, 2006) but, to the best of our knowledge, this is the first study to show an increase in MEF-2 after a bout of resistance exercise.

ERK1/2 is another member of the MAPK pathways. Like p38, ERK1/2 is known to be regulated by exercise (Widegren *et al.*, 2001). ERK1/2 phosphorylation increased by more than 50-fold in the sarcoplasm and by

about 8-fold in the nucleus, but the statistical significance was not reached due to the large inter-subject variability.

Some studies have reported that resistance exercise activates the PKB cascade (Creer *et al.*, 2005; Dreyer *et al.*, 2006); others have observed no changes (Creer *et al.*, 2005; Coffey *et al.*, 2006b; Deshmukh *et al.*, 2006; Eliasson *et al.*, 2006). It was thus surprising to observe a decrease in the phosphorylation state of PKB on Thr 308 and the downstream target 4E-BP1 on Thr 37/46. The same trend was found on PKB on Ser 473 and on $p70^{s6k}$ on Thr 389 but without reaching the statistical threshold. Recently, there have been few reports of an inhibition of 4E-BP1 immediately after exercise in human (Dreyer *et al.*, 2006; Koopman *et al.*, 2006). The decrease in the PKB phosphorylation state fits well with the findings that exercise in the fasted state decreases protein synthesis and increases protein breakdown (Phillips *et al.*, 1997). Moreover, the PKB pathway is very sensitive to nutrients, but since all biopsies were taken in the fasted state this could have blunted the activation of the PKB pathway generally observed during the recovery period (Cuthbertson *et al.*, 2006). It is likely that the results would have been different if the subjects were in a fed state.

In summary, it is generally accepted that the effects of exercise training are the results of incremental addition of repeated bouts of exercise. We found evidence to suggest that creatine induces changes in the expression of certain targeted genes as early as after 5 days of supplementation and in association with a single session of resistance exercise. The increase in collagen 1(α1) and MHC I-IIA mRNA by creatine might improve muscle framework providing a favourable environment for muscle mass accretion after a few weeks of training. However, we have no evidence of a modulation of translational signalling when resistance exercise is coupled to creatine supplementation.

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