Signal Transduction in Skeletal Muscle Mediating Responses to Phenotype Altering Signals

By

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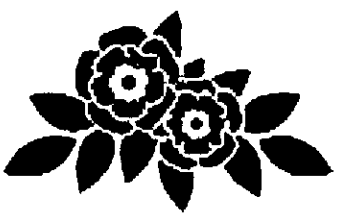

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Abstract

Skeletal muscle phenotype, size and function respond to exercise, disease and ageing. The aim of this thesis was to investigate the signal transduction pathways responsible for selected skeletal muscle phenotype and size changes. Myostatin, recently identified as a negative regulator of muscle mass was exposed at 10 ng ml^{-1} to C2C12 cells, and using cDNA genome-wide profiling, was shown to act as a transcriptional suppressor. Furthermore, in these cells myostatin significantly ($n=8$, $p<0.05$) reduced phosphorylation of components in the PI-3K pathway: PKB Ser473 ~30 %, mTOR Ser2448 ~50 %, p70 S6K Thr389 ~60 %, whereas 4E-BP1 Thr37/46 remained unaffected. These data provide insights in to the mechanisms by which myostatin controls muscle mass, through negatively affecting transcription and translation.

Differences in the concentrations of signalling proteins often alter cellular function and phenotype, as is evident from numerous heterozygous knock-out models. Whilst the levels of metabolic enzymes differ between fibre types, and are regulable by exercise, it is not known if this is also true of signal transduction proteins. Therefore, it was hypothesised that the relative levels of signalling proteins implicated in the adaptation to exercise in both fast rat extensor digitorum longus (EDL; 3% type I fibres) and slow Soleus (84% type I fibres) would be systematically different. Secondly, it was hypothesised that following 6 weeks of chronic electrical stimulation (CMNS) where the EDL undergoes a fast-to-slow transformation, the relative signalling protein concentrations between control EDL/stimulated EDL would mirror the differences shown in control EDL/Soleus. Finally, that CMNS would induce chronic signalling to produce, and maintain a slower phenotype. Western blots revealed that the concentrations of some proteins such as Calcineurin (2.6-fold) and

p38 MAPK (1.36-fold) were higher in EDL, whilst others such as PGC-1α (1.4-fold); and NFκB (3-fold) (all n=4, $p<0.05$) were higher in Soleus. CMNS of EDL also led to changes in protein levels between control EDL/stimulated EDL: AMPK which is higher in Soleus was actually 1.4-fold lower following stimulation of EDL, whereas other proteins such as PGC-1α moved in the direction of that of Soleus. CMNS was also able to induce chronic phosphorylation of proteins involved in fibre type and mitochondrial biogenesis, such as AMPK ~4 fold, and p38 ~4.5-fold. These data show that signal transduction protein concentrations vary between fast and slow muscles, presumably reflecting differences at a fibre level. Furthermore, signalling proteins are regulated by CMNS of EDL, but do not always change in the direction of slow Soleus. Chronic phosphorylation of many signalling proteins can explain the characteristic phenotypic change in response to CMNS.

Resistance training stimulates adaptive protein synthesis and hypertrophy whereas endurance training induces a partial fast-to-slow fibre phenotype transformation. To simulate these conditions, isolated rat muscles were stimulated at 25 °C with either high frequency (HFS; 6 x 10 repetitions, 3 s-bursts at 100 Hz to mimic resistance training) or low frequency (LFS; 3 h at 10 Hz to mimic endurance training). HFS significantly increased myofibrillar and sarcoplasmic protein synthesis 3 h after stimulation 5.3 and 2.7-fold, respectively (n=6, $p<0.05$). LFS had no significant effect on protein synthesis 3 h after stimulation, but increased UCP3 mRNA 11.7-fold, whereas HFS had no significant effect on UCP3 mRNA (n=6, $p<0.05$). Only LFS increased AMPK phosphorylation significantly at Thr172 by ~2-fold and increased PGC-1α protein to 1.3-fold of control. LFS had no effect on PKB phosphorylation but reduced TSC2 phosphorylation at Thr1462 and deactivated translational regulators. In contrast, HFS acutely increased phosphorylation of PKB at Ser473 5.3-fold and the

phosphorylation of TSC2, mTOR, GSK-3β at PKB-sensitive sites. HFS also caused a prolonged activation of the translational regulators p70 S6k, 4E-BP1, eIF2B, and eEF2 (all n=8, $p<0.05$). This behaviour has been termed the AMPK-PKB switch, and is hypothesised to mediate specific adaptations to endurance and resistance training, respectively.

Ageing is associated with a loss of muscle mass termed sarcopenia. Essential amino acids (EAA) are potent stimulators of muscle protein synthesis (MPS), and therefore defects in EAA-induced anabolism might affect ability to maintain muscle mass in ageing and disease. MPS and signalling responses to EAA-stimulation of 20 fasted young versus 24 elderly subjects (age 28 ± 6 and 70 ± 6; BMI 24 ± 3, 25 ± 4 $kg.m^{-2}$ respectively; means ± SD) and 8 fasted elderly versus 8 elderly with type II DM (age 66 ± 3 and 70 ± 6; BMI: 25 ± 4 vs. 32 ± 2 $kg.m^{-2}$, respectively means ± SD) were measured using gas combustion mass spectrometry and Western blotting methods. Basal MPS rates were indistinguishable, but the elderly displayed a reduced anabolic responsiveness of MPS to EAA, possibly due to decreased intramuscular phosphorylation after EAA, of amino acid sensing/signalling proteins mTOR, p70 S6 kinase, 4E-BP1 and eIF2Bε by ~50 %. This was further exacerbated in elderly with type II DM whom exhibited reduced Ser2448 phosphorylation of mTOR by ~50 %, reflecting decreased downstream signalling. Associated with the anabolic deficits were ~ 4-fold increases in NFκB protein, the inflammation-associated transcription factor, as well as ~50 % and ~20 % decreases in protein expression of p70 S6K of healthy elderly and elderly with type II DM, respectively. These results suggest that the elderly are unable to mount a full anabolic response to EAA and that this blunting is further pronounced in type II DM.

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Finally I would like to express my greatest appreciation to my parents for their continual support, love and encouragement over the years. Thankyou... this thesis is dedicated to you both.

Dedicated to:

Mum and Dad.....

.....Thanks for Everything

Declaration

I declare that whilst registered as a candidate for the degree for which this submission is made I have not been a registered candidate by any other awarding body

Declaration of Experimental work presented in this thesis but carried out without my involvement:

- ***In vivo*** **muscle stimulations utilised for studies in chapters 3 & 4 (University of Liverpool)**

- **Gas combustion mass spectrometry on human muscles presented in chapter 6 (University of Dundee)**

Definitions of Abbreviations

ACh	Acetylcholine
ADP	Adenosine diphosphate
AICAR	5-aminoimidazole-4-carboxamide ribonucleoside
ANOVA	Analysis of variance
AMP	Adenosine Monophosphate
AMPK	AMP-activated protein kinase
ATF-1	Activating transcription factor-1
ATP	Adenosinse triphosphate
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
CDK	Cyclin-dependent kinase
CMNS	Chronic motor nerve stimulation
CnA	Calcineurin A
CsA	Cyclosporine A
DHP	Dihydropyridine receptor
DNA	Deoxyribonucleic acid
EAA	Essential amino acid
E-C coupling	Excitation-contraction coupling
EDL	Extensor Digitorum Longus
eEF	Eukaryotic elongation factor
eIF	Eukaryotic initiation factors
eIF4E-BP1	Eukaryotic initiation factor 4E binding protein 1
ERK	Extracellular-signal regulated kinase
FSR	Fractional synthesis rate

FT	Fast twitch
GAP	GTPase activating proteins
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC-MS	Gas combustion mass spectrometry
GDP	Guanosine diphosphate
GSK3β	Glycogen synthase kinase 3 Beta
GTP	Guanosine triphosphate
HFS	High frequency stimulation
IGF-1	Insulin-like growth factor-1
IGFBP	Insulin-like growth factor binding protein
IL-15	Interleukin-15
JNK	c-Jun amino-terminal kinase (p54/p46)
LFS	Low frequency stimulation
MAFbx	muscle atrophy F-box
MAPK	Mitogen-activated protein kinase
MEF2	Myocyte enhancer factor-2
MGF	Mechano-growth factor
MHC	Myosin heavy chain
MLC	Myosin light chain
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
MuRF1	Muscle ring finger 1
NADH	Nicotinamide adenide dinucleotide phosphate

NADH-Tr	NADH, reduced form tetrazolium reductase
NBT	Nitroblue tetrazolium
NEB	New England Biolabs
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor kappa B
NRF	Nuclear respiratory factor
p38	protein of 38 KDa
p54/p46	p54/46 MAPK proteins of 54 and 46 KDa
p70 S6K	Ribosomal protein kinase p70
PCR	Polymerase chain reaction
PCr	Phosphocreatine
PDK	Pyruvate dehydrogenase kinase
PGC-1α	Peroxisome proliferator-activated receptor γ coactivator
PI-3K	Phosphatidyl inositol 3-kinase
PIP2	$PtdIns(3,4)P_2$
PIP3	$PtdIns(3,4,5)P_3$
PKB	Protein Kinase B
PKC	Protein Kinase C
PPARγ	Peroxisome proliferator-activated receptor-gamma
PRDX3	Peroxiredoxin 3
PTEN	Phosphatase and tensin homolog on chromosome ten
PVDF	Polyvinylidenedfluoride
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
RNase	Ribonuclease

rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
RyR	Ryanodine receptor
SAPK	Stress activated protein kinase (p54/p46)
SERCA	Sarco(Endo)plasmic Reticulum Ca^{2+}-ATPases
SSPE	NaCl, NaH_2PO_4, EDTA
SDS	Sodium dodecyl sulfate
Ser	Serine
SMAD	Mothers against decapentaplegic homolog
SR	Sarcoplasmic reticulum
ST	Slow twitch
TFAM	Mitochondrial transcription factor A
Thr	Threonine
TNFα	Tumour necrosis factor-alpha
TSC	Tuberosclerosis complex
Type II DM	Type II Diabetes Mellitus
Tyr	Tyrosine
UBF	Upstream binding factor
UCP-3	Uncoupling protein 3

Chapter 1

General Introduction

1.1 Characteristics of skeletal muscle

1.1.1 Skeletal muscle plasticity

Skeletal muscle constitutes 75-80 % of total lean body mass, accounts for ~50 % of body weight and is the largest component of the metabolically active cell mass of the body (Heymsfield *et al.*, 1995). Functionally, its purpose is to contract in order to move limbs and maintain posture. Skeletal muscle is a unique multi-nucleated tissue that shows remarkable plasticity and both mass and phenotype varies between individuals, and throughout life. Muscle also responds to disease, nutrition and physical activity (Fluck & Hoppeler, 2003). To illustrate this, elderly with advanced sarcopenia and cancer patients with severe wasting are examples of skeletal muscle atrophy (Glass, 2003) whereas the hypertrophy elicited through resistance exercise shows how much muscle mass can be gained through training (Evans, 1995a) (figure 1.1a). In addition, any form of physical training may influence the expression of myosin heavy chain isoforms (Harber *et al.*, 2002) and endurance training and chronic low frequency electrical stimulation produces highly oxidative muscles (figure 1.1b).

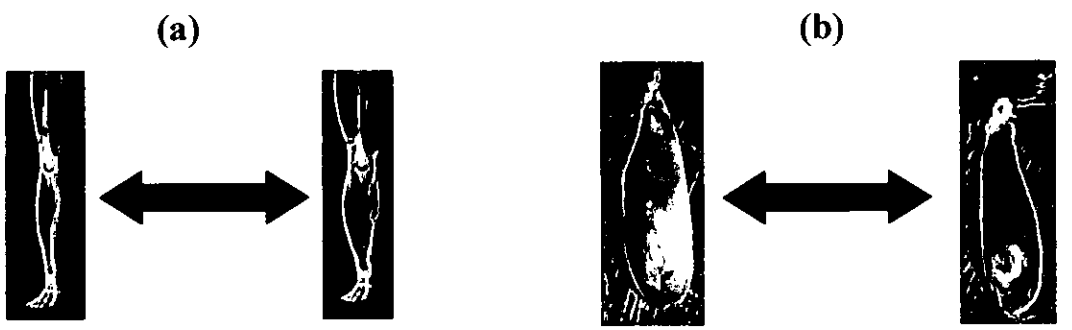


Figure 1.1. Images depicting the robust plasticity of skeletal muscle. (a) Example of muscle atrophy and hypertrophy in humans; (b) visual phenotypic transformation in rat tibialis anterior (paler to redder and weight loss) in response to 6 weeks of chronic stimulation. These changes are all reversible when the causative stimulus is removed.

1.1.2 Skeletal muscle structure

Both ends of most striated muscles articulate the skeleton and are thus called skeletal muscles. They are attached to the bones by tendons which have some elasticity provided by proteins of the extracellular matrix that surround them (Takala & Virtanen 2000). The extracellular matrix forms a complex dynamic architecture and is composed of glycoproteins, collagen, and proteoglycans. Collagen is the most abundant protein, and as muscle, is regulable by physical activity (Takala & Virtanen 2000). Proteoglycans have been suggested to play an important functional role in tissue differentiation. Proteoglycans affect the myogenic process by regulating collagen fibrillogenesis, modulating cell growth and controlling the response to growth factors. The extracellular matrix communicates information back to the cell through integrin receptors. Integrins are heterodimeric transmembrane glycoproteins that contain extracellular, transmembrane, and cytoplasmic domains (Velleman 1999).

The anatomy of skeletal muscles starting from the outside of the muscle and working inwards is as follows: the outermost layer that surrounds the entire muscle is called the epimysium. Connective tissue called the perimysium surrounds individual bundles of muscle fibres. These individual bundles of muscle fibres are called fascicules. Each muscle fibre within the fasciculus is surrounded by connective tissue called the endomysium. Each individual muscle fibre is a thin, elongated cylinder that generally extends the length of the muscle, thus making them potentially very large cells. The cell membrane surrounding the muscle cell is called the sarcolemma. Beneath the sarcolemma lies the sarcoplasm, which contains the cellular proteins, organelles, and myofibrils. Myofibrils are the numerous thread-like structures that contain the contractile proteins (Powers & Howley 1998) (figure 1.2).

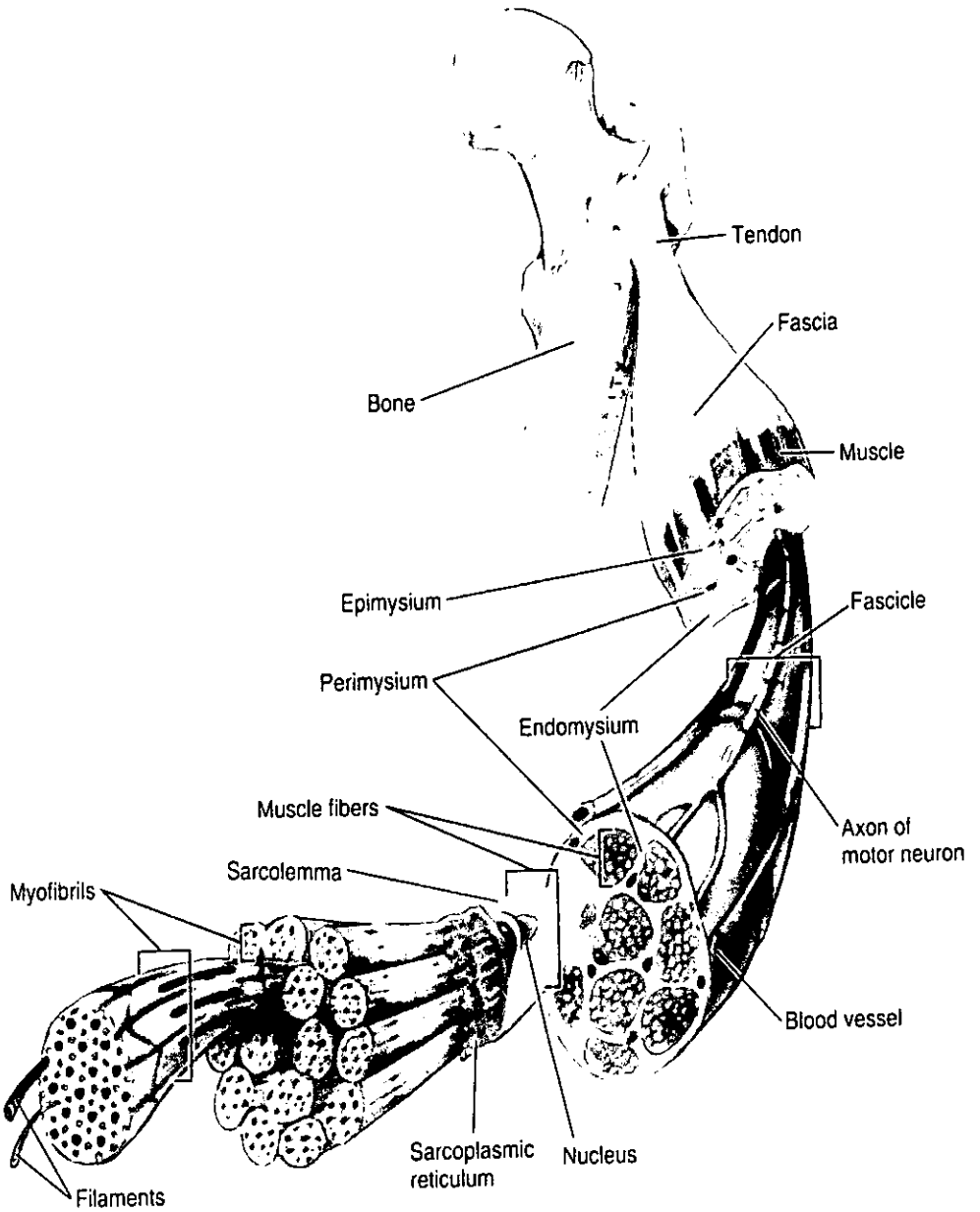


Figure 1.2. Diagram showing the structure of muscle. Tendons and bones support the muscle ends, which articulate them. The extracellular matrix surrounds the muscle cell. The epimysium surrounds the whole muscle, whilst the perimysium surrounds bundles of fibres. The endomysium surrounds individual fibres, as does the plasma membrane, the sarcolemma. The myofibrils contain the contractile proteins. Diagram taken from Powers & Howley (1998).

1.1.3 Skeletal muscle ultra-structure

In general, myofibrils are composed of two major types of protein filaments. Thick filaments composed of the protein myosin, and thin filaments composed primarily of actin (figure 1.3c). The arrangement of these two protein filaments gives skeletal muscle its striated appearance. Located on the actin filament are two additional proteins, troponin and tropomyosin (Powers & Howley 1998). These proteins play an important role in the regulation of the contractile process. Sarcomeres are separated from each other by a thin sheet of structural proteins called the Z-line which delineates the sarcomere (figure 1.3a). Myosin filaments are located primarily in the dark portion of the sarcomere which is called the A-band, whilst actin filaments occur principally in the light region of the sarcomere termed the I-band. In the centre of the sarcomere there is a portion of the myosin filament with no overlap of actin, this is termed the H-zone. The M-band bisects the central portion of the H-zone, which delineates the centre of the sarcomere (figure 1.3b).

Within the sarcoplasm of skeletal muscle is a network of membranous channels called the T- tubules, which are extensions in to the depths of the muscle cell provided by the sarcolemma. These T-tubule channels surround each myofibril. The sarcoplasmic reticulum (SR) forms a longitudinal system of calcium (Ca^{2+}) containing tubules and sacs and at localised regions called the terminal cisternae these come into close apposition to the T-tubules. The membranes of the two compartments form a structure in which two terminal cisternae sandwich a transverse tubule to give rise to a triad arrangement (Powers & Howley 1998).

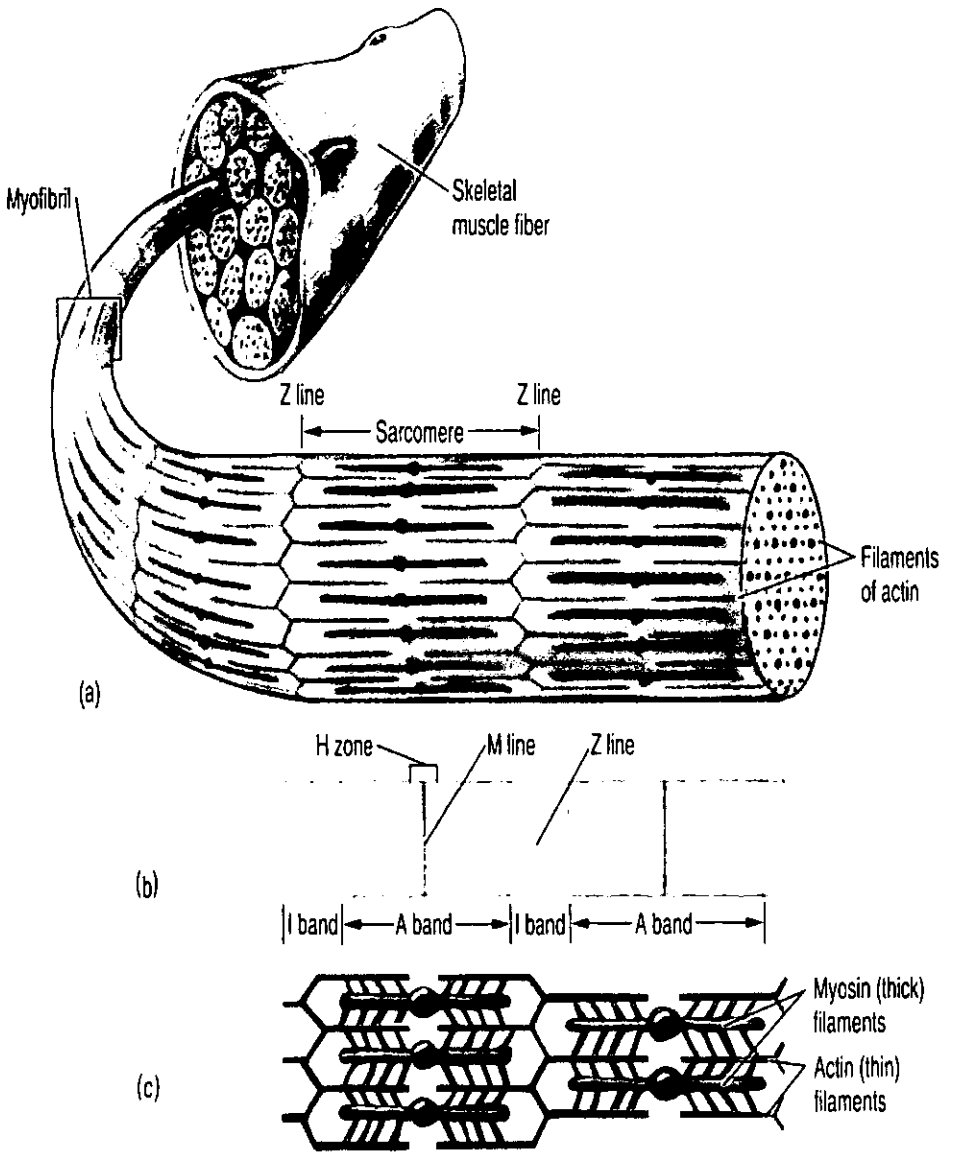


Figure 1.3. Diagram of skeletal muscle ultra-structure. (a) The portion of a myofibril between two Z-lines constitutes a single contractile unit, termed a sarcomere. (b) Further categories of areas within the sarcomere are: the A-band: area containing myosin; the H-zone: centre of A-band containing only myosin; I-band: the area containing actin, troponin, tropomyosin; the Z-line: centre of I-band, sarcomere border. (c) When actin and myosin interact they shorten each sarcomere. Diagram taken from Powers & Howley (1998).

1.1.4 Excitation-Contraction (E-C) coupling

Motor units consist functionally of three separate components: a single efferent neuron, the several muscle fibres that it innervates, and the synapses (neuromuscular junction) between the neuron and the muscle fibres (Clark *et al.*, 2002). The neurotransmitter substance acetylcholine (ACh) is received at receptor sites on the motor end plate. These end plate potentials are always excitatory, and a single end plate potential is induces an action potential in the muscle fibre (Powers & Howley 1998). A muscle action potential propagates into the T-tubular system, carrying excitation into the depths of the fibres axial core. The T-tubular action potential contributes a long-lasting (20-30 ms) after-depolarisation to the muscle (Powers & Howley 1998). This depolarisation of the skeletal muscle cell begins the process of E-C coupling. In the membrane of T-tubules are voltage sensors called dihydropyridine (DHP) receptors, which detect changes in the membrane potential. Structures that project between the SR membranes and the T-tubular membranes are hypothesised to form a mechanical link between DHP and the ryanodine receptors (RyR) (Bianchi, 1997). Depolarisation of the T-tubular membrane causes a conformational change in the DHP receptors which is thought to produce a conformational change in the SR Ca^{2+} channels through interaction with the RyR. This process leads to the opening of SR channels and subsequent Ca^{2+} release (Ikemoto & Yamamoto, 2000). Increased $[Ca^{2+}]_i$ leads to the subsequent opening of the Ca^{2+} sensitive RyR allowing Ca^{2+} ions to flow out of the SR down their electrochemical gradient, raising concentrations within the fibre cytoplasm, and at the same time causing more Ca^{2+} channels to open. This process is termed calcium-induced calcium release (Fabiato, 1983), although the precise necessity for this mechanism in muscle contraction is questionable.

1.1.5 The sliding filament theory

The physiological mechanism that explains contraction in response to elevated $[Ca^{2+}]_i$ is known as the sliding filament theory (Huxley, 1985) (figure 1.4). Under resting conditions, and low $[Ca^{2+}]_i$, tropomyosin inhibits actin and myosin interaction. The trigger for contraction to occur is when $[Ca^{2+}]_i$ is elevated through the aforementioned mechanisms (see 1.1.4), before Ca^{2+} binds to troponin and removes tropomyosin from the myosin binding site on actin. Myosin-ADP-P_i complex binds to actin, forming a cross bridge and causing the release of ADP and P_i. The myosin head rotates and pulls the thin filament towards the centre of the sarcomere; this is termed the power-stroke. When this is complete, the myosin in the actin-myosin complex develops a high affinity for ATP, and ATP binds to the myosin on the actin-myosin complex, causing the cross-bridge to break and yielding actin-myosin-ATP. The myosin ATPase partially hydrolyses the ATP in the myosin-ATP complex to yield myosin-ADP-P_i. This complex is now primed to form a new cross-bridge. Meanwhile the sarcoplasmic $[Ca^{2+}]_i$ is rapidly decreasing via the action of the Ca^{2+} ATPase increasing the SR stores. Since the Ca^{2+} bound to troponin is in equilibrium with the free sarcoplasmic Ca^{2+}, and since the pump has a greater affinity for Ca^{2+} than troponin, the amount of Ca^{2+} on troponin decreases. As more and more Ca^{2+} is re-sequestered, the troponin changes shape back to a conformation that moves the tropomyosin back over the binding site for myosin on actin (Tonomura & Oosawa, 1972). At this point the process of contraction ceases until the next stimulus arrives.

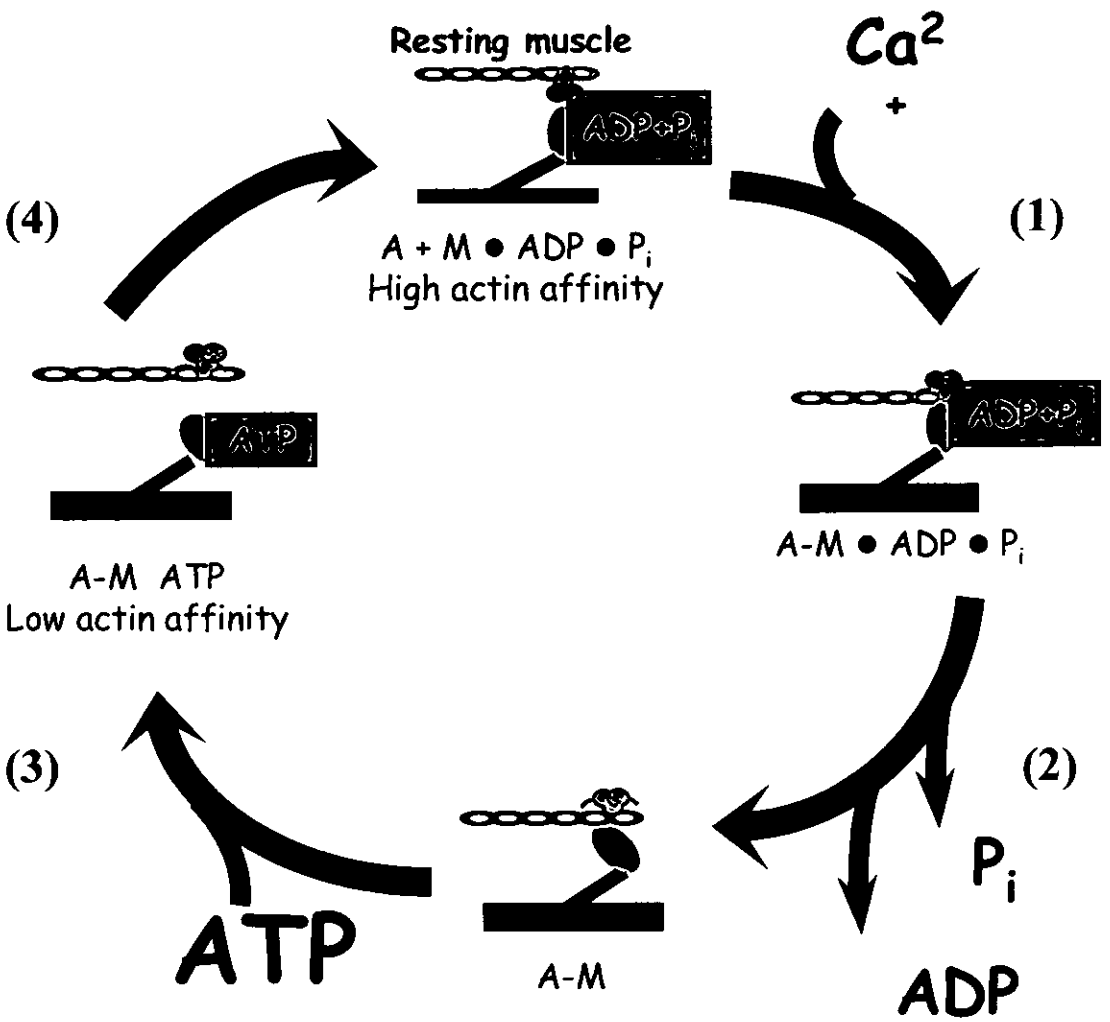


Figure 1.4. Schematic diagram of skeletal muscle cross-bridge cycling. The mechanism proposes the following chain of events: (1) Release of Ca^{2+}-dependent regulatory mechanisms allows binding of myosin head to the actin filament. (2) Release of ATP hydrolysis products causes a change in myosin head conformation that results in the power stroke and generation of increased tension. (3) Binding of ATP to the myosin head stimulates the release of myosin from the actin filament. (4) Rapid hydrolysis of ATP causes a change in the myosin head conformation to return to high actin affinity state. A= Actin, M= Myosin and P_i= inorganic phosphate.

1.2 Skeletal muscle fibre types

A Vastus Lateralis muscle of a young male has around 500,000-900,000 muscle fibres, and these are some of the largest cells in the body measuring up to 15-30 cm in length (Lexell *et al.*, 1988). Skeletal muscles are not identical, and are individually composed of different fibre type populations, which affect their functionality. These are largely defined as either slow twitch (ST) or fast twitch (FT) fibres.

1.2.1 Myosin Heavy Chain (MHC) isoforms

Distinct fibre types are mainly distinguished by the MHC isoforms expressed within them. The major component of the whole myosin molecule is the MHC protein which contains the ATP hydrolysing myosin head (Tonomura & Oosawa, 1972). The MHC isoform that is mainly expressed within a fibre type determines the muscle fibre's ATP hydrolysis rate and shortening speed (Barany, 1967). Two developmental (termed extraocular, developmental) and seven adult MHCs have been identified. The shortening speeds of adult MHCs decrease from extraocular > IIm > IIb > IIx > IIa > Iα (cardiac) > Iβ (Sciote & Morris, 2000). In humans, the extraocular, IIm and IIb isoforms are only expressed in eye and jaw muscles. Rodents and humans possess varying distributions of fibre types even within equivalent muscles (see table 1.1). Not all fibres are of mono-MHC isoform expression. There are also hybrid fibres co-expressing the type I and type II MHC isoforms MHC I and MHC IIa (I+IIA or I/IIA fibres). These fibres are referred to as IC or IIC fibres on the basis of the most abundant MHC isoform expressed (MHC I or MHC IIa, respectively) (Pierobon-Bormioli *et al.*, 1981). It is now widely accepted that the proportion of MHC hybrids

and their molecular complexity (as judged by the number of MHC isoforms co-expressed and the pattern of co-expression) is higher in muscles undergoing molecular and functional transformation than in normal muscles (Pette *et al.*, 1999). Each skeletal muscle contains specific ratios of fibre populations making them unique. The expression of MHC in developing and adult muscle is regulated by neural, hormonal, mechanical factors and exercise (Schiaffino & Reggiani, 1994).

Table 1.1. Myosin Heavy Chain (MHC) isoform composition of the Soleus and Vastus Lateralis muscles in rat and human. Taken from Talmadge, (2000).

Muscle	Species	MHC Iβ %	MHC IIa %	MHC IIx %	MHC IIb %
Soleus	Rat	89 ± 3	11 ± 3	0	0
Soleus	Human	67 ± 6	27 ± 6	6 ± 4	0
Vastus Lateralis	Rat	0	0	2	98
Vastus Lateralis	Human	39 ± 3	43 ± 2	18 ± 4	0

1.2.2 Physiological and metabolic differences between fibre types

Distinct muscle fibre types possess other unique properties aside from MHC isoforms. Electron microscopy of skeletal muscle fibres shows evidence of wider Z-bands and wider M-bands in slow twitch muscle of which this diminishing size pattern linearly narrows from ST>FTa>FTx, successively (Sjostrom *et al.*, 1982). The rate at which myosin-ATPase splits adenosine 5' triphosphate (ATPase activity) is a prime

determinant of muscle twitch property and is differentiated by the multi-molecular isoforms of motor proteins within each fibre (Pette & Staron, 1988). As a result of different expression of these isoforms, and of SR ATPase activity, ST fibres have a longer time to peak tension, whereas FT fibres reach peak tension in a shorter time (Termin *et al.*, 1989).

FT fibres reportedly possess ATP stores 60 % higher than ST fibres, and creatine phosphate content is reported to be up to 100 % higher in FT muscles (Kraus *et al.*, 1994). Triglyceride stores are also higher in ST fibres and glycogen substrate is also reported to be more abundant in these fibres (Ren & Hultman, 1990). ST fibres are well endowed with enzymes for oxidative phosphorylation, and possess a low anaerobic capability in tandem with low T-tubular, sarcoplasmic reticulum (SR) concentrations, and slow isoforms of the contractile proteins MHC, tropomyosin, and troponin which regulate contractile patterns of cross-bridge cycling (Kraus *et al.*, 1994). FT fibres have been shown to contain greater densities of glycolytic enzymes, T-tubular systems, and SR due to their superior Ca^{2+}-processing requirement and also possess FT isoforms of the contractile proteins, thus giving limited aerobic capacity and promoting anaerobic metabolism (Pette & Staron, 1990). To illustrate this, the activities of mitochondrial enzymes 3-hydroxyacyl-CoA dehydrogenase (β-oxidation), succinate dehydrogenase, and citrate synthase are ~50 % higher in ST than FT fibre groups (calculated by flux through the Krebs cycle); whilst lactate dehydrogenase, phosphorylase, phosphofructokinase activities are higher in FT muscle (Pette & Staron, 1990) (see table 1.2). These differences are equivocal in that an overlap between fibre type, enzyme activity and MHC, troponin and tropomyosin is existent between fibre species (Kraus *et al.*, 1994).

Table 1.2. Table illustrating the common characteristics denoting classification of skeletal muscle fibre types. Taken from Spangenburg & Booth, (2003).

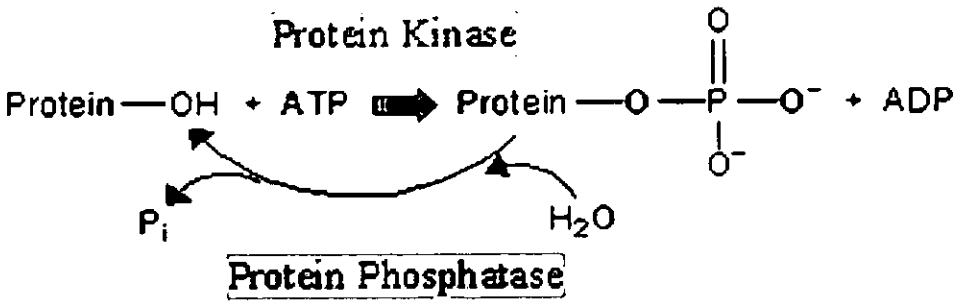
Characteristic	Type I	Type IIa	Type IIx	Type IIb
Contractile speed	Slow-twitch	Fast-twitch	Fast-twitch	Fast-twitch
Myosin heavy chain	Type I	Type IIa	Type IIx	Type IIb
Metabolic	Oxidative	Oxidative	Glycolytic	Glycolytic
Anatomical (colour)	Red	Red	White	White
Fibre CSA	Small	Medium	Large	Large

Type IIb fibres are not expressed in human locomotory muscles (Smerdu *et al.*, 1994).

1.3 Exercise, disease and nutrient-induced cell signalling- Mechanisms of signal transduction

A major focus in skeletal muscle research over the past few years has been the investigation of signal transduction pathways that regulate muscle mass and phenotype (Caffrey *et al.*, 1999). It is the sensing and signalling of specific signals which leads to genetic responses that produce characterised physiological changes within muscle. Exercise and disease lead to changes in intracellular and extracellular environments, and it is such changes that provide the stimulus to initiate signalling cascades. These stimuli can take many forms, and all the following are implicated initiation of signalling: cellular energy status (Frosig *et al.*, 2004), $[Ca^{2+}]_i$ (Chin *et al.*, 1998), free radical production (Jackson, 1999), mechanotransduction (Goldspink, 1999), growth factors (Goldspink & Yang, 2001), hormones (Smilios *et al.*, 2003), stretch (Baar *et al.*, 2000), cell swelling (Nygren & Kaijser, 2002), muscle damage (Clarkson & Hubal, 2002) and nutrients (Proud, 2004a).

Once initiated by stimuli such as those named above, signal transduction pathways are 'activated'. These pathways are usually cascades of proteins that alter the activity of their substrates via phosphorylation (kinases) or in other cases dephosphorylation (phosphatases). A kinase transfers the terminal phosphate of ATP to a hydroxyl group on a protein, whilst a phosphatase catalyses removal of the phosphate by hydrolysis:



Exercise has been shown to activate many such pathways and the increased activation of just one protein may stimulate multiple pathways. The following proteins are examples of those implicated in mediating responses to exercise and even disease: adenosine monophosphate kinase (AMPK) (Winder, 2001), calcineurin (Meissner *et al.*, 2001), calmodulin kinase (CaMK), peroxisome proliferator activated receptor gamma co-factor 1 alpha (PGC-1α) (Wu *et al.*, 2002), extracellular regulated kinase (ERK1/2), p38 MAPK (Widegren *et al.*, 2001), phosphatidyl inositol 3-kinase /protein kinase B/mammalian target of rapamycin (PI-3K/PKB/GSK3β/mTOR) (Turinsky & Damrau-Abney, 1999), and protein kinase C (PKC) (Richter *et al.*, 1987). Following activation of these proteins a signalling cascade will culminate in the activation of a transcription factor which has the potential to regulate the expression of genes by conveying the signal in to the myonucleus. Proteins that shuttle in and out of the nucleus usually have nuclear localisation signals or nuclear export signals (NES) (Cyert, 2001). Once inside the nucleus they may directly bind to the DNA or activate

other transcription factors via phosphorylation (Widegren *et al.*, 2001). Some proteins do neither of these things, instead acting as co-factors or co-activators. An example of this is PGC-1α which binds to transcription factors but not DNA directly (Knutti & Kralli, 2001). In fact, most genes are regulated by combinatorial control exerted by numerous signal transduction factors binding to their DNA regulatory elements (Arnone & Davidson, 1997). The activation of transcription will result in higher mRNA concentrations, which need to be translated. Protein synthesis may then be activated by the PI-3K/PKB/mTOR pathway (Bodine *et al.*, 2001), which directs the ribosome to increase protein translation of multiple RNA species.

1.3.1 Regulation of signalling proteins

Signalling proteins are of great importance in mediating cellular responses, yet their regulation is poorly understood. The output of a signal transduction pathway depends both on the strength of the input signal and on the signal amplification by the pathway (Rommel *et al.*, 2001). Since the amplification of a given signal is a result of the concentration and specific activity (control coefficient) of signal transduction proteins within a pathway, this means that signalling protein concentration has important consequences (Lion *et al.*, 2004). To illustrate this, increasing or lowering the concentration of a signal transduction protein often has effects on the function and phenotype of a cell. For example heterozygous mouse knockout models where the concentration of signal transduction proteins is lowered compared to wild-type can cause gross phenotype differences and even fatality. Cellular levels of many protein groups in muscle prove to be of key importance, and muscle metabolic enzymes are an example of proteins whose variations in content have gross effects upon muscle

metabolism. For example, individuals with higher concentrations of oxidative enzymes have enhanced aerobic capacity (Pette *et al.*, 1976). Moreover, enzyme concentrations are under regulation by physical activity, and an increase in concentration of oxidative enzymes following endurance training improves endurance fuel metabolism. If signal transduction proteins activated by contraction were also regulable and responded to exercise and disease then this could have important implications for adaptive processes and functionality, and thus warrants attention.

An interesting characteristic of signalling proteins is that they appear to have the unique ability to distinguish specific signals. Therefore, further to their role in mediating response to *any* exercise type, it could be that their selective activation could be linked to controlling responses, by discerning signals produced by specific type of stimulus. An example for this discerning activation was first demonstrated in a study which showed that signalling pathways could be selectively activated by different intensities of one signal. The authors found that a large, transient increase in $[Ca^{2+}]_i$ stimulated NFκB and JNK whereas a low, sustained $[Ca^{2+}]_i$ rise activated nuclear factor of activated T cells (NFAT) (Dolmetsch *et al.*, 1997). Before discussing the specific signalling which controls the responses of changes in muscle architecture and function, it is first necessary to systematically describe the typical physiological changes associated with these states. Therefore, the next section will focus upon the physiological adaptations to phenotype altering endurance training and then describe the signalling associated with producing these changes; whilst the section following this will follow the same pattern, but this time in regard to hypertrophy promoting, resistive training. Finally, the physiological changes in muscle atrophy, ageing and disease and the signalling controlling these changes will be discussed.

1.4 Physiological adaptations of skeletal muscle to endurance training

Skeletal muscle is a remarkably plastic tissue and MHC and fibre type characteristics have the potential to undergo phenotypic change in response to physical activity (table 1.2). The predominant physiological adaptation to endurance exercise is an incomplete fast-to-slow phenotype shift (Pette & Staron, 2001) with mitochondrial biogenesis (Freyssenet *et al.*, 1996). This is accompanied by increases in enzymes of the Krebs cycle such as succinate dehydrogenase, citrate synthase and malate dehydrogenase (Henriksson *et al.*, 1986). Given that the MHC isoform is the major determinant of fibre type, it has been under great scrutiny as to what extent this might be influenced by chronic endurance activity. There is evidence that increased contractile activity through electrical stimulation promotes a slow fast-to-slow phenotype conversion in a dose-dependent manner in rodents. However, in humans and in response to actual exercise, this remains a contentious area.

1.4.1 Do human skeletal muscle fibre types change with training?

Many studies and different groups have attempted to define the nature of any changes in fibre type produced by physical activity in humans. One group examined the fibre composition of biopsy samples from the Vastus Lateralis muscle of six subjects before and after a six month training programme, consisting of bicycle exercise at 70 % of VO_2, where the quadriceps muscle would undergo extensive employment (Gollnick *et al.*, 1973). The oxidative potential of the muscle was doubled following the 6 month period, although the actual fibre composition as determined by histochemical staining procedures was not altered by training. This study, however,

failed to further differentiate between sub-groups of FT muscles. Another group carried out a longitudinal study to examine the fibre composition of the Vastus Lateralis of four subjects after 11 weeks of endurance training and again after 18 weeks of training. During the initial endurance training period, the subjects ran 110 km/week, whilst the second training cycle included some high intensity exercise at 90-100 % VO_2 max. Unfortunately, no samples were taken prior to initialisation of endurance training so comparisons between sedentary and initial endurance training programmes were not available. However, following the second training bout there was a 17 % reduction in ST fibres, accommodated by an increase in type IIC hybrid fibres suggesting that a conversion away from ST, or at least hybridisation could occur as a result of mixed training (Jansson *et al.*, 1978). This change of ST to FT fibres due to high intensity exercise is not in agreement with another study, where each group exercised one leg with sprint, or endurance training and the other leg oppositely or not at all. Fibre composition of legs in untrained, endurance trained and sprint-trained had no significant differences (Saltin *et al.*, 1976).

Another group examined the effect of a 50-day programme of endurance training (skiing 18 miles/day) on the fibre composition of the triceps brachii muscle. There was no difference in the overall proportion of ST fibres after training cessation (pre-training proportionality of ST area and number withheld), although there was a reduction of about 6 % in the total population of type IIx fibres, which was in part compensated for by a 4 % increase in type IIa intermediate fibre type population (Schantz *et al.*, 1982), thus suggesting a shift to a slower phenotype from IIx to IIa. Another longitudinal study over 20 years assessed the fibre compositions between 11 highly trained athletes, 10 fitness trained and 7 untrained individuals. They found a

significant increase in the type I fibre percentage in the fitness trained and untrained groups. However, the type I fibre percentage did not increase further in the group of highly trained subjects where the type I fibre percentage was above 70 % at the baseline (Trappe *et al.*, 1995). The shift to slow fibres appeared attenuated in the highly trained athletes, although this may be attributable to some of the elite performers originally possessing up to 90 % ST fibre proportions. These data also suggest that an increase in type I fibre percentage is probably more an effect of ageing more than an effect of training, in agreement with another study (Short *et al.*, 2005).

Another cross-sectional study tested the muscle fibre composition and enzyme activities from the gastrocnemius of 14 elite male distance runners in comparison to untrained males. On average, it was found that the trained athletes showed evidence of significantly more ST fibres than the untrained males (Costill *et al.*, 1976), although this could of course be genetic predisposition. A longitudinal study assessed the effects on skeletal muscle fibre type alteration with high-intensity intermittent training consisting mainly of series of supramaximal exercise lasting 15 s to 90 s on a cycle ergometer. Their results indicated that sedentary subjects elicited an increase in the percentage of type I fibres and decreased type IIx fibres, whilst the proportion of type IIa remained unchanged (Simoneau *et al.*, 1985). These data are atypical, in denoting a shift from type IIx to I with high-intensity training.

Another study on seven young females subjected to 24 weeks of intensive endurance training found that the relative amount of type I fibres before and after the training period was unchanged and for type IIa fibres was 26.4 and 31.5 %, for type IIx fibres

9.2 and 3.4 % and for type IIC fibres 0.4 and 2.2 %. Therefore, showing interchange between fast twitch sub-groups with a decrease in type IIx and increase in type IIa (Ingjer, 1979). Another study examined orienteers, where comparisons were made between their trained leg muscles, against the leg muscles of untrained 16-18 years old boys. Whilst type I fibres were not different, the distribution of type II sub-groups in the leg muscles of the orienteers differed from the leg muscles of the controls, the relationship IIa/IIx being altered in favour of the more oxidative IIa. The leg muscles of the orienteers also showed an increased occurrence of IIC fibres (Jansson & Kaijser, 1977). These latter findings point at the possibility of a training induced alteration in the fast sub-group pattern, again reducing the population of IIx in favour of IIa, and an increase in group C hybrid fibres.

A more recent study examined subjects of different training background. Subjects were classified into three groups according to their physical activity and sport discipline: untrained students (group A), national and sub-national level endurance athletes (group B, 7.8+/-2.9 years of specialised training) and sprint-power athletes (group C, 12.8+/-8.7 years of specialised training). Significant differences regarding composition of muscle fibre types and MHC were found only between groups A (41.7+/-1.6 % of MHC I, 40.8+/-4.0 % of MHC IIa and 17.5+/-4.0 % of MHC IIx) and B (64.3+/-0.8 % of MHC I, 34.0+/-1.4 % of MHC IIa and 1.7+/-1.4 % of MHC IIx) and groups A and C (59.6+/-1.6 % of MHC I, 37.2+/-1.3 % of MHC IIa and 3.2+/-1.3 % of MHC IIx) (Zawadowska *et al.*, 2004). Interestingly, endurance athletes (group B) such as long-distance runners, cyclists and cross country skiers did not differ from the athletes representing short term, high power output sports (group C) such as ice hockey, karate, ski-jumping, volleyball, soccer and modern dance.

Furthermore, the relative amount of the fastest MHC IIx isoform in Vastus Lateralis muscle was significantly lower in the athletes from group C than in students (group A). Therefore it appears that probably any form of training reduces MHC IIx, but may also increase MHC I, at least as is evident from this cross-sectional study. In conclusion in human beings, endurance training usually does not cause type II fibres to turn into type I fibres (Gollnick *et al.*, 1973), but that does not eliminate the possibility after very long periods of training. It is most likely that there is a decrease in IIx MHC and an increase in IIa MHC (Jansson & Kaijser, 1977; Ingjer, 1979), and increases in hybrid fibres such as IIC. Furthermore, even power events probably changes fibre types from MHC IIx to MHC IIa. Many of these changes are also muscle-dependent, which might explain some variations in the reported data.

1.4.2 Chronic motor nerve stimulation promotes a fast-to-slow phenotype shift and provides a model mimicking endurance training

Chronic electrical motor nerve stimulation (CMNS) is a model that has been employed for studies within chapters 4 and 5 of this thesis. CMNS has been adopted to study the effects of muscle plasticity, and is used for its efficacy to mimic physiological responses to endurance training at the extreme end of the continuum. CMNS, carried out over several weeks can induce fibre type shifting from fast-to-slow twitch, and robustly reproduce adaptations elicited by endurance exercise (Henriksson *et al.*, 1989). CMNS is advantageous as a model since no habituation is required; stimulations are reproducible; stimulus is restricted to the muscles of choice thereby elminiating systemic effects; and all motor units are synchronously activated. Detailed below are the typical morphological, ultrastructural and performance

characteristics of changes at the muscle as a result of CMNS. When applied permanently for several weeks at 10 Hz, fast isoforms of MHC (discussed in 1.4.1) and myosin light chain are gradually replaced by slow varieties as a result of repression of fast isoform expression in conjunction with increased slow isoform expression. The 'nearest neighbour' theory proposes that fibre types will usually shift along the following continuum depending upon starting MHC: MHCI↔MHCI/IIa↔MHCIIa↔MHCIIa↔MHCIIa/IIx↔MHCIIx↔MHCIIX/Iib↔MHCIIb (Staron & Pette, 1993). This pattern of change also transpires for the other motor proteins such as troponin and tropomyosin (Kraus *et al.*, 1994). These increases in the expression of slow motor proteins, and especially the type I MHC consistently occurs in CMNS models, although to different extents dependent upon species and duration of stimulation. For example, rabbit EDL muscle undergoes a 100 % shift to type I MHC following 7 weeks of stimulation (Sutherland *et al.*, 1998), whereas rat and especially mouse muscle, are more resilient to CMNS and thus slower to transit (Jarvis *et al.*, 1996). There are also concomitant increases in mitochondrial volume and numbers with CMNS (Chi *et al.*, 1986), coupled with an increased number of nuclei per fibre (Kraus *et al.*, 1994) resulting in a decrease in the area managed by individual nuclei (Joplin *et al.* 1987). The decondensation of hetrachromatin in myonuclei and nucleolar enlargement suggest increased transcriptional activity (Joplin *et al.*, 1987). In support of this, increases in ribosome and polyribosomes in the sarcoplasm suggest increased transcriptional activity from DNA to RNA (Kraus *et al.*, 1994). CMNS leads to a rapid loss of force within a very short time period and ultimately there is an increase in time to peak twitch tension due to the underlying changes in myosin isoforms as the fast muscle begins to take on slow characteristics (Henriksson *et al.*, 1986). The Ca^{2+}-ATPase activity decreases, eaxplained by the shift

from fast SERCA1 to slow SERCA2a isoforms. There also appears a reduced capacity for Ca^{2+} uptake by the SR as well as a decrease in cytosolic Ca^{2+} buffering, secondary to a decline in parvalbumin content (Eisenberg & Salmons, 1981). Phospholamban levels also decrease, and since the phosphorylation-state of this protein is responsible for co-localising intracellularly with the slow isoform of Ca^{2+}ATPase, this further underlines a shift towards a slower phenotype (Kraus *et al.*, 1994). There is marked restructuring from an anaerobic to aerobic system shown by a collapse of the adenylate and creatine systems (PCr); although the muscle maintains a remarkable ability to recover from the initial insult on the ATP generating system which has been demonstrated by a temporary super-compensation in glycogen levels (+25 %) (Henriksson *et al.*, 1986). Enzymes of the Krebs cycle such as succinate dehydrogenase, citrate synthase and malate dehydrogenase increase their turnover (Henriksson *et al.*, 1986) to counteract increased contractile work-loads. Changes in enzymes involved in β-oxidation parallel this citric acid cycle modulation in showing an increase in 3-hydroxyacyl-CoA dehydrogenase, and carnitine acyltransferase which are coupled with increased extracellular albumin (fatty acid transporter), and an increase in fatty-acid binding proteins that function as intracellular lipid traffickers (Henriksson *et al.*, 1986). Increases in oxidative enzymes and machinery, are accompanied by an increase in myoglobin and capillarisation thus allowing oxygen utilisation to be maximised (Kraus *et al.*, 1994). These physiological changes mirror those encountered with chronic endurance training, yet on a much accelerated level.

However, aside from the rate in which CMNS elicits physiological change, there are other negative points as a physiological model. Under normal circumstances, Henneman's size principle dictates that smaller motor units (type I) are recruited at

low levels of force and are therefore active most often, whereas larger motor (type II) units are recruited less frequently (Henneman et al., 1965). However, if the force exerted by a muscle is increased from a sub-threshold level of CMNS, then there is a tendency for the larger axons to be depolarised first, which creates an inversion of normal recruitment patterns since larger motor units are permanently in use. Moreover, CMNS excites all the motor units at the same frequency and synchronously, resulting in a contraction which is grossly oscillatory and superficial to the asynchronous discharge rate seen in reality. In support of this it has been shown that CMNS produces more limited changes in myosin if applied intermittently or at a frequency lower than 10 Hz. Therrefore, artificially applied CMNS is often far and above any frequency/time scale likely to be training induced (Pette *et al.*, 1975). Nevertheless, CMNS is a useful model and produces changes in physiology that parallel those obseved with chronic endurance exercise, as discussed in section 1.4.

1.5 Cell signalling mediating physiological adaptations of skeletal muscle to endurance exercise training

Recent literature has implicated many candidate pathways that mediate the well characterised physiological responses to endurance training. These pathways are contraction responsive and have been shown to be involved in phenotypic change such as control of fibre type or mitochondrial biogenesis, the key muscular adaptations to endurance exercise. Below, each pathway implicated in these responses will be sequentially analysed in order to dissect the mechanisms by which activation of these proteins might create phenotype changes through changed genetic responses.

1.5.1 Control of mitochondrial biogenesis

Mitochondria are the 'cellular engines' that provide an ATP supply through oxidative phosphorylation and increase in their numbers and size is associated with endurance training (Freyssenet *et al.*, 1996). Endurance exercise activates signal transduction pathways that either stimulate or affect expression of transcription factors that regulate the expression of genes encoding mitochondrial proteins. One such protein is the adenosine monophosphate kinase (AMPK) which has been shown to be activated in response to contraction (Frosig *et al.*, 2004) and mediate many cellular responses to exercise (figure 1.5). AMPK is a heterotrimeric protein kinase with multiple isoforms for each subunit (alpha, beta, and gamma). Whereas only the alpha-1 and alpha-2 subunits are catalytic, the beta and gamma subunits are regulatory and necessary for full enzymatic activity. AMPK is activated by low glycogen, increased AMP levels and inhibited by phosphocreatine (PCr) aggregation (Winder & Hardie, 1996). These are situations which are common in endurance activity with the chronically elevated ATP turnover, and thus provide the stimulus for AMPK activation.

The link between AMPK and mitochondrial biogenesis comes from PGC-1α, since AMPK activation is known to induce PGC-1α (Suwa *et al.*, 2003). PGC-1α was first shown to be increased in response to cold and led to induction of nuclear respiratory factor 1 (NRF-1) and mitochondrial transcription factor A (TFAM) to control mitochondrial biogenesis (Wu *et al.*, 1999). Studies have shown that PGC-1α is upregulated as a result of exercise and that pharmacological activation of AMPK by 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) increases PGC-1α expression as well (Terada *et al.*, 2002). Finally, over-expression of the transcription

factor PGC-1α upregulates not only mitochondrial genes, but also non-mitochondrial genes such as myoglobin and slow troponin (Lin *et al.*, 2002) and muscles of transgenic mice over-expressing PGC-1α are visibly redder and contain a higher proportion of type I oxidative fibres. Therefore, activated AMPK is likely to be responsible for increases in NRF-1 which is a key factor involved in nuclear gene transcription (Wu *et al.*, 1999) and act in concert with PGC-1α and TFAM to regulate mitochondrial biogenesis and slow fibre formation. The protein p38 is also thought to induce PGC-1α (Barger *et al.*, 2001). Since p38 MAPK has shown to be significantly activated (through increased phosphorylation) in response to CMNS some of the effect is probably mediated through this MAPK. Thus, AMPK is a major activator of mitochondrial biogenesis when cellular energy metabolism is under increased stress (Bergeron *et al.*, 2001) as during endurance exercise.

Once nuclear genes are expressed, genes from mitochondrial DNA must also be, since mitochondrial biogenesis requires the coordinated expression of nuclear and mitochondrial genes in concert. It has been shown that TFAM is capable of activating mitochondrial DNA replication and expression (Fisher *et al.*, 1989). In support of this, TFAM expression increases as a response to chronic exercise, suggesting that TFAM is indeed involved in activation of mitochondrial biogenensis as a result of endurance exercise (Gordon *et al.*, 2001). TFAM knock-out mouse embryos die because of a complete absence of oxidative phosphorylation and a severe depletion of mitochondrial DNA, highlighting the essential role of TFAM in mitochondrial DNA replication (Larsson *et al.*, 1998). Therefore it appears that AMPK activated PGC-1α is the master controller of mitochondrial biogenesis through TFAM and NRF to control the expression of both nuclear and mitochondrial DNA.

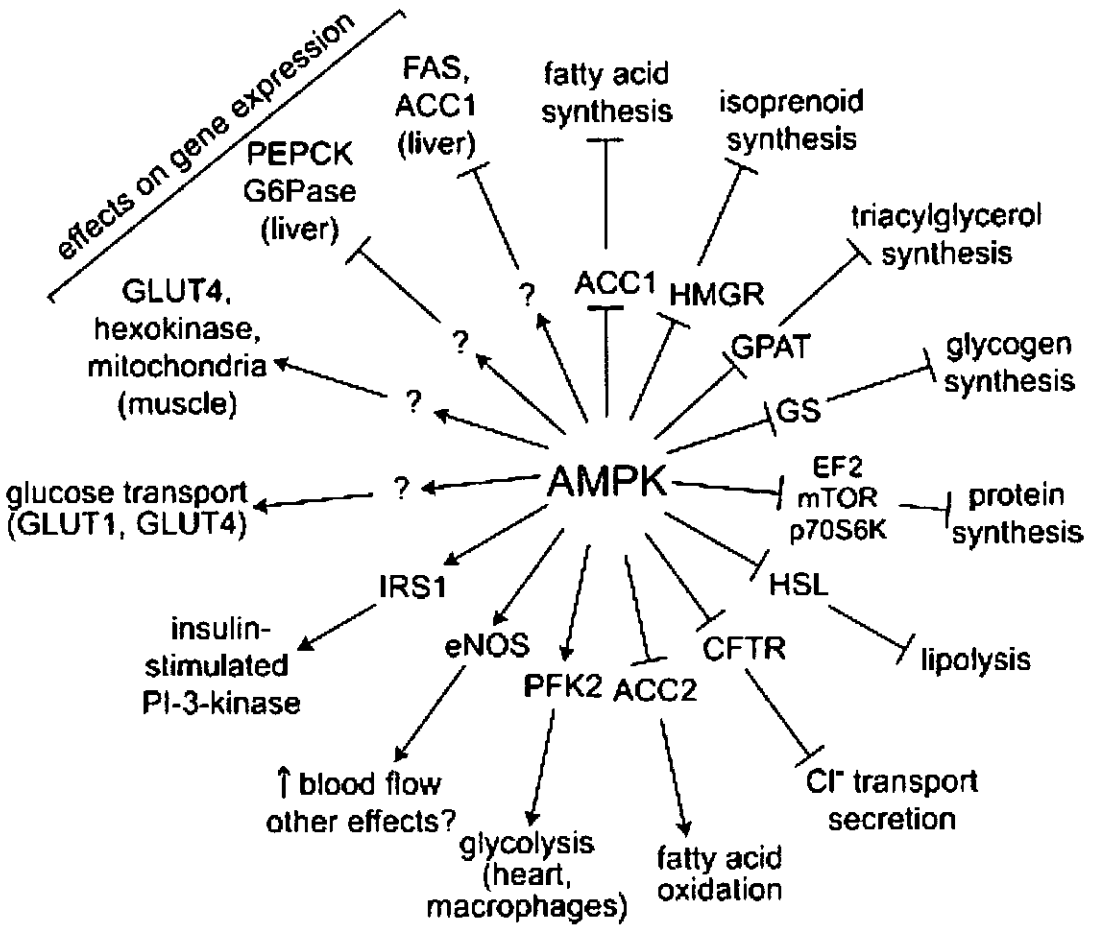


Figure 1.5. Diagram to show the known physiological target proteins and pathways regulated by the 'master regulatory' AMPK system. AMPK is switched on by cellular stresses that either interfere with ATP production (e.g. hypoxia, glucose deprivation, or ischemia) or by stresses that increase ATP consumption (e.g. muscle contraction). It is also activated by hormones that act via Gq-coupled receptors, and by leptin and adiponectin, via mechanisms that remain unclear. Once activated, the AMPK system switches on catabolic pathways that generate ATP such as through enhancing fatty acid oxidation, while switching off ATP-consuming processes that are not essential for short-term cell survival, such as the synthesis of lipids, carbohydrates, and proteins. Therefore AMPK activation by exercise is not only limited to affecting mitochondrial biogenesis and fibre type. Diagram taken from Hardie, (2003).

1.5.2 The Calcineurin (CnA) pathway and fibre type regulation

One of the major signals associated with adaptation to endurance exercise are contraction-induced intracellular free Ca^{2+} ($[Ca^{2+}]_i$) oscillations. Muscle fibre type characteristics are thought to be dependent on the frequency of motor nerve stimulation and some of this effect is proposed to be controlled by Ca^{2+}-dependent signalling. This is supported by the fact that increased $[Ca^{2+}]_i$ levels have been reported in muscles undergoing transition to a slower phenotype (Sreter *et al.*, 1987).

One such candidate pathway implicated in mediating response to Ca^{2+} signalling has been the calmodulin-dependent protein phosphatase, calcineurin (figure 1.6). The model proposes that tonic motor nerve activity sustains $[Ca^{2+}]_i$ at levels sufficient to activate the calcineurin-nuclear factor of activated T cells (NFAT) pathway. The protein phosphatase activity of calcineurin leads to dephosphorylation and nuclear localisation of NFAT proteins. In the nucleus, NFAT proteins bind DNA in conjunction with other transcriptional regulators, including myocyte enhancer factor 2 (MEF2). Binding sites for these regulators are clustered in promoter/enhancer regions controlling transcription of genes encoding proteins of the slow-fibre programme such as myoglobin and troponin I (Chin *et al.*, 1998). This is supposedly specific to endurance-like activity since high amplitude $[Ca^{2+}]_i$ transients stimulated by infrequent, phasic firing of the motor nerve are of insufficient duration to maintain calcineurin in the active state, so NFAT protein in remain phosphorylated and are excluded from the nucleus (Chin *et al.*, 1998), (figure 1.6). Therefore the calcineurin pathway is an example of a network which can be activated selectively depending upon the specific type of contractile activity.

It is, however, contentious as to the precise significance of calcineurin in control of phenotype. It appears that calcineurin is important for controlling fibre type since administration of the calcineurin inhibitor, cyclosporin A (CsA), reduced slow fibre gene expression *in vivo*, suggesting a key role of calcineurin in activation of the slow muscle fibre phenotype (Chin *et al.*, 1998). In support of this, the calcineurin specific inhibitor, FK506, was administered and whilst the control overloaded plantaris muscle showed fast-to-slow muscle fibre type transition and decrease in MHC IIb, increase in MHC IIa+d/x, and new expression of MHC I; in the FK506-administered group, overload-induced muscle fibre-type transition was completely prevented (Miyazaki *et al.*, 2004). Furthermore, in transgenic mice that express activated calcineurin there is an increase in slow muscle fibres (Talmadge *et al.*, 2004), whilst in calcineurin knock-out mice, overload-induced fibre type switch is inhibited (Parsons *et al.*, 2004).

However, in contrast to these findings it has also been shown that not only slow muscle promoters but also the fast muscle promoters for phosphoglycerate mutase, skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase, and MHC IIb were activated by over-expressed calcineurin in cultured muscle cells (Swoap *et al.*, 2000). These experiments suggested that calcineurin also activates some "fast" genes in skeletal muscle. Furthermore, the role of calcineurin operating in tandem with NFAT is unclear, since NFAT over-expression had no effect upon SERCA1 promoters in C2C12 cells, whilst constitutively active calcineurin increases SERCA1 promoter activity (Swoap *et al.*, 2000). Therefore, there remains some controversy over the importance of calcineurin and calcineurin-NFAT interactions in control of fibre type.

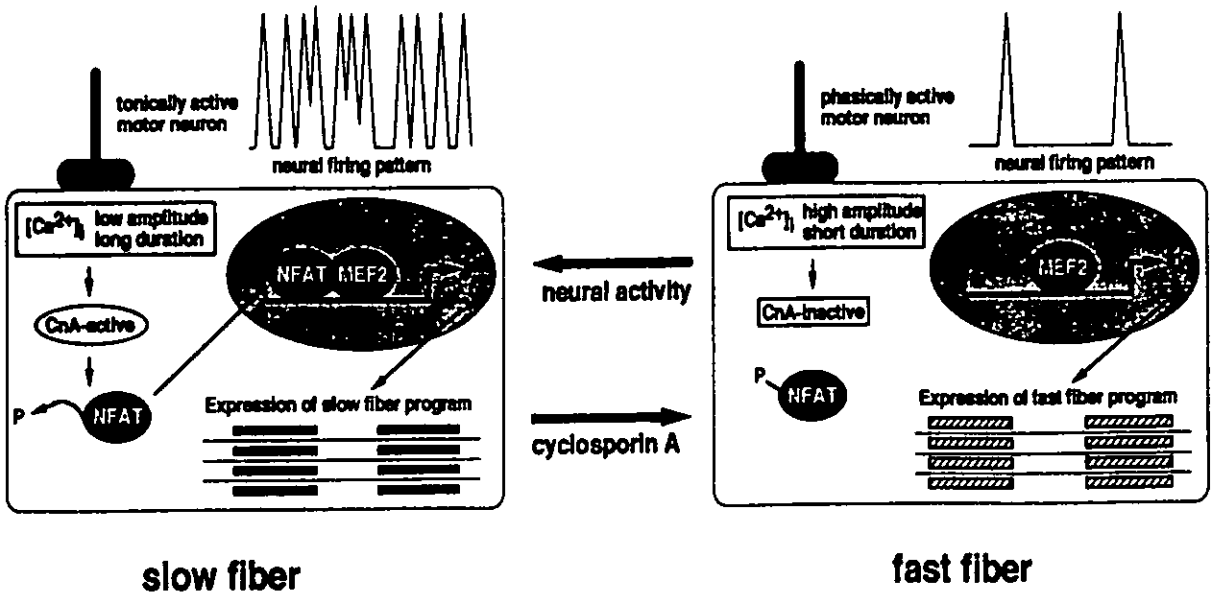


Figure 1.6. A summary model for the calcineurin (CnA)-dependent pathway which links contrasting motor nerve activity to gene expression that produces phenotypic differences between fibre types. Calcineurin, is activated by chronic elevation of $[Ca^{2+}]_i$ casued by the release of Ca^{2+} from the SR in response to contraction. Ca^{2+} binds to coth calmodulin and the B sub-unit of Calcineurin. Once activated, calcineurin has a phosphatase activity that dephosphorylates the nuclear factor of activated T cells (NFAT), unmasking the nuclear localisation signal on NFAT. Nuclear import of NFAT bound calcineurin leads to the collaboration with myocyte enhancer factor 2 (MEF2) to promote expression of the slow gene programme. The four MEF2 isoforms (A-D) belong to the super-family of MADS (MCM1, agamous, deficiens, and serum response factors nuclear phosphoproteins), which bind to DNA. The specific activation of calcineurin would be expected with endurance-like exercise. Conversely, high amplitude short duration $[Ca^{2+}]_i$ oscillations, such as in high intensity resistance-like intermittent exercise, means that calcineurin remains inactive and thus does not affect the expression of slow genes. Treatment of cells with cyclosporine inhibits calcineurin activation. Diagram taken from Chin *et al.*, (1998).

1.5.3 Ca^{2+}/calmodulin kinase dependent protein kinases and control of fibre type

Members of the Ca^{2+}/calmodulin dependent protein kinase (CAMK) family are serine/threonine protein kinases, and can be distinguished according to whether they have a single substrate such as phosphorylase kinase, CAMKIII and myosin-light chain kinase or are multi-functional having several substrates such as CAMK,I, II and IV (Hook & Means, 2001). Specifically, CAMKI and CAMKIV are implicated in regulating Ca^{2+} dependent transcription in cells (Hook & Means, 2001). In C2C12 myotubes, CAMKIV acts synergistically with calcineurin to increase transcription from MEF2-dependent enhancers through interactions with histone deacetylases (Wu *et al.*, 2000), thus suggesting effects on slow gene expression. Furthermore, transgenic mice that expressed a constitutively active form of CAMKIV in type II fibres, exhibited increased mitochondrial biogenesis and number of type I fibres, probably through increasing the expression of PGC1α (Soderling, 1999). These data suggest that CAMK isoforms may control fibre type, although much of the data centres around CAMKIV, which is not, or only lowly expressed in skeletal muscle (Soderling, 1999). However, CAMKI is expressed in skeletal muscle and whilst its role in fibre typing in unclear, it is possible that CAMK proteins have a role in fibre type regulation *in vivo*, although the precise mechanisms remain to be elucidated.

1.5.4 The ERK1/2 pathway and control of fibre type

The mitogen activated protein kinase (MAPK) ERK1/2 has also been shown to regulate fibre type, especially through controlling transcription of MHC isoforms and

muscle enzymes. A study using the MEK1/2 inhibitor U0126 on spontaneously twitching muscle cells significantly decreased type I MHC mRNA levels and significantly increased MHC IIx, MHC IIb, embryonal MHC and perinatal MHC mRNA levels (Higginson *et al.*, 2002). Another group used *in vivo* transfection in regenerating muscle, to show that constitutively active Ras and a Ras mutant that selectively activates the MAPK (ERK) pathway are able to mimic the effects of slow motor neurons on expression of myosin genes (Murgia *et al.*, 2000). Since ERK activity is increased by low-frequency electrical stimulation (Atherton *et al.*, 2005) and endurance exercise (Yu *et al.*, 2001), these results indicate that Ras-MAPK signalling is involved in promoting nerve-activity-dependent differentiation of slow muscle fibres *in vivo*.

1.5.5 The PKC pathway and control of fibre type

There are many distinct isoforms of Protein kinase C (PKC), including those that are Ca^{2+} and diacylglycerol sensitive (PKCα, PKCβ, PKCγ) and those which are only diacylglycerol responsive (PKCδ, PKCε, PKCη, PKCθ). Diacylglycerol stimulates PKC activity by greatly increasing the affinity of the enzyme for Ca^{2+} ions. Treatment of cultured avian muscle cells with staurosporine, a PKC inhibitor, increases the expression of slow MHC which was not expressed in control cells (DiMario & Funk, 1999). In a second experiment, PKCα and PKCτ were over-expressed and repressed slow MHC expression *in vivo* (DiMario, 2001). Therefore it appears that PKC inhibits slow MHC expression and thus the activation of PKC can not explain the endurance-training effect. Further studies are required on PKC isoforms in mammalian cells in order to clarify the effects, if any, of PKC on control of fibre type.

1.5.6 The AMPK- PGC-1α pathway and control of fibre type

The transcriptional co-activator PGC-1α which as explained previously controls mitochondrial biogenesis, also appears to have other effects on phenotype. When PGC-1α alpha is expressed at physiological levels in transgenic mice driven by a muscle creatine kinase promoter, a fibre type conversion is observed. Those muscles normally rich in type II fibres are redder. Notably, putative type II muscles from PGC-1α transgenic mice also express proteins characteristic of type I fibres, such as troponin I and myoglobin, and show a much greater resistance to electrically stimulated fatigue (Lin *et al.*, 2002). Interestingly, there is also a link between PGC-1α and calcineurin. One study found a simultaneous increase in PGC-1α mRNA which was associated with calcineurin activation (Garnier *et al.*, 2005). The results provide strong support for a central role for PGC-1α and calcineurin activation in mitochondrial biogenesis and control of fibre type in skeletal muscles.

1.6 Cell signalling mediating physiological adaptations of skeletal muscle to resistance exercise training

The predominant adaptation to resistance training is muscle hypertrophy. This adaptation is such in contrast to endurance exercise where the muscle sometimes actually loses mass and there is a more prominent phenotype shift. The vast majority of literature suggests that fibre hypertrophy (growth in size of existing fibres) is the main mechanism explaining muscle growth in response to resistance training (Gollnick *et al.*, 1981; Alway *et al.*, 1988; Frontera *et al.*, 1988), rather than

hyperplasia (increase in the number of fibres). Muscle hypertrophy is accompanied by only a limited fibre type effect and there is evidence of reduced IIx myofibre distribution with a concomitant increase in type IIa distribution (see 1.4.1). Resistance training-induced hypertrophy predominantly occurs in type II fibre sub-types and only little or not in type I fibres (Hather *et al.*, 1991). This physiological adaptation of increased muscle size is of primary importance since the ability to perform feats of strength and power is to a large extent, limited by muscle mass.

1.6.1 Protein synthesis, training and nutrition

Hypertrophy of skeletal muscle requires a net positive protein balance meaning that protein synthesis must exceed protein breakdown. In most models, including exercise, when protein synthesis increases so does protein breakdown and when protein synthesis falls protein breakdown also falls, adaptively (Cuthbertson *et al.*, 2004). Resistance exercise is a powerful way to increase protein synthesis and thus muscle mass, and in fact, after a single bout of resistance training, muscle protein synthesis (MPS) remains elevated for >48 h (Chesley *et al.*, 1992). Nutrients also stimulate protein synthesis, and feeding protein or amino acids alone without resistance training stimulates protein synthesis, although the effect lasts only for ~2 h (Bohe *et al.*, 2001). After a bout of resistance exercise, protein breakdown increases as well (Biolo *et al.*, 1995) and without food intake, net protein breakdown results. These studies show that both protein synthesis and breakdown increase in response to resistance training, however feeding is necessary to stimulate a chronic positive protein balance (Tipton *et al.*, 1999). A post-exercise diet of protein and carbohydrate will enhance protein synthesis through increased availability of amino acids whereas carbohydrate-induced

insulin increases will reduce protein breakdown (Rennie & Tipton, 2000). Therefore it is the combination of resistance exercise and nutrition that produces a long-lasting net positive protein balance, and thus potential for hypertrophy. Timing of nutrition also seems important and maximum effects are observed when feeding occurs immediately post-exercise in comparison to feeding 2 h post-exercise (Esmarck *et al.*, 2001).

1.6.2 Role of satellite cells in mediating hypertrophy

Much research has attempted to resolve the mechanisms of hypertrophy, and one major dynamic of this process involves satellite cells. During muscle hypertrophy the number of myonuclei tends to increase proportionally with the increase in fibre volume, thus maintaining the myonuclear domain : cytoplasm ratio (Roy *et al.*, 1999). Since skeletal muscle is a post-mitotic tissue and thus incapable of cell division, this additional genetic material must then be derived from satellite cells. Satellite cells are located in the basal lamina of muscle fibres (Kadi *et al.*, 2004a) and are myogenic cells attributed with the role of postnatal growth and regeneration in skeletal muscle.

Following proliferation and subsequent differentiation, these cells fuse with one another or with the adjacent muscle fibre, thereby increasing myonuclei numbers for fibre growth and repair (Allen & Rankin, 1990). Their importance in hypertrophy is highlighted since in response to synergist ablation (a growth stimulus for the antagonistic muscle), first satellite cells and then myonuclei increase in the hypertrophying muscle, adding further support to the hypothesis that satellite cells proliferate first and subsequently fuse with muscle fibres (Snow, 1990). There is further evidence that satellite cell proliferation is critical for the muscle growth

response. If mild γ-irradiation is used to block satellite cell proliferation then rat gastrocnemius muscle does not hypertrophy in response to synergist ablation (Rosenblatt *et al.*, 1994). In another study it was confirmed that γ-irradiation could prevent most hypertrophy achieved by overload in plantaris over the period of three months (Adams *et al.*, 2002). These data support the case that hypertrophy involving satellite cell proliferation is a major mechanism of muscle growth.

Several studies also suggest that satellite cell proliferation and nuclei uptake into muscle fibres occur in response to resistance training in human skeletal muscles. Subjects that undertake a resistance training programme to elicit muscle hypertrophy have been shown to possess a larger number of nuclei per fibre (Kadi *et al.*, 1999). Another study showed that after 10 weeks of resistance training in females, fibre hypertrophy is accompanied by both a ~70 % increase in the number of nuclei, and a 46 % increase in the number of satellite cells (Kadi & Thornell, 2000). This observation suggests that both satellite cell proliferation and fusion are associated with growing muscle fibres in humans. Finally, satellite cells increased by 19 % and 31 % after 30 and 90 days, respectively, following resistance training and decreased following cessation of training confirming that satellite cells respond with proliferation to resistance training, and that this is a reversible phenomenon in a situation of detraining (Kadi *et al.*, 2004b). Signalling controlling satellite cell proliferation is not well established, but probably involves the opposing actions of myostatin and IGF-1. Myostatin negatively regulates protein synthesis and the satellite cell cycle (McCroskery *et al.*, 2003), whereas IGF-1 (Barton-Davis *et al.*, 1999) not only increases muscle protein synthesis but also positively mediates satellite cell proliferation (Chakravarthy *et al.*, 2001; Barton-Davis *et al.*, 2001).

1.7 Signalling pathways controlling protein synthesis

Satellite cells might provide additional genetic material to assist increases in size, but hypertrophy requires gross increases in protein synthesis. In recent years there have been many muscle growth factors (myostatin, IGF-1, etc) and signalling pathways (PI-3K pathway, calcineurin etc) implicated in mediating control of muscle mass.

1.7.1 Muscle growth factors – Negative regulation by myostatin

The transforming growth factor (TGFβ) super-family is a group of proteins that regulate growth, differentiation and apoptosis. One subclass within this family are the growth and differentiation factors (GDFs). Myostatin is a GDF that was discovered in 1997, and genetic knock-out of myostatin in mice results in skeletal muscle hypertrophy and hyperplasia (McPherron *et al.*, 1997). Recently, a myostatin mutation was shown to promote gross muscularity in a child, thus confirming that a lack of functional myostatin can also increase muscle mass in humans (Schuelke *et al.*, 2004). The mouse myostatin knock-out model demonstrated the mechanisms by which myostatin exerts its effects through development, and this was shown to be through its inhibitory effects on the cell cycle. With functional myostatin during embryonic myogenesis, Myf-5 and MyoD specify cells to adopt the myoblast fate. Myoblasts then migrate and proliferate. In response to myostatin signalling, p21 is up-regulated, inhibiting cyclin-E·CDK2 activity, which causes retinoblastoma inactivation and G_1 arrest. Thus, myoblast number and, hence, fibre number, following differentiation, is limited. However, in the absence of functional myostatin, the signal for p21 up-regulation is lost and retinoblastoma remains in a hyperphosphorylated state, resulting

in increased myoblast proliferation and thus increased fibre number (Thomas *et al.*, 2000). Since myostatin has effects on the cell cycle, this means it may be important in hypertrophy through its effects on satellite cells. To illustrate this, there is evidence that myostatin has a negative effect on satellite cell proliferation (McCroskery *et al.*, 2003). This study showed that during development, myostatin up-regulated p21, a cyclin-dependent kinase (CDK) inhibitor, and decreased the levels and activity of CDK2 protein in satellite cells. Hence, myostatin negatively regulates the G1 to S progression and thus maintains the quiescent status of satellite cells.

This cell cycle effect has important implications given the aforementioned importance of satellite cell proliferation in hypertrophying muscle (Rosenblatt *et al.*, 1994) (see 1.6.2). Support for this comes from the facts that: 1) myostatin null mutants display muscle hypertrophy and hyperplasia, both of which may relate to elevated satellite cell activity and proliferation, 2) hind-limb unloading causes suppression of satellite cell proliferation, which coincides with increased myostatin expression, and 3) fast-twitch muscles have lower concentrations of satellite cells, which correlates with the higher level of expression of myostatin in fast muscle, and finally 4) the myostatin protein is concentrated at myotendinous junctions, where satellite cells are also present in greater densities than any other place within muscle cells (Wehling *et al.*, 2000).

1.7.1.1 Regulation of the myostatin gene

Several studies have attempted to investigate regulation of the myostatin gene expression on a molecular level. Putative binding motifs for glucocorticoids, androgens, thyroid hormones, myogenic differentiation factor 1, MEF2, PPARγ, and

NFκB were predicted in one study (Ma *et al.*, 2001) (figure 1.7). Of these, the predicted upregulation of myostatin expression by glucocorticoids was experimentally verified (Ma *et al.*, 2003). In another study it was shown that growth hormone suppressed the expression of myostatin in growth hormone-deficient patients and in cultured skeletal muscle cells (Liu *et al.*, 2003). The transcription factor and binding site by which the growth hormone effect is mediated are currently unknown and could be indirect via induction of IGF-1. Support for this hypothesis comes from the finding that IGF-1/IGFBP-3-complexes were shown to reverse the alcohol-induced increase in myostatin expression (Lang *et al.*, 2004).

In animal experiments, it was shown that myostatin mRNA increases in some muscles during atrophy-inducing hind-limb unloading (Carlson *et al.*, 1999) and space flight (Lalani *et al.*, 2000). In contrast, myostatin mRNA decreases during recovery and muscle re-growth after muscle injury (Kirk *et al.*, 2000). Surprisingly, in myostatin knock-out mice, hind-limb unloading leads to greater atrophy than in their wild-type controls (McMahon *et al.*, 2003), due to a repressed myogenic response to atrophy. In humans, myostatin mRNA levels have been shown to increase in disuse atrophy (Reardon *et al.*, 2001) and ageing (Yarasheski *et al.*, 2002). The role of myostatin in exercise adaptation is contentious. In rats, myostatin mRNA decreases in fast muscle in response to short-term endurance training consisiting of swimming 5 days/week (Matsakas *et al.*, 2005). In human beings some reports suggest that myostatin mRNA or circulating myostatin does decrease in response to resistance training (Zambon *et al.*, 2003) whereas others found no effect (Willoughby, 2004). Myostatin and endurance training has not yet been examined. Therefore the role of myostatin in controlling responses to training and disease in humans remains largely unidentified.

1.7.1.2 Myostatin regulates post-development muscle mass by affecting transcription and translation

Increasing systemic myostatin leads to cachexia (Zimmers *et al.*, 2002) and an increased muscle and serum myostatin concentration was found in HIV-infected men whom undergo muscle wasting (Gonzalez-Cadavid *et al.*, 1998). Therefore it appears that myostatin is not just solely controlling the developmental programme but is also regulated by other stimuli. Mechanisms by which myostatin might be regulated through exercise or disease could come through regulatory proteins linked to myostatin. Myostatin is under regulation from follistatin, the follistatin-related gene (FLRG) (Lee & McPherron, 2001), the myostatin propeptide and a protein named Gasp-1 (Hill *et al.*, 2003) all of which are proteins that can bind and inhibit myostatin. The formation of myostatin-inhibitor protein heterodimers is likely to prevent activin type II receptor binding of myostatin. This has been demonstrated for the genetically similar bone morphogenetic protein 7 (BMP7) and the protein Noggin, which dimerise and the resulting heterodimer cannot bind to the receptor (Groppe *et al.*, 2002). Perhaps these binding proteins may be affected by exercise or disease stimuli to affect myostatin signalling, either positively or negatively.

It appears then that myostatin can modulate adult skeletal muscle, yet the mechanisms controlling this remain to be elucidated. When not inhibited by its regulatory proteins in circulation, it is possible that myostatin may exert its effects through changing gene expression. Myostatin is a secreted protein that signals through TGFβ receptors which stimulate the phosphorylation of receptor-regulated mothers against decapentaplegic homolog (SMAD) proteins, which in turn form complexes with SMAD4 that

accumulate in the nucleus and regulate the transcription of target genes (ten Dijke & Hill, 2004). Inhibitory SMAD7, reduces myostatin-induced transcription and SMAD7 expression is stimulated by myostatin via the interaction between SMAD2, SMAD3, SMAD4 and the SBE (Smad binding element) in the SMAD7 promoter. These results suggest that the myostatin signal transduction pathway is regulated by SMAD7 through a negative feedback mechanism (Zhu *et al.*, 2004), and positively through SMAD 2,3,4. There is also evidence that myostatin has a negative effect on protein synthesis in cultured muscle, as cells treated with recombinant myostatin exhibit a decrease in protein synthesis (Taylor *et al.*, 2001). The mechanisms behind this effect are unclear although knowledge of this process would yield interesting information regarding the way in which myostatin negatively regulates growth.

In summary, myostatin is a secreted protein that acts as a negative regulator of skeletal muscle mass. During embryogenesis, myostatin is expressed by cells in the myotome and in developing skeletal muscle and acts to regulate the final number of muscle fibres that are formed by controlling the cell cycle. During post-mitotic adult-life, myostatin protein is produced and secreted by skeletal muscle, circulates in the blood, and acts to limit muscle fibre growth. The precise mechanisms by which myostatin inhibits muscle growth remain to be eulicidated, although involves the negative effect on satellite cell cycle and MPS. Interestingly, the existence of such circulating tissue-specific growth inhibitors of this type was hypothesised over 40 years ago to explain how sizes of individual tissues are controlled, but examples of such factors remained elusive. Skeletal muscle appears to be the first illustration of a tissue whose size is controlled by this type of negative regulatory mechanism, and thus myostatin appears to be the first example of the long sought 'chalone'.

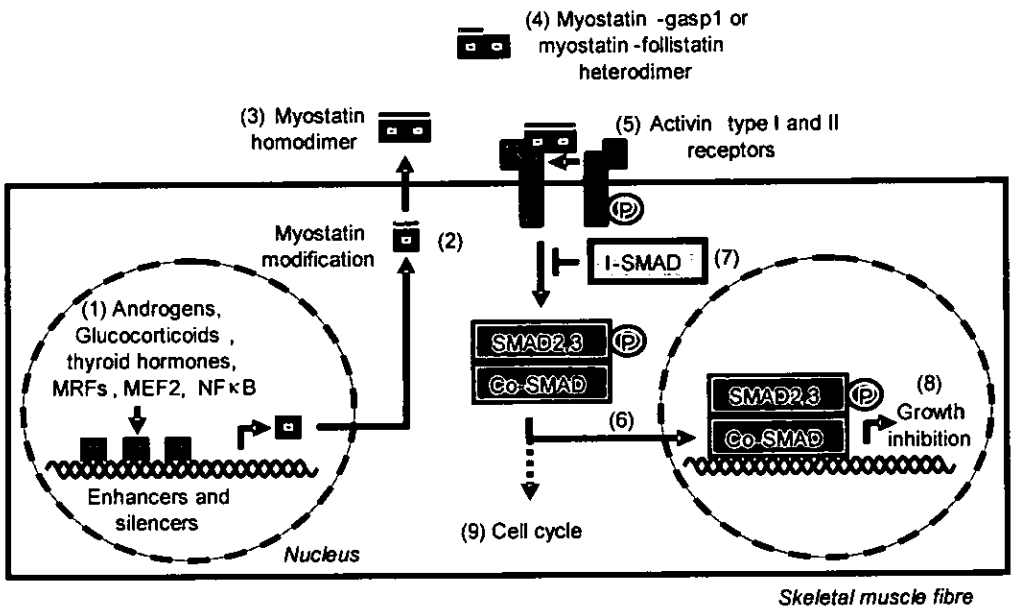


Figure 1.7. Schematical overview depicting the control of myostatin expression and subsequent effects on cell cycle and transcription. (1) Myostatin expression is regulated via DNA binding elements for glucocorticoid, androgen, thyroid hormone, myogenic regulatory factors (MRFs), MEF2, PPARγ, and NFκB (Ma *et al.*, 2001). Only the glucocorticoid binding site has been experimentally verified (Ma *et al.*, 2003). (2) Myostatin is modified post-translationally and secreted. (3) Secreted myostatin will be present as a homodimer or (4) heterodimer. Heterodimers of myostatin with either follistatin or gasp1 can not bind to receptors. (5) Myostatin homodimers bind to activin type RIIB and RIIA receptors. This is likely to lead to the recruitment of an activin type I receptor and receptor phosphorylation. (6) Receptor activation results in SMAD3 and/or SMAD2 phosphorylation. SMAD signalling can potentially be modified by Co-SMADs or (7) inhibitory I-SMADS. (8) Activated SMAD enters the nucleus and binds to SMAD binding elements. This is likely to mediate part of the growth inhibition possibly via repression of MyoD expression (Langley *et al.*, 2002). Cell cycle regulation occurs either via the expression of proteins regulating the cell cycle or (9) via regulation of cell cycle proteins.

1.7.2 Muscle growth factors – Positive control by Insulin like growth factor 1 (IGF-1) splice variants and the PI-3K pathway

IGF-1 is part of the system that regulates growth, and is a diametric opposite to myostatin in that it is a potent positive regulator of muscle mass. Growth hormone increases circulating IGF-1 as a second messenger through increasing IGF-1 expression in the liver (Butler & Le Roith, 2001). Apart from growth hormone, IGF-1 expression also increases in response to testosterone but decreases in response to the known atrophy-inducing factors such as glucocorticoids, TNFα and interleukin-1 (Frost & Lang, 2003). As in the case of myostatin, IGFs are found in association with a family of high-affinity insulin-like growth factor binding proteins (IGFBP 1-6) that affect their biological activity (Firth & Baxter, 2002). IGFBPs fulfill important functions demonstrated by the examples that only the IGFBP3-IGF-1 complex but not IGF-1 alone increased protein synthesis in muscles of alcohol-fed rats (Lang *et al.*, 2004); whilst binding of IGF-I to IGFBP-3 and the acid-labile subunit (ALS) forms a ternary complex that maintains circulating levels of IGF-I by slowing its rate of clearance (Svanberg *et al.*, 2000). Transgenic mice show the importance of IGF-1 since muscles and other organs of IGF-1 knockout mice are smaller than in the wild-type and few mice survive into adulthood (Liu *et al.*, 1993). In contrast, IGF-1 infusion into skeletal muscle results in skeletal muscle hypertrophy (Adams & McCue, 1998) and skeletal muscle specific over-expression of IGF-1 causes hypertrophy (Musaro *et al.*, 2001). IGF-1 is also synthesised in skeletal muscle and rats where hypophysectomy had been performed, still respond to muscle growth-inducing stimuli with a prolonged increase in IGF-1 expression and hypertrophy (DeVol *et al.*, 1990) thus showing the importance of local IGF-1.

Recently, a splice variant stretch-responsive isoform of IGF-1 was identified which was named mechano-growth factor (MGF). In one study, MGF mRNA increased for >40 h after exercise in resistance-trained rat muscles (Haddad & Adams, 2002). However, muscle stretch can also increase anabolic signalling without the activation of the PI-3K or PKB which probably excludes IGF-1 or MGF as the growth factors mediating this response (Hornberger *et al.*, 2004). The effect of resistance training on IGF-1 expression in human skeletal muscle is unexpectedly unclear where studies have found little or no increase in circulating IGF-1 and MGF in response to resistance training (Walker *et al.*, 2004; Borst *et al.*, 2001). It is therefore unclear as yet as to the importance of IGF-1 splice variants in human muscle hypertrophy.

1.7.3 IGF-1 and Forkhead (FOXO) transcription factors

The so-called forkhead (FOXO) factors have important relation to IGF-1 signalling. In cultured myotubes undergoing atrophy the activity of the PI-3K/PKB pathway decreases, leading to activation of FOXO transcription factors and atrogin-1 induction. IGF-1 treatment or PKB overexpression inhibits FOXO through nuclear exclusion, thus showing that activation of the PI-3K pathway can inhibit FOXO mediated expression of atrophy factors. Therefore, it appears that the forkhead family of transcription factors are inhibited by IGF-1 signalling and thus hypertrophy mediated through this pathway could be at least in part due to nuclear exclusion of FOXO transcription factors as well through the characterised effects on protein synthesis. This conclusion is further supported by the fact that PI-3K signalling is also required for transcriptional changes in muscle, demonstrated by the downregulation of the atrophy genes MuRF-1 and MaFbx in response to IGF-1 (Latres *et al.*, 2005).

1.7.4 Role of the IGF-1/PI-3K pathway in hypertrophy

1.7.4.1 PI-3K, PDK1 and PKB

IGF-1 exerts its growth effects through the PI-3K pathway, and even though the role of IGF splice variants in human hypertrophy in response to exercise is contentious, the importance of this pathway is nevertheless critical in mammalian protein synthesis. The following discussion assumes the chain of events following the binding of IGF-1 to the insulin/IGF-1 receptor in order to layout the process by which stimulation of this pathway results in enhanced protein synthesis. Once the receptor has been stimulated, insulin receptor substrate (IRS) proteins, which possess binding sites for PI-3K (White, 2002), phosphorylate the phosphoinositols (PtdIns) (Vanhaesebroeck *et al.*, 2001). PI-3K uses ATP to phosphorylate PtdIns(3,4)P_2 (PIP2) to PtdIns(3,4,5)P_3 (PIP3) (Vanhaesebroeck *et al.*, 2001). The increased production of PIP3 stimulates interaction between pyruvate dehydrogenase kinase 1 (PDK1) and PKB. Both PDK1 and PKB have a PIP3 binding site, named the pleckstrin homology domain (Vanhaesebroeck & Alessi, 2000). The production of PIP3 stimulates translocation of PDK1 and PKB from the cytosol to the cell membrane. This localisation of PKB enables PDK1 to phosphorylate PKB at Thr308 and an unknown kinase (sometimes termed PDK2) to phosphorylate PKB at Ser473 (Vanhaesebroeck & Alessi, 2000). Both PDK1 and PKB regulate muscle growth as is evident from the phenotypes of PDK1 knockout (Lawlor *et al.*, 2002) and PKB transgenic mice (Yang *et al.*, 2004). The effect of PKB activation on skeletal muscle growth was demonstrated in an experiment where a constitutively active PKB construct was expressed in regenerating skeletal muscle (Pallafacchina *et al.*, 2002) and the fibres that expressed the construct were much larger. There are three PKB isoforms - PKBα

(Akt1), PKBβ (Akt2) and PKBγ (Akt3) (Yang *et al.*, 2004). The most important isoform regulating growth appears to be PKBα since knockout mice have defects in foetal and postnatal growth that persist into adulthood (Cho *et al.*, 2001) and furthermore, the activation of PKBα signalling in mouse gastrocnemius muscle is sufficient to promote myofibre hypertrophy (Takahashi *et al.*, 2002). Once the stimulus activating PI-3K has gone, a lipid phosphatase called phosphatase and tensin homolog on chromosome ten (PTEN) converts of PIP3 into PIP2 thus reversing the effect of IGF-1 regulated PI3-kinase and "switching" off this pathway.

1.7.4.2 PKB and AMPK signalling via TSC2

PKB is a major upstream kinase which is capable of directing activation of protein synthesis by phosphorylating tuberin (TSC2), mTOR and GSK-3β, to affect translational regulators, of which each will be discussed in turn. Recently TSC2 has been identified as a link to growth regulation by indirectly connecting PKB to mTOR and thus translational regulation. The complex of hamartin (TSC1) and TSC2 were originally shown to cause the autosomal dominant disorder tuberous sclerosis complex when mutated (Kwiatkowski, 2003). PKB phosphorylates TSC2 at Ser939 and Thr1462 resulting in the inhibition of TSC2 (Manning *et al.*, 2002). The TSC2 protein has a domain that is termed a GTPase-activating protein (GAP). GAPs catalyse the activity of GTPases of G proteins which convert guanosine triphosphate (GTP) to guanosine diphosphate (GDP). The G protein Rheb was recently shown to be the TSC2 substrate (Inoki *et al.*, 2003a), and without stimulus, TSC2 acts as a GAP to inactivate Rheb by directly stimulating GTP hydrolysis. However, when IGF-1 activates the P-I3K cascade, and PKB phosphorylates TSC2 on Thr1462 and Ser939,

there is an inhibition of TSC2 which leaves Rheb in its GTP-bound active state. Rheb is not a kinase but the TSC2-Rheb downstream target, mTOR is phosphorylated at residues such as Ser2448 in response to insulin and muscle loading (Reynolds *et al.*, 2002). Therefore, Rheb probably activates an unknown kinase that phosphorylates mTOR on Ser2448 unless the Ser 2448 site is phosphorylated directly by PKB as was reported in the time before the identification of TSC2 of a PKB downstream target (Nave *et al.*, 1999). Interestingly, TSC2 does not only activate protein synthesis in response to IGF-1 but also inhibits protein synthesis in response to energy stress. It was recently shown that AMPK could directly phosphorylate TSC2 at the PKB independent sites of Thr1227 and Ser1345 (Inoki *et al.*, 2003b). Phosphorylation at these sites is predicted to stimulate GTP hydrolysis on Rheb and thus provide a mechanism preventing energy-consuming protein synthesis when a cell is experiencing chronic energy stress.

1.7.4.3 mTOR, p70 S6K, 4E-BP1 (PHAS-1) and eEF2

The mammalian target of rapamycin (mTOR) is the next substrate in the pathway, and is a large >200 kDa serine/threonine kinase controlling cell growth. mTOR detects IGF-1, IGF-2 or insulin receptor binding (PKB-TSC2 signalling); energy stress (AMPK-TSC2 signalling); and amino acid availability (via an unknown pathway). The requirement for mTOR mediating signalling in hypertrophy has been demonstrated pharmacologically; blockade with rapamycin decreases muscle hypertrophy *in vitro* and *in vivo* and blunts the hypertrophy-associated phosphorylation of p70 S6K and 4E-BP1 (Bodine *et al.*, 2001). An important regulatory mechanism controlling mTOR activity appears to be the phosphorylation

of Thr2446 and Ser2448 (Cheng *et al.*, 2004). Phosphorylation of Thr2446 increases when AMPK is activated and is low in response to nutrients and insulin. In contrast, Ser2448 phosphorylation is decreased in response to AMPK but increased in response nutrients and insulin (Cheng *et al.*, 2004). Thus, Thr2446 and Ser2448 seem to be phosphorylated in opposite directions and the more Ser2448 is activated the greater the effect of mTOR on protein synthesis; whereas the opposite is true for Thr2446 phosphorylation. It is unclear whether the phosphorylation state of Thr2446 and Ser2448 are both mediated by TSC2 or whether PKB or AMPK have independent effects on both TSC2 and mTOR. mTOR is also activated by amino acids as is evident from an amino acid-induced phosphorylation of Ser2448 phosphorylation (Nave *et al.*, 1999), although it is not currently known how amino acids stimulate phosphorylation of mTOR. In response to increased phosphorylation (at least of PKB-mTOR and PKB-TSC2 phosphorylation sites) mTOR is capable of activating downstream effectors of protein synthesis. mTOR directly or indirectly phosphorylates and activates the translational regulators 4E-binding protein 1 (4E-BP1/PHAS1) and the 70 kDa S6 phosphorylating ribosomal protein (p70 S6K), resulting in increased protein synthesis.

As mentioned, p70 S6K is a downstream target of mTOR and another protein indirectly stimulated by IGF-1. In mammalian cells a short sequence of 5–15 pyrimidine nucleotides immediately following the m^7GTP cap (the 5'-terminal oligopyrimidine tract or 5'TOP) is an invariant feature of ribosomal mRNA species (Shah *et al.*, 2000). This sequence has been demonstrated to be essential for translational regulation of these mRNAs in response to different growth stimuli, including mitogens, amino acid starvation and differentiation, and mutation of this

sequence abrogates this regulation. p70 S6K phosphorylates the ribosomal protein S6 and has been shown to regulate the selective translation of mRNAs with this 5'TOP sequence, and thus specifically regulates translation of the ribosomal machinery (Shah *et al.*, 2000). It has been demonstrated that p70 S6K and protein synthesis are increased concomitantly for up to 24 h after a bout of resistance exercise, and correlates with muscle protein synthesis (Hernandez *et al.*, 2000) thus highlighting the importance of this pathway in growth response. Translation elongation also depends indirectly on mTOR (Browne & Proud, 2002). mTOR activates p70 S6K which in turn can phosphorylate and deactivate eEF2 kinase (eEF2K, formerly known as Ca^{2+}/calmodulin dependent kinase III) which decreases the phosphorylation of eEF2 at Thr56. Dephosphorylated eEF2 is more active and promotes the elongation of the amino acid chain during translation. Similar to the regulators of translation initiation, eEF2 activity is inhibited when AMPK is activated (Horman *et al.*, 2002; Browne *et al.*, 2004), again suggesting a way in which at times of chronic high energy consumption, protein synthesis is reduced.

Another protein downstream of mTOR and activated indirectly by IGF-1 named 4E-BP1 also controls protein synthesis. 4E-BP1 controls the regulatory step involving the binding of mRNA to the 43S pre-initiation complex, which is mediated by eIF4F, a complex of several subunits (Proud, 2002). One of the subunits, eIF4E, binds the 7-methylguanosine 5'-triphosphate cap structure present at the 5'-end of eukaryotic mRNAs to form an eIF4E · mRNA complex. During translation initiation, the eIF4E · mRNA complex binds to eIF4G and eIF4A to form the active eIF4F complex. The binding of eIF4E to eIF4G is controlled by the translation repressor protein 4E-BP1. Binding of 4E-BP1 to eIF4E is hypothesised to limit eIF4E availability for formation

of an active eIF4E · eIF4G complex (Proud, 2002). Therefore, the phosphorylation of 4E-BP1 at various sites including thr37/46 removes 4E-BP1 from eIF4E, allowing eIF4E to participate in initiating translation by the process described (Proud, 2002). 4E-BP1 has a role in exercise-induced hypertrophy and its phosphorylation has been shown to increase following acute resistance exercise (Bolster *et al.*, 2003).

1.7.4.4 PKB, GSK-3β, and eIF2B

PKB regulates protein synthesis not only via the TSC2-Rheb-mTOR-p70 S6K-4E-BP1 connection but also via PKB-GSK3β-eIF2B pathway. PKB inhibits GSK-3β by phosphorylating it at Ser9 and this can be activated by IGF-1 in skeletal muscle cells (Rommel *et al.*, 2001). The inhibition of GSK3β in cultured muscle cells has been shown to induce hypertrophy (Vyas *et al.*, 2002), suggesting the importance of this section of the pathway in protein synthesis. The phosphorylation and inhibition of GSK3β leads to a decreased phosphorylation of eukaryotic initiation factor 2B (eIF2B) (Kimball, 2001). eIF2B is an important factor regulating the first step controlling peptide-chain initiation: binding of initiator methionyl-tRNA to the 40S ribosomal subunit to form the 43S pre-initiation complex. This reaction is mediated by eukaryotic initiation factor 2 (eIF2) and is regulated by the activity of eIF2B. The ε sub-unit of eIF2B has a catalytic activity and is a guanine nucleotide-exchange factor which mediates the exchange of GDP bound to translation initiation factor eIF2 for GTP (Kimball *et al.*, 1998). Decreased phosphorylation of eIF2B at Ser535 increases the activity of eIF2Bε and promotes initiation of translation (Proud, 2002). This mechanism provides more scope for increases in global protein synthesis through stimulation of the PI-3K pathway by IGF-1 splice-variants.

In summary, a required mediator of skeletal myofibre enlargement is increased protein synthesis, which is controlled at the levels of translation initiation and elongation. Therefore the IGF-1/PI-3K pathway is a necessary mediator of myofibre enlargement through its characterised effects upon protein synthesis.

1.7.5 Role of the Calcineurin pathway in hypertrophy

It was first suggested that IGF-1 induced skeletal muscle growth via the calcineurin pathway (Semsarian *et al.*, 1999) which a year earlier was shown to promote hypertrophy in the heart (Molkentin *et al.*, 1998). A paper in Nature reported that IGF-1 induces calcineurin-mediated signalling and activation of GATA-2, a marker of skeletal muscle hypertrophy, which co-operates with NFATc isoforms to activate gene expression programmes (Musaro *et al.*, 1999). Later to this it was reported that administration of cyclosporine A, (CsA, a calcineurin inhibitor) abolishes the increase in fibre size seen in mouse plantaris due to overload synergist ablation (Dunn *et al.*, 1999). However, recently controversy has surrounded the role of calcineurin in mediating skeletal muscle cell hypertrophy. One study examined the ability of calcineurin-deficient mice to undergo skeletal muscle hypertrophy following mechanical overload stimulation or IGF-1 stimulation. Two distinct models of calcineurin deficiency were employed which show approximately 50% and 80 % reductions in total calcineurin in skeletal muscle. Calcineurin deficient mice demonstrated no defects in muscle growth in response to IGF-1 treatment or mechanical overload stimulation. Both groups of gene-targeted mice show normal increases in PKB activation following mechanical overload or IGF-1 stimulation (Parsons *et al.*, 2004). Furthermore, calcineurin knock-in transgenic mice show no

muscle hypertrophy in skeletal muscle (Naya *et al.*, 2000). The role of calcineurin has been further refuted since pharmacological inhibition with CsA and transgenic blockade of calcineurin does not prevent hypertrophy in response to synergist ablation (Bodine *et al.*, 2001). In agreement with this, CsA does not block IGF-1-induced hypertrophy of C2C12 myotubes (Rommel *et al.*, 2001). It therefore seems likely that the major effect of calcineurin is regulation of the fibre phenotype as was previously discussed (see 1.5.2), and reported (Chin *et al.*, 1998; Parsons *et al.*, 2003).

1.7.6 Role of interleukin-15 in hypertrophy

Interleukin-15 (IL-15) is a growth factor which is highly expressed in skeletal muscle, and has been shown to be a novel growth factor. IL-15 used at concentrations of 10 or 100 ng ml^{-1} increased MHC accumulation five-fold in myoblast cultures and 2.5-fold in primary bovine myogenic cultures. Moreover, myotubes formed in the presence of IL-15 appeared larger than controls (Quinn *et al.*, 1995). These findings indicate IL-15 can stimulate differentiated myocytes and muscle fibres to accumulate increased amounts of contractile proteins. Another study agreed finding that IL-15 overexpression induced a hypertrophic myotube morphology similar to that described for cultured myotubes which overexpressed IGF-1 (Quinn *et al.*, 2002). However, in contrast to IGF-I, the hypertrophic action of IL-15 on skeletal myogenic cells does not involve stimulation of skeletal myoblast proliferation or differentiation. Recently it was found that IL-15 protein was increased immediately after acute resistance exercise but did not change with chronic training (Riechman *et al.*, 2004). The significance and mechanisms of IL-15 in muscle hypertrophy warrants further research and could be a good target for treatment of muscle wasting.

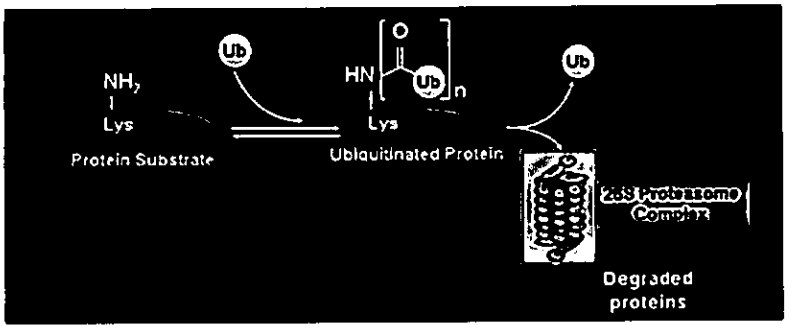
1.8 Pathways mediating skeletal muscle atrophy

Muscle atrophy is the opposite of hypertrophy in that it is the reduction in muscle fibre size and volume. In some cases loss of muscle fibres attributes to overall wasting, although this is not technically, atrophy. In contrast to hypertrophy, skeletal muscle atrophy is mediated by a chronic net protein breakdown (Latres *et al.*, 2005). There are many circumstances in which muscle undergoes atrophy and this may be in response to ageing (sarcopenia), AIDS, HIV, severe diabetes, sepsis, inactivity, chronic bed-rest, space flight (Glass, 2003) and perhaps even chronic endurance-like physical activity. During these conditions, cellular environments change, and there are many factors that have been implicated in mediating atrophy including TNFα-NFκB, angiotensin II, glucocorticoids, reactive oxygen species, myostatin and other cytokines; all of which can induce muscle protein loss under specific conditions.

1.8.1 Atrophy requires net protein breakdown

Atrophy is characterised by increases in protein degradation processes, particularly the ATP-dependent proteolytic ubiquitin-proteasome pathway (Latres *et al.*, 2005). Therefore, recent studies have focused on molecular cascades that control the activation of ubiquitin ligases, and have commonly indicated that an induction of the ubiquitin proteasome is common to a range of atrophic stimuli. The ubiquitin-proteasome pathway functions widely in intracellular protein turnover and plays a central role in degradation of short-lived and regulatory proteins important in a variety of processes, including regulation of the cell cycle, modulation of cell surface receptors and ion channels, and antigen processing and presentation (Lecker, 2003).

The ubiquitin-proteasome pathway employs an enzymatic cascade by which multiple ubiquitin molecules are covalently attached to a protein substrate. Binding of the ubiquitinating enzyme complex at the N-terminus of targeted cytosolic proteins facilitates the polyubiquitination of the ε-amino moieties on the internal lysine residues (see diagram below). The ubiquitin molecules serve as tags that allow recognition of the targeted protein by the 19S regulatory sub-unit on the proteasome, leading to eventual funneling into the proteasome where it is proteolytically degraded (Lecker, 2003). To highlight the importance of this pathway, inhibitors of the proteasome decrease protein breakdown in atrophy conditions (Tawa, Jr. *et al.*, 1997).



Recently, a microarray study examined the transcriptional responses to various muscle atrophy-associated states. They looked at fasted mice, rats with cancer cachexia, streptozotocin-induced diabetes mellitus, and uremia induced by sub-total nephrectomy, thus covering a number of disease-induced atrophy states (Lecker *et al.*, 2004). From the cDNA arrays, they identified a common set of genes that were induced or suppressed in muscles in these four catabolic states. Among the strongly induced genes were many involved in protein degradation, including polyubiquitins, ubiquitin fusion proteins, the ubiquitin ligases atrogin-1, muscle atrophy F-box proteins (MAFbx) and muscle ring finger 1 (MuRF-1), multiple (but not all) subunits of the 20S proteasome, it's 19S regulator, and cathepsin L. These atrophy induced-

genes constituted a major portion of the ubiquitin-proteasome pathway. The authors termed these genes 'atrogins' and upregulation of these indicate an increase in proteolysis in all of the muscle wasting states examined. Several growth-related mRNA species were also down-regulated, including p311, c-jun and IGF-1 binding protein 5, which is important in mediating IGF-1-induced hypertrophy (Musaro & Rosethal, 1999). Therefore, it appears that during many disease states there is a common change in gene expression, primarily involving upregulation of proteolytic mechanisms. It must be stated that actual increases in these proteins of the ubiqutin-proteasome pathway, as has been suggested in many states, does not necessarily induce a larger net protein breakdown. Bearing in mind the fact that muscle protein breakdown (MPB) is facilitative to changes in MPS; in situations of cancer and HIV atrophy, it is thought that MPS is depressed and if this is the case then MPB is probably actually also suppressed in these situations. However, even though MPB is lower than normal it still exceeds MPS, leading to a net negative protein balance. Conversely, in patients with burns and renal failure, there is a hypermetabolic state which is indicated by an increase in MPS in an attempt to minimise the effect of large increases in MPB, and since MBP still exceeds MPS, a negative net balance occurs.

1.8.2 Sarcopenia in the healthy elderly

Whilst most forms of atrophy described in disease seem to follow a common programme and often involve gross changes in MPS/MPB ratios (Jackman & Kandarian, 2004), sarcopenia is a unique, gradual, but progressive situation that appears not to involve significant changes in these synthesis/breakdown ratios (Cuthbertson *et al.*, 2004). Also, whilst disease related atrophy only affects a small

percentage, sarcopenia is a unique biological situation that affects or will affect the entire population to some extent. Sarcopenia is specifically the gradual loss of muscle mass with advancing age, and wasting in the elderly is associated with atrophy and a ~50 % loss of muscle fibres (particularly type II) between 20-80 y (Lexell *et al.*, 1988). Physically inactive adults exhibit a greater loss of muscle mass than physically active adults as has been shown by numerous studies identifying the benefits of exercise in the elderly (Hunter *et al.*, 2004; Rogers & Evans, 1993). However, sarcopenia is not completely prevented by exercise, as it is also evident, but to a lesser degree, in physically active individuals (Evans 1995a). Some of this effect may be because the physical activity is not of sufficient intensity as to recruit FT fibres thus not inhibiting the preferential atrophy of these fibres and development of sarcopenia. Muscle weakness induced by sarcopenia reduces general activity levels in elderly individuals which in turn elevates the risk of osteoporosis and frailty due to a decrease in overall mechanical loading of the skeleton; as well as many other complications.

Current research is finding that the development of sarcopenia is a multifactorial process. Many factors including, inflammatory factors (Grimble, 2003), physical inactivity (Evans, 1995b), decreased hormone levels (Butterfield *et al.*, 1997), deranged protein synthesis/breakdown (Welle *et al.*, 1993), inadequate nutrition (Campbell & Evans, 1996) and disease conditions may all contribute to sarcopenia.

Of all of these factors, perhaps the most potentially influential mechanism would be a shift to prolonged negative net protein balance. There is disagreement in whether basal protein synthesis falls with ageing with studies by (Yarasheski *et al.*, 1993), (Balagopal *et al.*, 1997; Hasten *et al.*, 2000) suggesting a decrease. However, it seems

unlikely that the rates of basal, post-absorptive MPS rates are diminished by as much as 20-30 % (Volpi *et al.*, 2001; Paddon-Jones *et al.*, 2004) or muscle protein breakdown elevated by ~50 % (Chevalier *et al.*, 2003; Trappe *et al.*, 2004) because the rates of muscle wasting should be much greater than are noted. Other studies disagree and these results seem more cogent in finding no changes in MPS in the elderly compared with the young (Volpi *et al.*, 2001; Volpi *et al.*, 1999). Since most gradual changes in muscle protein mass are mainly due to alterations in MPS with alterations in MPB usually being adaptive to the changes in synthesis (Halliday *et al.*, 1988), there is little evidence that MPB is elevated either. Therefore, the lack of unification in agreement of these studies makes, coupled with the fact that the rate of atrophy is very gradual, it unlikely that this is the primary mechanism for sarcopenia.

Deranged hormone/growth factor levels have been shown to have potential effect on sarcopenia. As an example of this, the anabolic growth factor, IGF-I has been shown to be significantly reduced in aged rats, 60 % in females and 21 % in males as was circulating IGF-binding protein 3 (IGFBP-3) (Severgnini *et al.*, 1999). Reduced levels of growth hormone, thyroid hormone and testosterone are usually also noticed in advancing age suggesting that anabolic signalling may be reduced (Roy *et al.*, 2002). Consistent with this, decreases in lean body mass, increased fat, and other changes consistent with hormone deficiencies occur during ageing (Harman & Blackman, 2004). Therefore, reduced growth factor levels might partly contribute to sarcopenia. Since exercise is such an important stimulus for protein synthesis, perhaps the typical reduction in physical activity with advancing age could contribute to sarcopenia. In older age, physical activity usually declines, and whilst those who remain more active elicit reduced sarcopenia it is nevertheless still evident in this population (Roubenoff,

2003). Therefore, whilst being a contributory factor it is not causative. The physiological responses to exercise in elderly is still positive although blunted somewhat. This could be related to compromised satellite cell activation, proliferation and differentiation, and blunted responses to hypertrophic stimuli (Kadi *et al.*, 2004a).

There is also an inflammatory theory of ageing. Pro-inflammatory cytokines such as interleukin-6 (IL-6) and TNFα are found at elevated levels in sarcopenia and are associated with lower muscle mass and lower muscle strength in well-functioning older men and women (Visser *et al.*, 2002). TNFα is implicated in skeletal muscle atrophy through activation of NFκB, a protein known to induce atrophy responses (Ladner *et al.*, 2003). Since these cytokines are partly derived from the adipose tissue bed, those with increased fat mass such as type II diabetics, are at further risk of chronic inflammation that occurs during ageing. There are often other predisposing factors for the chronic inflammation that occurs during ageing. These include increased oxidative stress, a decrease in ovarian function (women), a decrease in stress-induced glucocorticoid sensitivity of pro-inflammatory cytokine production, obesity, and an increased incidence of asymptomatic bacteriuria (Grimble, 2003). Inflammation is a key factor in the progressive loss of lean tissue and impaired immune function is observed in ageing. Polymorphisms in the promoter regions of pro- and anti-inflammatory cytokine genes influence the level of cytokine production and the ageing process (Grimble, 2003). Thus, a genotype for high pro-inflammatory cytokine production results in high cytokine production and may accelerate the rate of tissue loss. Conversely, polymorphisms in genes for anti-inflammatory cytokines may result in a slowing of tissue loss. Another study suggested that the increased serum myostatin may be a biomarker of sarcopenia (Yarasheski *et al.*, 2002), giving a

potential role for this negative muscle mass regulator in age-related muscle loss. It could be suggested that some of the discrepant findings upon MPS/MPB and, cytokines and growth factors that are reported are probably due to the failure of experimenters to differentiate healthy elderly from those with additional conditions

The blunting in anabolic responses to nutrients has recently been implicated in age-related atrophy. Amino acids are powerful stimulators of MPS, and in rat, leucine-dependent stimulation of translation initiation *in vivo* occurs via a rapamycin-sensitive pathway, suggesting a role for mTOR in nutrient signalling (Anthony *et al.*, 2000). Initial studies examining the responsiveness of MPS to essential amino acids (EAA) indicated there may be a deficit in anabolic response in aged animals. Measurements made on isolated muscles from young and elderly rats showed that the sensitivity and responsiveness of MPS to the EAA leucine, is diminished in the elderly rats (Dardevet *et al.*, 2000). These data underline the importance of EAA in mediating protein synthesis provide another area which may be under altered regulation with ageing.

1.8.3 Sarcopenia in elderly with type II Diabetes Mellitus

It has been suggested that since muscle disease symptoms and myopathies are not uncommon in the elderly, the associated inflammatory and non-inflammatory myopathies lead to proximal extremity or axial weakness and are superimposed on the intrinsic changes that occur in muscle with ageing (sarcopenia) (O'Rourke, 2000). An example of such a situation is the metabolic disease, type II Diabetes Mellitus (type II DM). Type II DM is common in the elderly population and by the age of 75, approximately 20 % of the population are afflicted with this illness (Meneilly &

Tessier 2001). Typically, type II DM develops in the elderly through the following steps: natural ageing leads to a loss in muscle mass which accounts for the age-associated decreases in basal metabolic rate, muscle strength, and activity levels, which, in turn is the cause of the decreased energy requirements of the elderly. In sedentary individuals, the main determinant of energy expenditure is fat-free mass, which declines by about 15 % between the third and eighth decade of life (Evans, 1998). Furthermore, declining caloric needs are not matched by an appropriate decline in caloric intake, with the ultimate result an increased body fat content with advancing age. Increased body fatness along with increased abdominal obesity are thought to be directly linked to the greatly increased incidence of Type II DM among the elderly (Evans, 1998). Type II DM, characterised by insulin resistance, is a typical condition of such a muscular disease state which leads to many complications (Sartorelli & Fulco, 2004). Individuals displaying type II DM commonly suffer peripheral neuropathy due to their chronic hyperglycaemic state. This neuropathy is characterised by severe loss of synaptic vesicles, electron-dense bodies, and myelin-like figures as well as degeneration of mitochondria (Sartorelli & Fulco, 2004). Interestingly, these ultrastructural changes in neuromuscular junctions are similar to those that have been described in ageing. Therefore, it remains to be shown whether the effects of disease states such as type II DM lead to greater susceptibility to sarcopenia and do in fact, overlay the progression of age-related atrophy. It should be stated that in such situations of metabolic disease and ageing it is widely accepted that increased mechanical loading of the musculoskeletal system (e.g. resistive exercise) can have a beneficial effect on both skeletal muscle and the supporting skeleton resulting in a significant reduction in the risk of developing age-related problems such as type II DM, and can also counteract sarcopenia (Clarke, 2004).

1.9 Research Objectives

The main hypothesis of the study was that specific and distinct cell signalling is responsible for the change in phenotype and muscle growth/atrophy in response to exercise, sarcopenia and disease. The work encompassed novel studies investigating many levels of regulatory control to answer the specific aims, which were:

(1) Investigating the regulation of myostatin, and how it altered gene transcription and translation to affect post-mitotic muscle mass.

A cell culture model was used to examine the effects of various growth factors on myostatin expression using Northern blotting and RT-PCR; microarray experiments were performed on cells treated with recombinant myostatin to identify genome wide changes in gene expression; Western blotting was employed to examine how recombinant myostatin affects the phosphorylation of elements of pathways controlling translation, and ultimately protein synthesis.

(2) Investigating contraction activated signalling proteins to ascertain if they were present at different concentrations between fast and slow muscles. Secondly, to investigate whether they are regulable by contractile activity.

Western blotting of total proteins between fast EDL and slow Soleus were used to detect differences in relative protein concentrations; CMNS (fast-to-slow model) was used to stimulate EDL in order to assess the effect of CMNS on signalling proteins, and see if they were brought in line to relative concentrations of control EDL/Soleus.

(3) Investigating the matching of cellular signalling with known physiological responses; and examining the specificity in signalling, that mediates the contrasting physiological responses to endurance and resistance-like training.

CMNS was used in attempt to match the cellular signalling produced in this model to the known physiological adaptations, using phospho-specific immunoblots. *In vitro* systems of either high frequency intermittent (HFS) to simulate resistance-like exercise; or low frequency chronic stimulation (LFS) to simulate endurance-like exercise, were employed in order to examine the selective activation of signalling pathways between protocols using phospho-specific immunoblotting. The validation of HFS was checked by identifying specific increases in MPS using GC-MS. LFS validity was assured by measuring specific increases in UCP3 mRNA using RT-PCR.

(4) Investigating whether sensitivity to amino acids in sarcopenia and disease (type II DM) could explain loss (or accelerated loss) of muscle mass.

MPS using stable isotopes and GC-MS were examined between healthy elderly/young and healthy elderly/elderly with Type II DM, both in the post-absorptive period and following infusion of essential amino acids. Western blotting of phospho-proteins within translation controlling pathways would be used in order to identify, and explain, any differences in muscle protein synthesis. The capacity of the system would be further examined by using native antibodies against the same targets to asses any concentration differences present.

Each specific aim stated, formed a chapter of results in this thesis (chapters 3-6).

Chapter 2

Materials and Methods

2.1 Cell culture and muscle dissection

L6 cells were grown in 8 % FBS αMEM supplemented with 2.2 g/l^{-1} NaHCO3, amphoteracin B (1:1000) and Penicillin/Streptomycin (1:100). All dishes containing myotubes were treated with 5 μM arabinofuranosidase C, for 24 h prior to application of treatment to remove the effect of any dividing myoblasts remaining. Four 90 mm diameter plates were treated for 24 h with four separate 10-fold incremental concentrations (Molar, unless stated) of either: insulin-like growth factor 1 (10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}), clenbuterol (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}), growth hormone (10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}), testosterone (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}), dexamethasone (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}), cyclosporine A (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}), and finally with creatine for 24 h and 48 h at 5 mM, 10 mM, 15 mM and 20 mM. Cells were harvested using cell scrapers and directly homogenised in 1 ml Tri-Reagent by pippeting up and down several times before being placed in RNAse free safelock Eppendorf tubes and frozen in liquid N_2. For raw skeletal muscles, male Wistar rats were killed by a blow to the head and then cervical dislocation, before a selection of muscles of mixed fibre proportions, were excised and frozen in liquid N_2. Samples were stored at – 80 °C awaiting assay. All animal procedures were in line with governmental law and the relevant ethics committees.

C2C12 murine myoblasts were cultured to confluence in Dulbecco's modified Eagle's medium containing 10 % FBS, and supplemented with amphoteracin B (1:1000) and Penicillin/Streptomycin (1:100) and formation of myotubes triggered by switching to 2 % horse serum. Eight 90 mm dishes were subjected to 10 ng ml^{-1} recombinant myostatin for 6 h, whilst eight dishes served as controls. Proteins were extracted and stored at – 80 °C ready to be used for Western blotting as described under section 2.8.

2.2 RNA extraction and preparation

Total RNA was extracted from the L6 cultured cells and skeletal muscle using Tri-Reagent, based on the acid guanidinium thiocyanate-phenol-chloroform RNA extraction method (Chomczynski & Sacchi, 1987). After defrosting, the samples were further homogenised with a hand-held polytron and the sample was left at ambient temperature for 5 min. Thereafter, 100 µl of 1-bromo-3-chloropropane was added to each tube and then vortexed for 15 s and left at room temperature for 15 min. Tubes were then centrifuged at 12,000 g for 15 min at 4 °C. During centrifugation, the mixture separates into an upper aqueous phase containing the RNA, an interphase containing the DNA and a red lower organic phase containing the proteins, including the ribonucleases. Precisely, 500 µl of the upper phase was carefully transferred to a fresh 1.5 ml Eppendorf tube and 500 µl of isopropanol added to the aqueous phase to precipitate the RNA. This solution was left at room temperature for 10 min before being centrifuged at 12,000 g for 8 min at 4 °C. The supernantant was then collected and 1 ml 75 % ethanol RNAse free (ribonuclease) was added to the tube and then spun at 7,500 g for 5 min at 4 °C. This process was repeated a further two times to ensure the guanidinium salts are eliminated. The visible RNA pellet was then air-dried for 5 min and resuspended, in 20 µl RNase-free water. A 10 µl aliquot of the RNA was then dissolved in Tris-EDTA (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and the absorbance measured at 260, 280 and 240 nm. The concentrations were calculated as C= 40 ng/µl • A_{260}. The RNA samples were mixed with formaldehyde loading buffer then incubated for 15 min at 65 °C and placed on ice for 2 min. Incubation was then repeated for 15 min at 65 °C and the samples placed at – 80 °C.

2.3 Northern blotting - Generation of myostatin probe

The following constituents were added to a PCR tube: 10 µl 10 × *pfx* buffer, 2 µl 50 mM $MgSO_4$, 10 µl 10 × PCR enhancer solution, 6 µl 5 µM dNTP, 3 µl 10 µM sense primer, 3 µl 10 µM antisense primer 0.5 µl cDNA (skeletal muscle), 1.5 µl 2.5 U/µl *Pfx* polymerase and 64 µl H_2O. The PCR reaction was run at 94°, 5' → {94°, 15" → 55°, 30" → 68°, 1'}x 40 → 68°, 15' → 4°. Precisely 10 µl of the PCR reaction was checked on a 3 %, 3:1 agarose gel, whilst the remaining 90 µl of the PCR reaction was purified with the Boehringer High Pure PCR purification kit according to the manufacturers protocol. Precisely, 50 µl was eluted in Tris-EDTA (10 mM Tris-HCl, 1 mM EDTA pH 7.5).

2.3.1 Preparation of the vector

The following components were mixed in an Eppendorf tube: 2.5 µg pBlueScriptII SK(+), 5 µl 10 × reaction buffer, 1 µl 10 U/µl *SmaI*, to a total of 50 µl with H_2O. The plasmid was incubated at room temperature for 1 h to cut the plasmid, before being heated to 70 °C to denature the *SmaI* enzyme. The vector was then checked on an agarose gel, where a band of 3 kb was detected, confirming cutting of the vector. For the ligation, two controls were made, one without the PCR product to check for false positives and one ligation without the ligase to check that the vector has been sufficiently cut. The purified PCR product was substituted for H_2O in the aforementioned controls. Next, an Eppendorf tube was loaded with 6 µl of the purified PCR product, 2 µl 5 × ligase buffer, 1 µl vector cut with *SmaI*, 1 µl 1 U/µl T4

DNA ligase. Sample and controls were incubated for 4 h at room temperature and then at 70 °C for 15 min to destroy the ligase.

Precisely, 50 µl of competent DH5α cells was added to 5 µl aliquots of the ligations and incubated on ice for 30 min. Then tubes were incubated at 42 °C for 45 s in a water bath and then quickly put back on ice. Cells were plated on plates with ampicillin, X-Gal and IPTG and incubated at 37 °C overnight. Providing that there were few colonies on the negative ligase control; mainly blue colonies on the positive ligase control; and mainly white colonies on the PCR ligated plate then (positive) white colonies were streaked onto new plates to isolate single colonies. These new plate colonies containing the PCR product incorporate into the vector were incubated in 8 ml LB with ampicilin and grown overnight at 37 °C with gentle shaking. The next morning, 900 µl outgrown cell culture was mixed with 600 µl 50 % sterile glycerol in a 2 ml cryotube to preserve the cells at -80 °C. A volume of 4 ml of the remaining outgrown culture was then purified with the Boheringer High Pure Plasmid Isolation Kit according to the protocol. Thereafter, 100 µl was eluted in Tris-EDTA, and the plasmids checked by cutting with restriction enzymes and separating the fragments on an agarose gel.

2.3.2 Hot long single-stranded DNA probe by PCR and biotinylation-DNA probe synthesis

A PCR mix containing 2 µl 10 µM M13 primer1 bio, 2 µl 10 µM M13 primer 2, 2 µl 1 ng μl^{-1} template plasmid, 1 µl 10 x PCR buffer excluding Mg^{2+}, 10 µl 50 mM $MgCl_2$, 4 µl 5 mM dNTP's, 1 µl 5 U/µl Platinum Taq polymerase and then 78 µl H_2O

was used to make up a final volume of 100 µl. The PCR cycle was then run as follows, 94°, 4' → {94°, 5" → 56°, 30" → 72°, 1'} x 30 → 72°, 15' → 4°. In preparation, 5 µl Dynabeads M280 Streptavidin (10 mg ml^{-1}) were washed twice in 20 µl 6 x SSC, and resuspend in 20 µl 6 x SSC. Precisely, 5 µl of the PCR product was added to the pre-washed dynabeads and incubated at room temperature for 5 min. Next, the dynabeads were collected with immobilised template by placing tube in magnet stand, and after 30 s the supernatant was removed with a pipette. Next, the dynabeads were resuspended in 100 µl melting solution (0.125 M NaOH, 0.1 M NaCl) and incubated at room temperature for a further 5 min. Following this, the supernatant was removed and the beads were washed twice in 100 µl 1 x SSC. After the first washing step, the dynabeads were transferred to a new tube to ensure no NaOH droplets were left on tube walls before being resuspended in 10.5 µl H_2O. Addition of 1 µl 5 µM antisense primer was put in to the mix and incubated at 42 °C for 5 min. On ice, 5.0 µl 5 x Strip-EZ buffer -dATP/-dCTP, 2.5 µl 10 x Modified dCTP, 5.0 µl α^{32}P-dATP (3000 Ci/mmol), 1.0 µl Exonuklease-free Klenow were mixed and added to the DNA/primer mixture which was incubated for 15 min at 42 °C. Finally, the beads were washed twice with 100 µl 1 x SSC and 40 µl melting solution was added and left to incubate for 3 min at room temperature. Supernatant (the probe) was collected and 1 µl of the probe counted for radioactivity.

2.3.3 Formaldehyde agarose gel

An amount of 0.8 g Gibco agarose was dissolved in 68.8 ml distilled water through boiling until the agarose dissolved and then cooled to 55 °C in a water-bath before the

addition of 8 ml 10 × morpholineopropanesulfonic acid buffer. A volume of 3.2 ml of 37 % (12.3 M) formaldehyde was then added and swirled gently. Thereafter the gel was poured into a gel tray (12 ×14 cm) and combs put in place to create wells.

2.3.4 Electrophoresis and blotting

Following polymerisation, the solidified gel was placed in the electrophoresis tank and the combs removed before 1 L 1 × morpholineopropanesulfonic acid was poured into the tank, so that the buffer could reach the top of the gel, but not cover it. A volume of 10 µl of each sample was loaded per well amounting to 2 µg RNA per sample. The electrophoresis was then run at 100 V until the bromophenol blue die marker had migrated 4.5 cm in the gel. Following completion of electrophoresis, the gel short was then rinsed in deionised water and then in a tray with SYBR green II (diluted in deionised water 10,000 times) on a tilt table for 30 min. The gel was then placed in saran wrap and photographed on an ultra-violet illuminator and then scanned on a phosphorimager to detect SYBR green II. Following acceptance of RNA quality (see 3.2.1) the formaldehyde gel was then rinsed in a tray with deionised water before addition of 1 litre of 25 mM freshly prepared NaOH and shaken for 10 min. Three sheets of 3 mm paper were placed on top of a stack of paper towels with a further three sheets of 3 mm paper pre-wetted in 25 mM NaOH positioned on top. The Appligene nylon membrane pre-wetted in 25 mM NaOH was then placed on top before placing the formaldehyde gel on the membrane. Any air bubbles were removed by rolling a pipette gently over the gel, and then the stack covered with saran wrap and left for 1 h. After completion of transfer, the blot was rinsed in 2 × SSPE to remove agarose and neutralise.

2.3.5 Hybridisation - Long single-stranded DNA probe from PCR

Hybridisation solution was first pre-heated to 68 °C to dissolve the precipitates. Following transfer, the membrane was rolled and placed in a hybridisation tube filled with distilled water. Addition of 5 ml hybridisation solution to the tubes followed before tubes were put into a hybridisation oven at 50 °C to avoid precipitation. Blots were incubated with rotation at 50 °C for 1 h. An appropriate amount of probe was diluted in 1 ml hybridisation buffer to allow 7000 counts per blot and centrifuged for 1 min at full speed to precipitate any residual beads before 900 µl of the labelled probe was transferred to the hybridisation tube. The membrane was then incubated with rotation at 50 °C overnight. The following morning, the membrane was washed with 250 ml 2 x SSPE and 0.1 % SDS at room temperature and incubated on a tilt table for 5 min before pouring off the wash buffer and repeating these steps. Washing buffer was then poured off and incubated with 250 ml 0.1 x SSPE and 0.1 % SDS preheated to 60 °C and incubated for 15 min at 60 °C, before pouring the wash buffer off and repeating these steps. Following washing, the membrane was removed, wrapped in two layers of plastic film and exposed to a phosphorscreen after 24 h.

2.4 RT-PCR

2.4.1 Semi-quantitative RT-PCR for myostatin

RNA was extracted using Tri-reagent according to the Manufacturer's protocol, and as described (see 2.2). Reverse transcription was performed with 1 µg of RNA as template using first strand cDNA synthesis kit. PCR amplification was carried out on

a 2 µl aliquot of the resultant cDNA using the primer sequences below. For internal standards, primers for the 'housekeeping' gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were used.

Rat myostatin forward: 5'-ATGAGGACAGTGAGAGAGAGG-3'

Rat myostatin reverse: 5'-GCACAAGATGAGTATGCGG-3'

GAPDH forward: 5'-ATCAGTGCCACCCAGAAGACT-3'

GAPDH reverse: 5'-CATGCCAGTGAGCTTCCCGTT-3'

Thermal cycling conditions were denaturation at 94 °C for 2 min, amplification (94°C→30 s, 58°C→30 s, 72°C→50 s) and a final elongation at 72 °C for 10 min for 30, 35 and 40 cycles. Amplification products were separated by electrophoresis on 2 % agarose gels and then stained with ethidium bromide prior to scanning.

2.4.2 Quantitative RT-PCR for UCP-3

Quantitative RT-PCR for uncoupling protein-3 (UCP-3) was assayed in order to verify that LFS could induce a marker gene for the adaptive response to endurance training (Stoppani *et al.*, 2002). RNA was extracted using Tri-reagent according to the manufacturer's protocol, as described (see section 2.2). Reverse transcription was performed with 1 µg of RNA as template using first strand cDNA synthesis kit (Roche: 1 483 188). PCR was carried out for 35 cycles using the lightCycler FastStart DNA master^{PLUS} SYBR Green 1 kit, following melting curve analysis to ensure specific gene amplification. The primer sequences for rat UCP3 and GAPDH were designed using rat-specific sequence data, and are listed below.

Rat UCP-3 forward primer: 5'-GAACCATCGCCAGGGAAGAAGGAGTCAG-3'

Rat UCP-3 reverse primer: 5'-GGGGGAGCGTTCATGTATCGGGTCTTTA-3'

GAPDH forward: 5'-ATCAGTGCCACCCAGAAGACT-3'

GAPDH reverse: 5'-CATGCCAGTGAGCTTCCCGTT-3'

Conditions were 35 cycles at 92 °C→60 s, 60 °C→30 s and 72 °C→110 s. A final 5-min extension step at 72 °C was performed. Amplification products were separated on a 2.5 % agarose gel and stained with ethidium bromide for visualisation. Changes in UCP-3 expression were normalised to changes in GAPDH following RT-PCR, in order to allow for differences in efficiency of cDNA synthesis following RT.

2.5 Electrical stimulation

2.5.1 Chronic *in vivo* electrical stimulation (CMNS)

Miniature neuromuscular stimulators were implanted in the peritoneal cavity of 16 adult male, ~ 200 g Wistar rats, with electrodes placed near to the common peroneal nerve in the left hind limb. The devices were activated remotely after surgery to provide continuous activation of the muscles of the anterior compartment of the lower hind limb at 10 Hz for six weeks. In all cases stimulation caused palpable oscillatory dorsiflexion of the foot throughout the experimental period showing that stimulation was effective. Following the stimulation period, the rats were humanely killed by a blow to the head followed by cervical dislocation before muscles were harvested rapidly with sterile instruments, frozen in liquid N_2 cooled isopentane and stored below –80 °C. This part of the study had ethical clearance from the University of Liverpool Ethics Commitee.

* Electronic stimulators were constructed, implanted and performed by members of Professor Salmons Laboratory, University of Liverpool. Muscle was then dissected at Liverpool following 6 weeks of *in vivo* stimulation, frozen in liquid N_2 and sent on dri-ice to University of Central Lancashire for further analyses.

2.5.2 Isolated Muscle Stimulation

Young adult male Wistar rats were maintained on a constant 12:12 h light-dark cycle. Animals were between 8-10 weeks old and weighed 220 ± 12 g in all experiments. Food and water were available ad libitum. The rats were humanely killed by a blow to the head and then cervical dislocation and then either one EDL or Soleus, and their contralateral muscles, were dissected and placed in Krebs Henseleit buffer (118.5 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 25 mM $NaHCO_3$, 3.4 mM $CaCl_2$, 35 mM Mannitol, 5 mM glucose, 1 g/l^{-1} bovine serum albumin) gassed with 95% O_2 and 5 % CO_2. Muscles were incubated at 25 °C to aid tissue oxygenation (Bonen *et al.*, 1994) and set to their resting length. Pilot tests showed that EDL and Soleus twitch:tension ratios did not change when left resting in the organ bath up to 12 h. Maximal contraction voltage was determined in preliminary experiments using a Grass isometric force transducer, Grass stimulator and Student Harvard chart recorder. Stimulation began 30 min after incubation of the muscle. The high-frequency stimulation (HFS) protocol was chosen based on its efficacy in inducing skeletal muscle hypertrophy whilst the low frequency stimulation (LFS) protocol has been shown to be effective in inducing endurance-like adaptations when applied 5 days per week for 3 weeks in rats (Nader & Esser, 2001). LFS protocol was performed using isometric contractions at 50 V and 10 Hz with a 90 ms delay and 10

ms duration, continuously for 3 h. HFS protocol was performed with isometric contractions involving 10 sets of 6 repetitions at 50 V and 100 Hz with a 7 ms delay on contractions. Each repetition was 3 s in duration and there was a 10 s recovery between repetitions and a 1 min rest period between sets. EDL and Soleus muscles were either control (incubated in a separate organ bath for the same time period as their stimulated equivalent) or LFS and HFS at 0 h post stimulation or at 3 h post stimulation. At the end of the experiment, samples were quickly frozen in liquid nitrogen and stored at -80 °C prior to processing. This study had ethical clearance from the Ethics Committee of University of Central Lancashire.

2.6 Protein synthesis measurements

2.6.1 Myofibrillar and sarcoplasmic fractional protein synthesis rates in isolated rat muscles

In order to verify that HFS was capable of increasing protein synthesis relative to control and LFS, the fractional protein synthesis rate was measured in 6 control EDL, 6 EDL 3 h after LFS and 6 EDL 3 h after HFS. The incubation media contained amino acids at fasting physiological levels (Taylor *et al.*, 1996) and protein synthesis was measured using a flooding dose of ^{13}C labelled proline (20 atoms percent) over 15 min (Garlick & Cersosimo, 1997). The labelling of proline in the incubation medium measured by gas chromatography-mass spectrometry and the incorporation of proline into myofibrillar and sarcoplasmic fractions was carried out as described in detail elsewhere using our standard techniques (Schwenk *et al.*, 1984). Briefly, muscle (30-40 mg) was ground in liquid nitrogen to a fine powder and hand

homogenised in a low salt buffer; the myofibrils and collagen were pelleted by centrifugation. The sarcoplasmic fraction was aspirated off and the myofibrils separated from the collagen by dissolving in 0.7 M KCl. The incorporation of proline into the myofibrillar and sarcoplasmic protein fractions was then measured by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS), after hydrolysis, purification and derivatisation as their N-acetyl-*n*-propyl ester (NAP) derivative. The fractional synthetic rate (FSR) was calculated as the rate of increase of labelling of protein-derived ^{13}Cproline compared to the average labelling of the proline in the incubation media.

2.6.2 Myofibrillar and sarcoplasmic fractional protein synthesis rates in human subjects

Two groups of 20 and 24 healthy young and elderly males (28 ± 6, 70 ± 6 y; BMI 24 ± 3, 25 ± 4 respectively; means ± SD) participated in a study examining differences between young an elderly. In the second study, 8 elderly males with type II DM were compared with 8 healthy age and near weight-matched controls (means ± SD: 66±3 vs. 70 ± 6; BMI: 25 ± 4 vs. 32 ± 2 kg.m^{-2}). The subjects received a primed constant infusion of [1-^{13}C] ketoisocaproic acid (KIC) (8.8 μmol kg^{-1}, 13.2 μmol kg^{-1} h^{-1}) over 3.5 h in the post-absorptive state. Insulin and glucose were clamped at basal values. Octreotide (1.8 μg kg^{-1} h^{-1}) and insulin (360 μU m^{-2} h^{-1}) were infused throughout and maintained insulin (~10 μU ml^{-1}) and glucose (~4-5 mM) at post-absorptive values. A medical doctor then took muscle biopsies from the Vastus Lateralis before and 3 h after ingestion of a solution in the first study containing 0, 2.5, 5.0, 10.0, 20.0 and (in elderly only) 40.0 g of mixed EAA in 500 ml of water; whilst in the second study

diabetic only 0 g and 20 g. Fractional synthetic rates (FSR) of myofibrillar and sarcoplasmic proteins were determined in quadriceps biopsies by measuring the rate of incorporation of [^{13}C]leucine during a primed, constant infusion of [1-^{13}C]α-ketoisocaproic acid over 3 h after they drank water or the EAA solution. Plasma was separated by centrifugation at 1600 x g and stored at -80 °C until analysis. An aliquot of 200 μl was taken for determination of amino acid labelling. Derivatised leucine was separated by gas combustion (GC) and its $^{13}C/^{12}C$ isotope ratio determined by selected ion monitoring MS (Bohe *et al.*, 2003). Muscle samples were homogenised in a high ionic strength buffer containing inhibitors of phosphatases and proteases. Myofibrillar and sarcoplasmic protein fractions were separated, acid hydrolysed and derivatised as NAP esters before measurement of bound leucine $^{13}C/^{12}C$ by GC-Combustion IRMS (Finnigan DeltaPlus XL). Fractional synthetic rates (FSR) were calculated as the rate of increase of labelling of muscle protein bound leucine compared to the average plasma KIC labelling. This study had ethical clearance from Ethics Committees of the University of Dundee and University of Nottingham.

* EAA infusions and human muscle protein synthesis experiments completed without my involvement by members of Professor Rennie's Laboratory, University of Dundee and University of Nottingham.

2.7 ATP-ase fibre type staining and NADH-Tr mitochondrial staining

The Soleus and Extensor Digitorum Longus (EDL) muscles were excised from the hind limb of a Sprague-Dawley rat. Muscles were then frozen using liquid N_2 cooled isopentane, and after obtaining 10 μm sections of the muscle, heated gently for 5 min.

Pre-incubation medium of (100 mM glycine, 1 % $CaCl_2$, 10 % formaldehyde, pH 7.25) was dropped onto the sections for 5 min at 25 °C, and then rinsed, three times with distilled water. Slides were then placed in a staining jar with incubation medium (2.8 mM ATP, 100 mM sodium barbital, 2 % $CaCl_2$ anhydrous) and incubated for 90 min at 37°C in a water bath. A 1 % $CaCl_2$ solution was then placed on the sections three times for 2 min. A 2 % Cobalt Chloride solution was then placed on the sections, again three times for 2 min before washing with distilled water three times for 2 min. Next, a drop of 2 % ammonium sulfide solution was placed on the sections for 90 s, before washing with distilled water, and then under running tap water for 10 min in the staining jars. Finally, the sections were dehydrated in ascending alcohols (50 %, 60 %, 70 %, 80 %, 90 %, and 95 %) with a drop of each placed on the section for 30 s. The slides were then cleaned, and cleared with two changes of histoclear, before being mounted with histomount. Under the microscope, Type I fibres have no staining, type IIa fibres have heavy staining and type IIb fibres show light staining.

The NADH-Tr stain was performed on chronically stimulated and control EDL muscles. Muscles were quickly excised and then frozen using isopentane cooled in liquid N_2, and after obtaining 10 μm sections of the muscle, heated gently for 5 min. A tris buffer (0.2 mM of tris-HCL, trizma base, pH 7.4) was made up with 1 mg ml^{-1} of nitro blue tetrazolium (NBT), before readjusting to pH 7.4. Then 1 mg ml^{-1} of NADH was added to the tris buffer. The sections were then covered with incubation solution and placed above the water bath at 37°C for 25 min. Under the microscope, the darkest fibres indicate higher mitochondrial densities and are thus theoretically type IIa fibres or type I, with type IIb fibres yielding little staining. Light microscopy was used to examine the results of both the ATP-ase and NBT histochemistry.

2.8 Western Blotting

2.8.1 Protein extraction

Proteins were extracted from rat EDL and Soleus muscles. Approximately, 30 mg of muscle was homogenised on ice in 0.6 ml of homogenisation buffer (50 mM Tris-HCL; 0.1 % Triton-X; 1 mM EDTA; 1 mM EGTA; 50 mM NaF; 10 mM β-glycerophosphate; 5 mM Na pyrophosphate; 0.1 % 2-mercaptoethanol; 100 nM okadaic acid; 50 μM sodium orthovanadate; 1 tablet of a protease inhibitor cocktail). Samples were rotated for 60 min at 4 °C, before being centrifuged at 13,000 g for 10 min. Protein concentration was measured using the Bradford assay (Bradford, 1976) and adjusted to 2 mg ml^{-1} by diluting in SDS sample buffer (3.55 ml deionised water, 1.25 ml 0.5 M Tris-HCL, pH 6.8; 2.5 ml glycerol; 2 ml 10 % (w/v) SDS; 0.2 ml 0.5 % (w/v) bromophenol blue).

2.8.2 Electrophoresis and Blotting

Protein samples (20 μg) were electrophoresed in running buffer (1 % SDS; 192 mM glycine; 25 mM tris-base; pH 8.3) on a 10 % (13.5 % high-bis gel for 4E-BP1) SDS-polyacrylamide gel electrophoresis gel at 100 V for 30 min through the stacking layer and then 200 V until the dye marker reached the bottom of the gel. Precisely, 10 μl of a rainbow molecular size marker was loaded in to the final lane in order to later confirm detection of the correct protein size. Following conclusion of electrophoresis, the polyvinylidene difluoride (PVDF) membrane was permeabilised in 100 % methanol for 1 min before both the gel and pre-wetted membrane were equilibrated in

transfer buffer (192 mM glycine; 25 mM Tris base; 20 % w/v methanol; pH 8.3) for 30 min. The transfer was run for 2 h at a constant 100 V. Upon completion of transfer, the uniformity of loading was checked with Ponceau S before the membrane was incubated in 30 ml of blocking buffer (TBS with 0.1 % Tween-20; 5 % w/v non-fat milk powder) for 2 h. Following incubation with the blocking buffer the membrane was washed 3 times for 5 min with wash buffer (TBS with 0.1 % Tween-20) in 30 ml with gentle agitation. Stimulated samples were exposed to the following pan antibodies: anti-calcineurin (Sigma C1956; 1:2000); anti-NFκB (NEB3034; 1:2000); anti-PKB/AKT (Courtesy of Prof Sir P Cohen; 1:2000); anti-p70 S6K (NEB9202; 1:2000); anti-GSK3β (Chemicon AB8687; 1:2000), anti-eIF2B and anti eEF2 (both courtesy of Prof CG Proud; 1:1000) anti-p44/42 (NEB9102; 1:2000); anti-p38α,β/SAPK2 (Biocompare S0096-01A; 1:2000); anti-p38γ (Upstate 07-139; 1:2000); anti-AMPK NEB2532; 1:2000); anti-SAPK/JNK (NEB9252; 1:2000); anti-myostatin (Santa Cruz sc-6885; 1:500); anti-4E-BP1 (courtesy of Prof CG Proud; 1:1000); anti-ubiquitin (NEB3936; 1:2000) or anti- SMAD2/3 (Santa Cruz sc-6033; 1:500) overnight at 4°C. Phosphorylation status was measured using: phospho-AMPK (NEB2531; Thr172; 1:2000); phospho-PKB (NEB9271; Ser473; 1:2000); phospho-p70 S6K (NEB9205; Thr389; 1:2000); phospho-ERK1/2 (p44/p42) (NEB9101; Thr202/Tyr204; 1:2000); phospho-p38 (NEB9211; Thr180/Tyr182; 1:2000); phospho-SAPK/JNK (NEB9251; Thr183/Tyr185; 1:2000); phospho-GSK3αβ (NEB9331; Ser21/9; 1:2000); phospho-SMAD2/3 (Santa Cruz sc-11769; Ser433/435; 1:500); phopsho-UBF (Santa Cruz sc-21639; Ser637; 1:500); phospho-eEF2 (courtesy of Prof CG proud; Thr56; 1:2000); phospho-eIF2Bε (Courtesy of Prof CG Proud; Ser535; 1:2000) and phospho-4E-BP1 (NEB9459; Ser37/46; 1:2000) also overnight at 4 °C. The following morning, the membrane was rinsed in wash buffer 3 times for 5

min each time in 30 ml. The membrane was then incubated for 1 h at ambient temperature with gentle agitation in 30 ml of blocking buffer containing the appropriate secondary antibody, either: horseradish peroxidase linked anti-mouse immunoglobulin (NEB7072; 1:2000); anti- rabbit immunoglobulin (NEB7074; 1:2000) or anti-sheep immunoglobulin (courtesy of Prof Chris Proud; 1:5000). The membrane was cleared 3 times for 5 min in 30 ml wash buffer. Membranes were exposed to ECL chemiluminescent detection reagents mixed 1:1 in 10 ml for 1 min. Membranes were partially dried, wrapped in saran and exposed to X-ray film.

2.8.3 Densitometry

X-ray films were scanned using a Biorad Imaging densitometer (Model GS-670) to detect the relative band intensity. Each band was identified and the optical density volume adjusted by subtraction of the background. All values obtained from one blot were normalised to the average resting control band intensity which was set to 1.

2.9 Microarray analysis

Spotted microarrays of *Mus musculus* (about 15,000 cDNAs on two slides) were obtained from The Human Genome Mapping Project Resource Centre Microarray Programme (Harwell, UK). Description of the arrays and links to the cDNA clone information can be found on:

http://www.hgmp.mrc.ac.uk/Research/Microarray/HGMP-RC_Microarrays/description_of_arrays.jsp#2

2.9.1 Experimental design

C2C12 mouse myoblasts were cultured to confluence in DMEM containing 10 % FBS, supplemented with amphoteracin B (1:1000) and Penicillin/Streptomycin (1:100), and formation of myotubes was triggered by switching to 2 % horse serum. Five 90 mm dishes were subjected to 10 ng ml^{-1} recombinant myostatin for 6 h whilst 5 dishes served as controls. Mouse NIA Clone Set Arrays (~15k cDNAs) from the Rosalind Franklin Genomic centre for Research were used which consisted of both 'A' and 'B' slides. Therefore, five biological repetitions were performed.

2.9.2 RNA extraction, cleanup and cDNA synthesis

Following treatment, total RNA was extracted from C2C12 cells according to the manufacturer's protocol, as described (see 2.2). RNA was then purified by Qiagen RNeasy cleanup as according to the manufacturers protocol. Eluted RNA was then vacuum centrifuged and adjusted to 2 μg $μl^{-1}$ in preparation for cDNA synthesis using the FairPlay Microarray labelling kit. For generation of cDNA 1 μl of 500 ng $μl^{-1}$ oligo(dT) was added to a 12 μl aliquot of the RNA preparation, mixed and heated at 70 °C for 10 min. Samples were then allowed to cool for 10 min to allow primers to anneal to template. For each reaction the following was added: 2 μl 10 × Statascript reaction buffer; 1 μl 20 × dNTP mix; 1.5 μl 0.1 M DTT; 0.5 μl RNase block (40 U $μl^{-1}$) and finally 1 μl Statascript reverse transcriptase (RT). This mixture was then vortexed briefly and incubated at 48 °C for 25 min. Following this 1 μl of of reverse transcriptase (RT) was added and incubated at 48 °C for a further 35 min. RNA-DNA

hybrid bonds were then hydrolysed by addition of 10 µl sodium hydroxide and incubation at 70 °C, before being neutralised with 10 µl HCl. Following this, the following constituents were added to the tube: 4 µl 3 M sodium acetate, pH 5.2; 1 µl 20 mg ml^{-1} glycogen; 100 µl 100 % ethanol. The mixture was then briefly vortexed and stored at -20 °C overnight.

2.9.3 cDNA labelling, purification and quality control

The following morning, the reaction mixture was then spun at 15,000 g for 30 min, supernatant removed and washed with 250 µl of 70 % ice-cold ethanol before centrifugation at 15,000 g for a further 30 min. The supernatant was removed and pellet allowed to air dry for 15 min. Precisely, 5 µl of 2 × coupling buffer was added to each tube and heated at 37 °C for 15 min to ensure resuspension. To each control sample, 5 µl of Cy3 red fluourescent dye was added and to each myostatin treated sample 5 µl of Cy5 green fluourescent dye was added before being thoroughly mixed and incubated at room temperature for 1 h in the dark. To each reaction 90 µl of DNA grade water was added before purification of DNA fragments from dye-labelled reactions was performed using the Qiaquick PCR purification kit as according to manufacturers instructions. The quality of labelling was checked on a 2 % agarose gel where 5 µl labelled cDNA was mixed with 5 µl xylene cyanol FF/bromphenole blue buffer diluted in glycerol. Scanning on a proXPRESS imager on Cy3 and Cy5 specific filters would reveal a bright 'smear' along the vertical stream of cDNA if labelling of cDNA was successful. Once the quality of labelling was ensured, samples were placed in a vacuum centrifuge to concentrate the sample, before resuming under section 2.9.5.

2.9.4 Preparation of microarray slides

Prehybridisation of microarray slides was performed for 1 h at 42 °C in 1 % bovine serum albumin, 5 × SSC, 0.1 % SDS. Slides were then washed in water and then isopropanol before centrifugation at 500 g for 5 min.

2.9.5 Hybridisation of cDNA to arrays

Labelled cDNA was then resuspended in 50 µl of hybridisation buffer (50 % formamide; 5 % dextran sulphate; 3 × SSC; 1 % SDS; 5 × Denhardt's solution) before Cy3 and Cy5 fluourescent dyes were combined. Samples were mixed and 2 µg/µl polyA was added. Combined samples were placed at 100 °C for 5 min, briefly centrifuged and cooled to 25 °C. Following this, the samples were carefully applied to the microarray slides using a pipette, before being protected with a plastic cover-slip. Finally, the microarray slides were then incubated in a 3 × SSC humid chamber for 18 h at 42 °C.

2.9.6 Washing

Cover-slips were removed from slides by immersion in 1 × SSC, 0.2 % SDS. Slides are then immersed in this same buffer and washed 2 × 5 min. Slides were then washed again for 2 × 5 min in 0.1 × SSC, 0.1 % SDS, and further for 2 × 5 min in 0.1 × SSC. Slides were then stored in the dark at ambient temperature awaiting scanning and analysis.

2.9.7 Analysis of microarrays

Microarrays were scanned with arrayWoRx^e scanner and resulted images analysed by arrayWoRx Software Suite 2.0 (all from Applied Precision Instruments, USA). Data were generated for spot and their background intensities as means and standard deviations. Spot intensity data were imported into locally installed BioArray Software Environment, BASE (Saal *et al.*, 2002) and pre-processed by local background subtraction, filtering out spots with unreliable intensities (SNR<3), lowess normalisation, averaging normalised intensities across replicated spots on array, and choosing only genes with intensity data for two or more array replicates.

For analysis, gene's experiment to control ratios were imported into TIGR Multiexperiment Viewer (MeV) software (Saeed *et al.*, 2003), $\log_2$-transformed and normalised. Differentially expressed genes were determined as those with ratios deviated from expected ratio of one (one-sample t-test, $p<0.05$) and having unusual ratio (z-test, $P<0.05$). No multiple comparisons corrections were applied.

* Microarray scanning and spot analysis was performed by Dr Viacheslav Bolshakov, University of Dundee without my involvement.

2.10 Statistics

All statistical data in chapters 3 and 4, and the first study examining protein phosphorylation following *in vivo* muscle stimulations in chapter 5, are displayed as mean ± standard error (±SE) in the figures. Analysis for statistical significance between treatments (chapter 3); EDL and soleus muscle types (chapter 4); between stimulations *in vivo* (chapter 5), was done using the Student's t-test. Differences were considered significant when $p<0.05$.

All statistical data for the *in vitro* HFS/LFS stimulations in chapter 5 are displayed as mean ± standard error of the mean (±SEM) in the figures. Changes in phosphorylation were normalised to total levels of both phosphorylated/unphosphorylated forms using native antibodies. Means were compared using an independent, three-factorial ANOVA (muscle: EDL, Soleus; stimulation: HFS, LFS; time: control, directly after stimulation, 3 h control, 3 h after stimulation). A one-factorial ANOVA (control; 3 h after LFS; 3 h after HFS) was used to analyse the protein synthesis and RT-PCR data (chapters 5 & 6). Tukey's test was used as a post hoc test and $p<0.05$ was used as a threshold for statistical significance.

Chapter 3

Role of myostatin in the control of muscle mass: regulation of transcription and translation

3.1 Introduction

Myostatin is a powerful negative regulator of skeletal muscle mass. However, it remains largely unknown how myostatin might affect muscle mass in post-mitotic situations, even though there is evidence that systemic myostatin causes cachexia (Zimmers *et al.*, 2002), and is elevated in disease atrophy states (Jackman & Kandarian, 2004). Myostatin may also be subject to regulation by resistance exercise (Walker *et al.*, 2004), and endurance exercise, although the latter has not been examined to date. Therefore, myostatin is an important component in the control of muscle mass in relation to both exercise and disease.

However, precisely how myostatin affects transcription and translation to control muscle mass has not been elucidated, and is poorly understood. Also inadequately characterised is the regulation of the myostatin gene. For although myostatin regulation has been linked putatively by many factors (Ma *et al.*, 2001), only glucocorticoids have been experimentally verified to increase myostatin expression.

Therefore, the aims of these studies were to perform a battery of experiments in order to gain insight in to both how myostatin exerts its effects in adult muscle, and what factors regulate the myostatin gene. Specifically, these studies aimed to examine how various growth factors and hormones including IGF-1, clenbuterol, growth hormone, testosterone, dexamethasone, cyclosporine A, and finally creatine would affect myostatin gene expression in L6 cultured muscle cells using Northern blotting and RT-PCR. This should yield important information about the regulation of expression of the myostatin gene. Secondly, to elucidate the signalling mechanisms behind the inhibitory effect of myostatin on protein synthesis by using Western blotting to

examine the phosphorylation of components of the IGF-1-PI-3K-PKB-mTOR pathway in the presence of recombinant myostatin in C2C12 skeletal muscle cells. Finally, to examine the transcriptional effects of recombinant myostatin on C2C12 skeletal muscle cells using genome-wide cDNA microarrays in order to identify genes involved in mediating the negative effect of myostatin on muscle mass.

The specific hypotheses of these studies being:

1. Treatment of L6 skeletal muscle cells with growth factors can suppress myostatin mRNA expression, whereas glucococortioids, such as dexamethasone will increase myostatin gene expression.

2. Myostatin can inhibit elements of the PI-3K pathway responsible for directing increases in muscle protein synthesis.

3. Recombinant myostatin treatment of C2C12 skeletal muscle cells can affect genes involved in controlling muscle mass.

Methods

As described in Chapter 2.

3.2 Results

3.2.1 RNA quality

RNA viability of C2C12 and L6 skeletal muscle cells was checked by spectrophotometry and was within recommended levels, indicating minimal DNA, protein and guanadinium salt contamination (absorbance 260 / absorbance 280 = 1.8-2). Furthermore, when electrophoresed and stained with ethidium bromide on an agarose gel, RNA had a 2:1 ratio of 28s : 18s ribosomal RNA (rRNA), as determined by densitometry. Finally, there was no evidence of RNA degradation or DNA contamination as shown by 'streaking' between ribosomal bands.

3.2.2 Northern blots detected no myostatin transcript in L6 cells

Northern blots of all L6 samples at 2 μg RNA, showed no expression of myostatin following exposure to a phosphorscreen after a 24 h probe hybridisation period. Therefore, the time before exposure to the phosphorscreen was increased for up to 72 h, and RNA the quantity electrophoresed on agarose gels was increased to between 4 and 20 μg of RNA, at 2 μg increments. RNA at 15-20 μg was not able to migrate on a horizontal gel due to the high viscosity, although concentrations between 4 and 14 μg electrophoresed effectively. These modifications however, yielded only ribosomal RNA (rRNA) banding, thus indicating non-specific binding of the myostatin probe to rRNA. Consequently, no myostatin mRNA was detected in any of the L6 samples, regardless of treatment; and this was also true for control L6 cells. Lack of myostatin mRNA using Northern blotting detection methods was therefore regardless of RNA concentration and probe exposure times.

Following this failure to detect expression of myostatin mRNA in the L6 cells, it was decided to examine myostatin mRNA of various skeletal muscles, heart, primary skeletal muscle cells, and the L6 cells at higher RNA concentrations (14 μg), including those treated with Dexamethasone (known to increase myostatin mRNA). Again, myostatin mRNA was not detected in L6 cells, whilst also undetected in the primary skeletal muscle cells. However, myostatin mRNA was detected in rat muscle extracts and was present at higher levels in muscle with a greater proportion of type II muscle fibres such as EDL, tibialis anterior (TA), epitrochlearis and gastrocnemius than in slower muscles such as Soleus (figure 3.1). These data showed that the myostatin probe generated was successful in detecting native myostatin mRNA and could not be responsible for the lack of detection in the L6 cells.

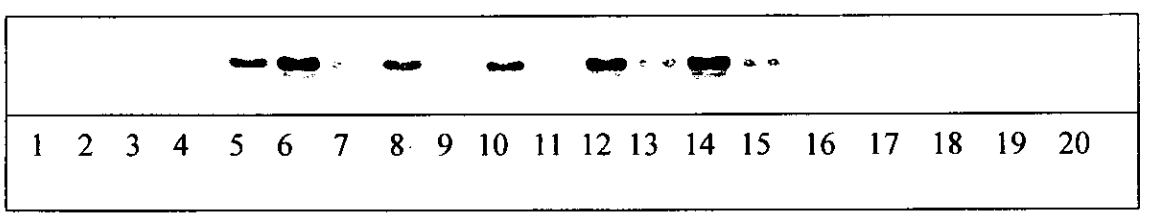


Figure 3.1. Example of myostatin Northern blots at 2 μg RNA of primary muscle cells, 2 μg various muscle tissue extract and 14 μg of Cyclosporine A and dexamethasone treated L6 cells. Lanes 1-4 primary cultured muscle cells; lane 5 plantaris; lane 6, EDL; lane 7, heart; lane 8, tibialis anterior; lane 9, Soleus; lane 10, epitrochlearis; lane 11, vastus intermedius; lane 12, gastrocnemius; lane 13, Vastus Lateralis lane 14, tibialis posterior; lane 15, flexor digitorum longus; lanes 16-18 Cyclosporine A treated L6 cells; lanes 18-20 dexamethasone treated L6 cells. Blots are a typical example of 4 such experiments, (n=4).

Next, it was necessary to check the viability of the L6 cells since although the RNA was checked, there remained a possibility of sample contamination and therefore it was decided to attempt Northern blotting using a different probe, other than myostatin. Figure 3.2 shows Northern blots for GLUT1 on the L6 treated cells. GLUT1 was chosen as an additional probe since it was previously shown by GeneCards to be expressed highly and ubiquitously in skeletal muscle. Furthermore, it is also known from the literature to be regulated by IGF-1 and dexamethasone, two of the treatments exposed to the L6 cells. As expected from the literature, GLUT1 was upregulated mainly in response to IGF-1 and dexamethasone (figure 3.2). These results indicated that the treatments of the L6 cells were effective and the RNA from the L6 cells was apparently viable and not contaminated.

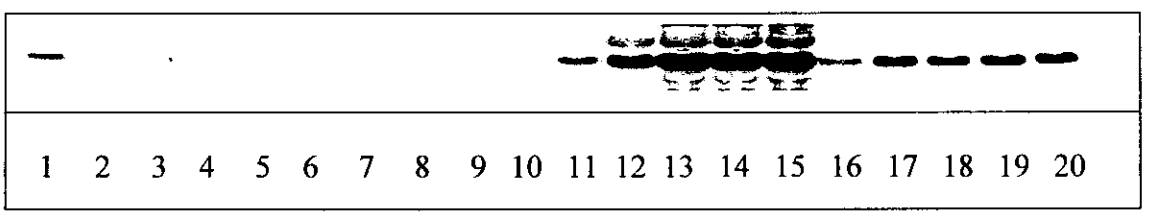


Figure 3.2. Example of GLUT 1 Northern Blot of L6 cells. Treatment of L6 cells with growth hormone (lanes 1-4), creatine 24 h (lanes 5-8) and Cyclosporine A (lanes 9-12) showed small increases (and a larger increase for CsA at high concentrations) in GLUT1 mRNA following exposure to phosphorscreen after 24 h. Treatment of cells with IGF-1 (lanes 13-16), and dexamethasone (lanes 17-20) robustly upregulated GLUT1 mRNA at various concentrations, except IGF-1 at the highest concentration, lane 16) which had limited effect. Blots are a typical example of 4 such experiments, (n=4).

3.2.3 RT-PCR detected no myostatin transcript in L6 cells

The RT-PCR shown in figure 3.3 was attempted since it could give more sensitive detection of myostatin mRNA, and moreover it could confirm the negative results of the Northern analyses. Reverse transcription (RT) was checked through completing the L6 RT alongside various other muscle homogenates. Furthermore, the samples were spiked with cDNA from fresh skeletal muscles in order to ascertain whether the L6 cells contained any substance inhibiting the detection of myostatin. Muscle homogenates alone showed myostatin expression, as did L6 cells 'spiked' with cDNA from skeletal muscle homogenates. However, L6 cells treated with dexamethasone and all other treatments showed no myostatin expression, thus further confirming the lack of myostatin expression in these cells (note the mis-primings in L6 cells).

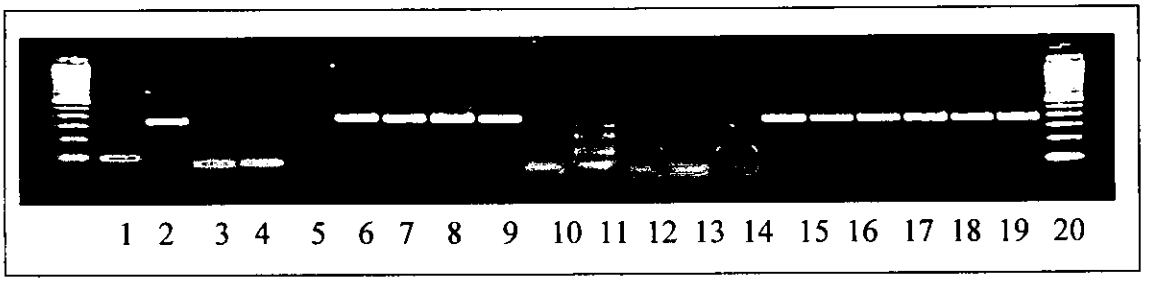


Figure 3.3. Myostatin RT-PCR in various tissue types. Lanes 1-5, dexamethasone treated L6 cells; lanes 6-9, L6 samples 'spiked' with cDNA positive control to ensure there was nothing in the L6 samples which inhibited detection of myostatin; lanes 10-14 mixed L6 samples; lanes 15-19, various muscle homogenate RNA reverse transcribed alongside L6 samples to ensure RT reaction was successful; lane 20 positive control cDNA. End lanes are 100 Kb size markers. Primary and L6 cells again showed no presence of myostatin transcript. *Lane 2 not at the same size as myostatin detected in skeletal muscles. Image typical of 4 such experiments, (n=4).

3.2.4 Myostatin increases SMAD2 phosphorylation and inhibits the phosphorylation of elements of the PI-3K pathway

Treatment of C2C12 cells with recombinant myostatin causes an increase in phosphorylation of SMAD2 (~35 %) (figure 3.4). Since SMAD proteins are implicated in myostatin signalling, the increased phosphorylation is presumably indicative of an increased SMAD2 signalling in response to recombinant myostatin.

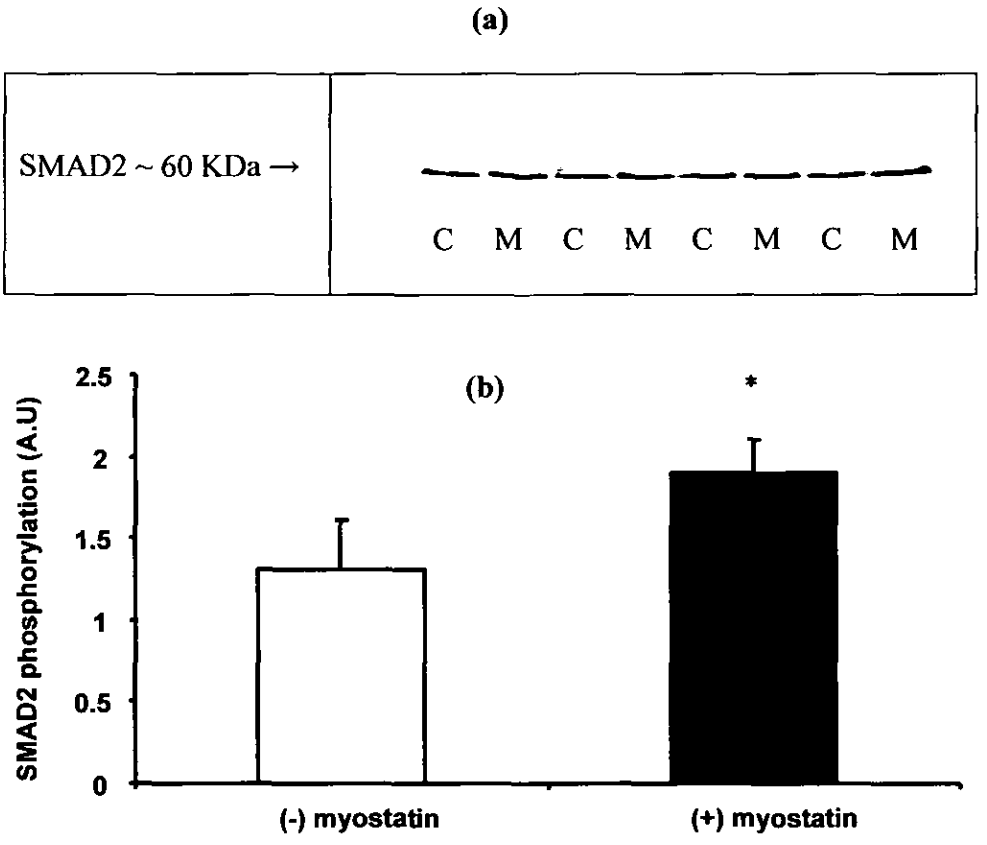


Figure 3.4. Immunoblots (a) and representative bar chart (b), showing that SMAD2 phosphorylation is increased in response to recombinant myostatin. Representative Western blots above, C= Control cells, M = myostatin, 10 ng ml^{-1} (data are mean ±SEM n=8, * p = <0.05).

Treatment of the murine C2C12 skeletal muscle cells with 10 ng ml^{-1} recombinant myostatin (+) reduces the phosphorylation of many key signalling proteins in the insulin/IGF pathway (figure 3.5), suggesting a mechanism for suppression in protein synthesis. PKB phosphorylation decreased by ~30 %, mTOR by ~50 %, p70 S6K by ~60 % and 4E-BP1 remained unaffected.

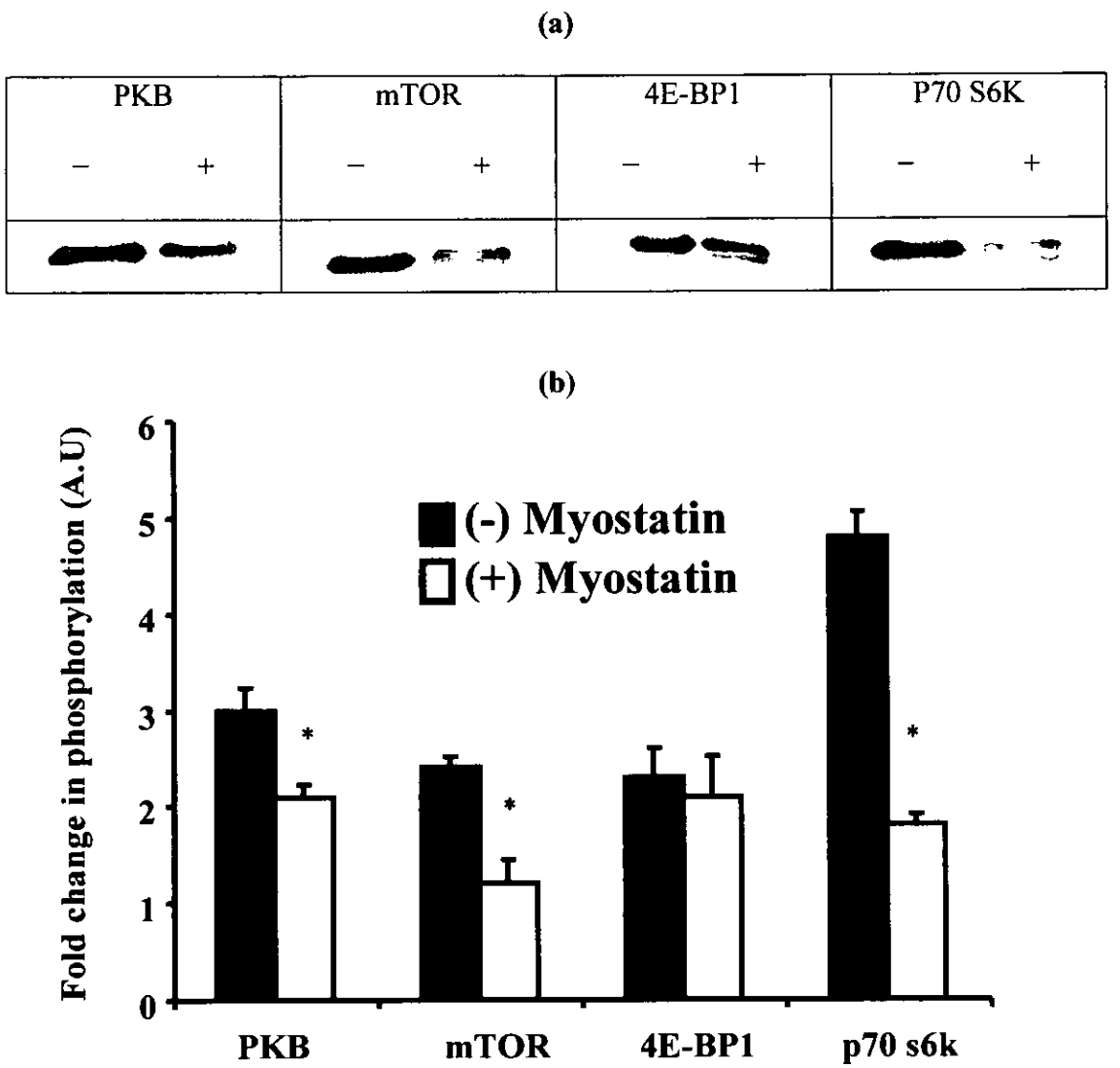


Figure 3.5. Immunoblots (a) and representative bar chart (b), showing that myostatin inhibits signalling through PKB, mTOR and p70 S6K in C2C12 cells (data are mean ±SEM n=8, * p = <0.05).

3.2.5 cDNA microarrays – Myostatin inhibits transcription

The Rosalynd Franklyn Centre provided the cDNA microarrays on which probes from the National Institute of Ageing 15K Mouse cDNA clone set have been printed on an A and B array. The quality of cDNA labelling with Cy3 and Cy5 dyes was indicated to be very good as shown by the high intensity of the 'smear' on an agarose gel. Following analysis of the data, the major finding of this study is that recombinant myostatin leads only to a down-regulation of genes in C2C12 skeletal muscle cells. Therefore, myostatin is indirectly, a transcriptional inhibitor. Gene lists have been produced using two levels of stringency. The first list contains genes likely to be downregulated by myostatin and the second list genes less likely to be downregulated. Only the first list of genes was systematically analysed. For known genes, information regarding the differentially expressed genes was obtained using GeneCards to obtain information on expression in skeletal muscle compared to other tissues (Rebhan *et al.*, 1998; Safran *et al.*, 2002). If available, links to Mendelian Inheritance in Man (MIM or OMIM) or LocusLink were followed.

A selection of genes, were initially identified that are potential regulators of the atrophy/anti-proliferation effect of myostatin. It is very likely that many other genes in this list mediate the negative effects of myostatin on muscle mass, although it was beyond the scope of this thesis to investigate all of these. For unknown genes expressed sequence tags (ESTs) and other unnamed genes, the identifier was entered into the National Institute of Ageing 15 K mouse cDNA clone set website (these clones have been used to print the arrays) to search for updated information. Listed below are two sets of data from the microarray data.

(a) List of genes likely to be down-regulated by myostatin:

Identifier	*Gene description*
Mm_NIA_H3004A06	Tetratricopeptide repeat domain
Mm_NIA_H3010D06	Caseinolytic protease, ATP-dependent, proteolytic subunit homolog (E. coli)
Mm_NIA_H3025C07	G0103G11-3 NIA Mouse E7.5 Embryonic Portion cDNA Library Mus musculus cDNA clone G0103G11 3', mRNA
Mm_NIA_H3028G01	DNA segment, Chr 16, ERATO Doi 454, expressed
Mm_NIA_H3012E12	RIKEN cDNA D030054H19 gene
Mm_NIA_H3015D03	ESTs
Mm_NIA_H3009F07	Kinesin family member 5B
Mm_NIA_H3034F11	ESTs
Mm_NIA_H3062H06	RIKEN cDNA 1190006A08 gene
Mm_NIA_H3050G07	ESTs
Mm_NIA_H3025G01	Splicing factor, arginine/serine-rich 3 (SRp20)
Mm_NIA_H3029A07	Nuclear protein 220
Mm_NIA_H3074E11	RIKEN cDNA 2210401J11 gene
Mm_NIA_H3011E06	H3011E06-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3011E06 3', mRNA sequence.
Mm_NIA_H3029G08	Neural-salient serine/arginine-rich
Mm_NIA_H3017B03	C0176A09-3 NIA Mouse E7.5 Extraembryonic Portion cDNA Library Mus musculus cDNA clone C0176A09 3', mRNA sequence.

Mm_NIA_H3051A01	C85250 Mouse fertilized one-cell-embryo cDNA Mus musculus cDNA clone J0204H03 3', mRNA sequence.
Mm_NIA_H3050B12	ESTs
Mm_NIA_H3011F07	Protein tyrosine phosphatase 4a2
Mm_NIA_H3051E01	Hypothetical protein MGC12070
Mm_NIA_H3001E07	ESTs
Mm_NIA_H3020F07	RIKEN cDNA 3110001H15 gene
Mm_NIA_H3062E08	Epoxide hydrolase 2, cytoplasmic
Mm_NIA_H3076D12	Similar to erythrocyte protein band 4.1-like 4b
Mm_NIA_H3001F01	Ephrin A1
Mm_NIA_H3029F01	P53 apoptosis effector related to Pmp22
Mm_NIA_H3002A03	RIKEN cDNA 1110002B05 gene
Mm_NIA_H3013H07	RIKEN cDNA 1810010A06 gene
Mm_NIA_H3031E02	RIKEN cDNA 2810410M20 gene
Mm_NIA_H3010H12	PHD finger protein 5A
Mm_NIA_H3073C09	H3073C09-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3073C09 3', mRNA sequence.
Mm_NIA_H3042E06	Protein tyrosine phosphatase, receptor type, A
Mm_NIA_H3029H05	Teratocarcinoma-derived growth factor
Mm_NIA_H3026F04	RIKEN cDNA 6430573H23 gene
Mm_NIA_H3065F10	Chromosome condensation protein G
Mm_NIA_H3023G01	Heat shock protein 1, alpha
Mm_NIA_H3049A07	H3049A07-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3049A07 3', mRNA sequence.

Mm_NIA_H3004A05	Transforming growth factor beta regulated gene 4
Mm_NIA_H3060G12	ESTs
Mm_NIA_H3020G02	RIKEN cDNA 2310081H14 gene
Mm_NIA_H3017B01	H3017B01-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3017B01 3', mRNA sequence.
Mm_NIA_H3001B05	Pan hematopoietic expression
Mm_NIA_H3025B07	EST's
Mm_NIA_H3003A01	ESTs, Moderately similar to hypothetical protein FLJ23311 [Homo sapiens] [H.sapiens]
Mm_NIA_H3001B04	DNA segment, Chr 9, Wayne State University 20, expressed
Mm_NIA_H3001B02	Mus musculus 2 days neonate thymus thymic cells cDNA, RIKEN full-length enriched library, clone:E430020E19 product:ROD1 homolog [Rattus norvegicus], full insert sequence.
Mm_NIA_H3002B04	CUG triplet repeat, RNA binding protein 1
Mm_NIA_H3018F12	RIKEN cDNA 1110017C15 gene
Mm_NIA_H3006B03	Interleukin 13 receptor, alpha 1
Mm_NIA_H3001A03	DNA segment, Chr 11, Wayne State University 99, expressed
Mm_NIA_aaaaaM28723	Peroxiredoxin 3
Mm_NIA_H3001B01	RIKEN cDNA 9430023L20 gene
Mm_NIA_hhhhhU49720	Opsin 1 (cone pigments), short-wave-sensitive (color blindness, tritan)
Mm_NIA_H3001A06	ESTs

Mm_NIA_H3153E06	B-cell receptor-associated protein 29
Mm_NIA_H3104G01	Myosin light chain, phosphorylatable, fast skeletal muscle
Mm_NIA_H3131D06	L0212C09-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0212C09 3', mRNA sequence.
Mm_NIA_H3130F01	Serologically defined breast cancer antigen 84
Mm_NIA_H3110H05	RIKEN cDNA 1110023P21 gene
Mm_NIA_H3098D04	RIKEN cDNA 1300004C11 gene
Mm_NIA_H3127D09	RIKEN cDNA D330050P16 gene
Mm_NIA_H3081A02	Eukaryotic translation initiation factor 4A2
Mm_NIA_H3138F12	Syndecan 4
Mm_NIA_H3147E02	S-adenosylmethionine decarboxylase 1
Mm_NIA_H3095E07	H3095E07-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3095E07 3', mRNA sequence.
Mm_NIA_H3131C02	RIKEN cDNA 2410016O06 gene
Mm_NIA_H3147F09	RIKEN cDNA D130072O21 gene
Mm_NIA_H3156B04	RIKEN cDNA 0710001E19 gene
Mm_NIA_H3105B04	Bone morphogenetic protein 15
Mm_NIA_H3147D03	ESTs
Mm_NIA_H3132B10	ESTs
Mm_NIA_H3095A05	Estrogen receptor 1 (alpha)
Mm_NIA_H3092C01	Eukaryotic translation initiation factor 4, gamma 2
Mm_NIA_H3138C06	L0235C10-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0235C10 3', mRNA sequence.
Mm_NIA_H3118E03	RIKEN cDNA 2810442I22 gene

Mm_NIA_H3083A04	V-crk sarcoma virus CT10 oncogene homolog (avian)
Mm_NIA_H3126D06	H3126D06-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3126D06 3', mRNA sequence.
Mm_NIA_H3119C06	Nucleolar protein 5
Mm_NIA_H3083H12	Synaptosomal-associated protein 23
Mm_NIA_H3138B03	RIKEN cDNA 1110017P05 gene
Mm_NIA_H3131A03	Heat shock protein 1, alpha
Mm_NIA_H3133G10	RIKEN cDNA 4933402K10 gene
Mm_NIA_H3137F09	Hypothetical protein MGC36997
Mm_NIA_H3107F02	Cyclin-dependent kinase 4
Mm_NIA_H3084A06	Spindling
Mm_NIA_H3112D06	RIKEN cDNA 2610027L16 gene
Mm_NIA_H3105B10	Cyclin B1, related sequence 1
Mm_NIA_H3156E01	ESTs, Moderately similar to I58401 protein-tyrosine kinase (EC 2.7.1.112) JAK3 - mouse [M.musculus]
Mm_NIA_H3088E08	ESTs
Mm_NIA_H3138D07	Voltage-dependent anion channel 2
Mm_NIA_H3094E12	RIKEN cDNA 1190002A17 gene
Mm_NIA_H3120C07	Tribbles homolog 1 (Drosophila)
Mm_NIA_H3114C06	L0011F07-3 NIA Mouse E12.5 Female Mesonephros and Gonads cDNA Library Mus musculus cDNA clone L0011F07 3', mRNA sequence.
Mm_NIA_H3139H03	ESTs
Mm_NIA_H3146H12	RIKEN cDNA 1700021F05 gene

Mm_NIA_H3114H09	Nucleosome assembly protein 1-like 4
Mm_NIA_H3109H08	ESTs, Weakly similar to RIKEN cDNA 5730493B19 [Mus musculus] [M.musculus]
Mm_NIA_H3139G01	L0240E12-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0240E12 3', mRNA sequence.
Mm_NIA_H3100G02	ESTs
Mm_NIA_H3084H07	RIKEN cDNA 6430573H23 gene
Mm_NIA_H3084G05	RIKEN cDNA D230034E10 gene
Mm_NIA_H3100B07	Forkhead box O1
Mm_NIA_H3129F05	Syntaxin 3
Mm_NIA_H3103C07	Icos ligand
Mm_NIA_H3103D04	Budding uninhibited by benzimidazoles 1 homolog, beta
Mm_NIA_H3083B05	Neural-salient serine/arginine-rich
Mm_NIA_H3145G11	ESTs
Mm_NIA_H3128B12	L0204B09-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0204B09 3', mRNA sequence.
Mm_NIA_H3131G07	ESTs
Mm_NIA_H3087F09	H3087F09-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3087F09 3', mRNA sequence.
Mm_NIA_H3131H09	Expressed sequence AW011752
Mm_NIA_H3083H03	ESTs
Mm_NIA_H3126G12	RIKEN cDNA 2500001H09 gene
Mm_NIA_H3128D12	L0204F02-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0204F02 3', mRNA sequence.

Mm_NIA_H3147E11	Mus musculus, clone IMAGE:4161424, mRNA
Mm_NIA_H3140B06	L0242A05-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0242A05 3', mRNA sequence.
Mm_NIA_H3092G11	Limb region 1
Mm_NIA_H3139C11	Membrane-spanning 4-domains, subfamily A, member 6D
Mm_NIA_H3083A05	DNA segment, Chr 5, ERATO Doi 605, expressed
Mm_NIA_H3138A12	RIKEN cDNA 1810019E15 gene
Mm_NIA_H3148D12	L0267E10-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0267E10 3', mRNA sequence.
Mm_NIA_H3115B02	L0015A01-3 NIA Mouse E12.5 Female Mesonephros and Gonads cDNA Library Mus musculus cDNA clone L0015A01 3', mRNA sequence.
Mm_NIA_H3151C01	Mitogen-activated protein kinase 8 interacting protein 3
Mm_NIA_H3152F12	L0281F06-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0281F06 3', mRNA sequence.
Mm_NIA_H3140D06	Zinc finger protein 106
Mm_NIA_H3082H10	RIKEN cDNA 1700021P22 gene
Mm_NIA_H3126B09	H3126B09-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3126B09 3', mRNA sequence.
Mm_NIA_H3127C08	DnaJ (Hsp40) homolog, subfamily A, member 1
Mm_NIA_aaaaaM28723	Peroxiredoxin 3
Mm_NIA_H3124B06	Brain cDNA 7
Mm_NIA_hhhhhU49720	Opsin 1 (cone pigments), short-wave-sensitive (color blindness, tritan)

Mm_NIA_H3142G03	DnaJ (Hsp40) homolog, subfamily C, member 3
Mm_NIA_H3141G02	Eukaryotic translation initiation factor 4A2
Mm_NIA_H3109B12	RIKEN cDNA 5830484A20 gene
Mm_NIA_H3099B07	Hypothetical protein A130042E20
Mm_NIA_H3148D06	Inosine triphosphatase (nucleoside triphosphate pyrophosphatase)
Mm_NIA_H3117G02	ESTs
Mm_NIA_H3082B01	Natural killer tumour recognition sequence
Mm_NIA_H3138E07	RIKEN cDNA 1810038N08 gene
Mm_NIA_H3084B05	ESTs
Mm_NIA_H3131B07	Max dimerization protein 4
Mm_NIA_H3084B03	T7 gp4-like protein with intramitochondrial nucleoid
Mm_NIA_H3144F07	Purinergic receptor P2X, ligand-gated ion channel 4
Mm_NIA_H3082B04	RIKEN cDNA E330010H22 gene
Mm_NIA_H3103D06	ESTs
Mm_NIA_H3088H08	ESTs
Mm_NIA_H3139C06	Mus musculus embryo cDNA, full- clone:3002006F17
Mm_NIA_H3087B04	Expressed sequence AI182287
Mm_NIA_H3155F07	RIKEN cDNA 2810008M24 gene
Mm_NIA_H3083A06	RIKEN cDNA E330013P04 gene
Mm_NIA_H3104H07	ESTs
Mm_NIA_H3135C02	Lectin, galactose binding, soluble 7
Mm_NIA_H3088A05	RIKEN cDNA A430079H05 gene
Mm_NIA_H3103E09	Solute carrier family 37 (glycerol-3-phosphate transporter)

(b) List of genes less-likely to be downregulated by myostatin:

Identifier	*Gene description*
Mm_NIA_H3148D06	Inosine triphosphatase (nucleoside triphosphate pyrophosphatase)
Mm_NIA_H3030E02	Pan hematopoietic expression
Mm_NIA_H3030E02	GABA(A) receptor-associated protein like 2
Mm_NIA_hhhhhU49720	Opsin 1 (cone pigments), short-wave-sensitive (color blindness, tritan)
Mm_NIA_H3001B03	Mus musculus 0 day neonate kidney cDNA, RIKEN full-length enriched library D630017N12 product, clone: hypothetical Zinc finger, C2H2 type containing protein, full insert sequence.
Mm_NIA_H3001B02	Mus musculus 2 days neonate thymus thymic cells cDNA, RIKEN full-length enriched library, clone:E430020E19 product: ROD1 homolog [Rattus norvegicus], full insert sequence.
Mm_NIA_H3152F12	Mm_NIA_H3002B02; H3002B02-3 NIA Mouse 15K cDNA Clone Set cDNA H3002B02 3', mRNA sequence.
Mm_NIA_H3001B01	RIKEN cDNA 9430023L20 gene
Mm_NIA_H3003A06	RIKEN cDNA 2700002L06 gene

(b) Table continued.....

Identifier	*Gene description*
Mm_NIA_H3007A01	RIKEN cDNA C920006C10 gene
Mm_NIA_H3002B05	RIKEN cDNA 9430065L19 gene
Mm_NIA_H3001B01	RIKEN cDNA 9430023L20 gene
Mm_NIA_H3006B04	RIKEN cDNA 5730466P16 gene
Mm_NIA_H3046A01	RIKEN cDNA 2210008F15 gene
Mm_NIA_H3126G12	RIKEN cDNA 2500001H09 gene
Mm_NIA_H3082B04	RIKEN cDNA E330010H22 gene
Mm_NIA_H3001A06	ESTs
Mm_NIA_H3001A06	ESTs
Mm_NIA_H3002A02	ESTs
Mm_NIA_H3092B09	ESTs
Mm_NIA_H3084B05	ESTs
Mm_NIA_H3012G05	DNA segment, Chr 13, Wayne State University 177, expressed
Mm_NIA_H3001A03	DNA segment, Chr 11, Wayne State University 99, expressed

- No genes were detected to be upregulated in response to 10 ng ml^{-1} recombinant myostatin treatment, in the murine C2C12 skeletal muscle cell line.
- ESTs = Expressed sequence tags.

3.3 Discussion

3.3.1 Myostatin was not detected in L6 myotubes

In this study, myostatin mRNA was not detected in the L6 muscle cells using either RT-PCR or Northern blotting (figures 3.1-3.3). Therefore, it is likely that myostatin is not expressed in this cell line, at least at the point where the myotubes were harvested. However, this is contrary to a study where myostatin expression was shown to be present in myotubes, but not myoblasts (Artaza *et al.*, 2002). This discrepancy might be explained by the fact that this was in the murine C2C12 mouse cell line and not the L6 rat line that were used in the present study. Failure to detect myostatin is unlikely to be methodological error since several control experiments were performed to ensure this. For example, raw skeletal muscle extract was processed alongside L6 samples and also used to 'spike' L6 cells to ensure that there was nothing within the culture sample responsible for inhibiting detection of myostatin. Therefore, it is also unlikely that probe, reverse transcription, or PCR failure could be responsible for the lack of detection since skeletal muscle extracts showed positive for myostatin mRNA with both RT-PCR and Northern blotting. It may also be possible that the level of myostatin was too low to detect, although magnification by RT-PCR is capable of detecting very small quantities of mRNA transcripts so this is unlikely. In addition, cells treated with dexamethasone, which is known to upregulate myostatin mRNA (Ma *et al.*, 2001), still failed to show detection of myostatin. When different rat skeletal muscles homogenised and assayed, the presence of myostatin mRNA was highest amongst predominantly fast muscles in agreement with another study which came later (Artaza *et al.*, 2002) associating myostatin localisation with type II MHC. Since the expression of myostatin is fibre-type specific, this may have some effect

upon how slow and fast muscles respond to exercise training. The failure to detect myostatin in the L6 cells leaves an unfortunate gap in information regarding the regulation of the myostatin gene, although this could not have been predicted prior to the initiation of this study.

3.3.2 Myostatin inhibits phosphorylation of translation proteins

Myostatin is a potent negative regulator of muscle mass and whilst it has been shown to inhibit protein synthesis, the signal transduction mediating this effect had not been previously identified. It had been reported that myostatin signals through the SMAD family of proteins, via binding activin II receptors (Zhu *et al.*, 2004). The present results (figure 3.4) support these findings in finding that SMAD2 phosphorylation increases in response to myostatin treatment, and it can therefore be assumed that recombinant myostatin bound to the receptor. A major pathway directing changes in protein synthesis is the insulin/IGF (PI-3K-PKB-mTOR) pathway. The PI-3K-PKB-mTOR pathway is known to be important in IGF-1 mediated hypertrophy (Bodine *et al.*, 2001), and therefore the identified reduction in phosphorylation of signalling components within this cascade may explain how myostatin inhibits muscle growth, even in the presence of mitogenic factors. In response to mitogenic signals PI-3K activation leads to the phosphorylation of PKB whose target is mTOR. mTOR, then directs increases in protein synthesis through phosphorylation of 4E-BP1 which increases the activity of eIF4E the translation elongation factor by releasing its inhibitory effect. Secondly, mTOR phosphorylates p70 S6K which targets the S6 subunit of the ribosome to increase ribosome assembly. These processes act to increase cap-dependent/independent translation of multiple mRNA species, thus

increasing global protein synthesis (see section 1.7.4). The inhibition of PKB-mTOR-p70 S6K signalling by myostatin is a possible means by which the negative effect of myostatin on protein synthesis could be explained. However, the exact mechanism remains to be elucidated, although may involve interactions of SMAD proteins with PKB or other downstream constituents of this pathway (Conery *et al.*, 2004).

These findings on myostatin have important functional importance for both exercise and disease. If myostatin was suppressed in response to resistance training then this would reduce the inhibitory effect of myostatin upon the PI-3K pathway and thus increase IGF-1/MGF-induced hypertrophy. Conversely, if myostatin was to be upregulated during endurance training then this may be able to explain the atrophy associated with long term training regimes and the inhibition of protein synthesis observed in chronic aerobic exercise (Atherton *et al.*, 2005). In wasting states where myostatin is systemically increased, neutralisation of the protein in circulation would function by removing any inhibitory effect on growth though the PI-3K pathway. It remains to be seen if activating elements of this pathway, by IGF-1 for example, in these conditions could remove the inhibitory effect of myostatin.

3.3.3 Myostatin microarrays – Myostatin inhibits transcription

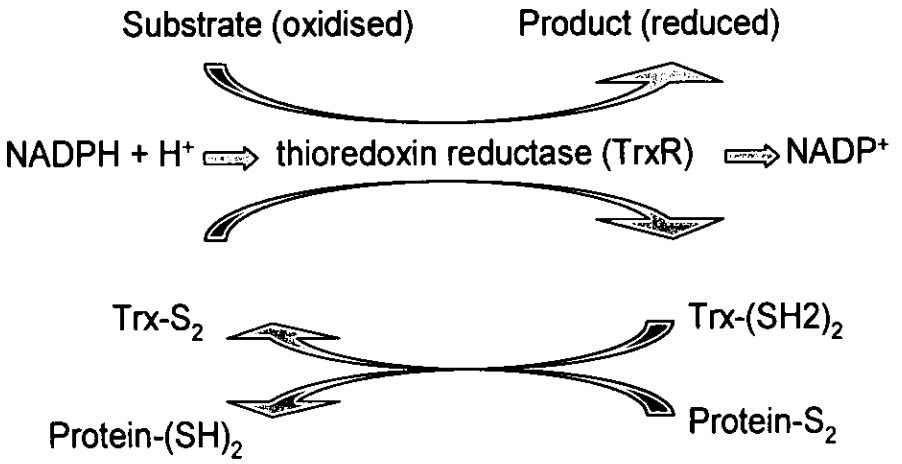
The first observation from these gene arrays is that no genes are upregulated but several genes appear to be downregulated. Thus, the first novel data of this study reveal that myostatin exerts its effects through inhibition of transcription. The most interesting genes related to growth regulation have been further investigated, whilst those that are lesser downregulated have been listed under section 3.2.5.

The first gene of interest is Mm_NIA_H3002B04; CUG triplet repeat, RNA binding protein 1 (CUGBP1). The expression of CUGBP1 in muscle is average compared to other tissues according to GeneCard information obtained with Affymetrix microarrays in human tissues (Rebhan *et al.*, 1998). The protein is linked to myotonic dystrophy, a disorder among other associated with muscle wasting. Skeletal muscle cells from myotonic dystrophy patients fail to induce CUGBP1 while normal differentiated cells accumulate CUGBP1 in the cytoplasm. Furthermore, patients with myotonic dystrophy, display a 70% reduction in expression of CUGP1 compared with healthy individuals (Watanabe *et al.*, 2004). Alterations in the activity of CUGBP1 causes disruption of p21-dependent control of cell cycle arrest (Timchenko *et al.*, 2001). The down-regulation of CUGBP1 by myostatin might therefore cause a CUGBP1-dependent cell proliferation defect.

Another gene of interest is Mm_NIA_H3029H07; myristoylated alanine rich protein kinase C substrate (MARCKS). The expression of MARCKS is low compared to other tissues according to GeneCard information obtained with Affymetrix microarrays in human tissues (Rebhan *et al.*, 1998). MARCKS has been implicated in the regulation of muscle function. In cultured skeletal muscle, MARCKS is a substrate for several PKC isoforms and a component of an integrin-related signalling pathways (Disatnik *et al.*, 2002). Integrins have been hypothesised to be possibly involved in mediating skeletal muscle hypertrophy (Carson & Wei, 2000), through detecting changes in mechanical stress (mechanotransduction). Therefore, a potential hypothesis is that the myostatin-induced down-regulation of MARCKS inhibits integrin signalling which might be linked to myostatin-induced atrophy through yet unknown mechanisms.

Another gene of potential interest is Mm_NIA_H3025C12; interleukin enhancer binding factor 3 (ILF3; NFAR-2). Expression of this gene in skeletal muscle is average compared to other tissues according to GeneCard information obtained with Affymetrix microarrays in human tissues (Rebhan *et al.*, 1998). The protein was identified as a protein that could bind double stranded RNA and as a protein that was phosphorylated by the RNA protein kinase (PKR) (Saunders *et al.*, 2001). In muscle cells, PKR has been shown to be capable of regulating myogenesis (Kronfeld-Kinar *et al.*, 1999). A potential hypothesis is that myostatin downregulates ILF3 which then affects PKR-related myogenic reactions.

The gene Mm_NIA_aaaaaM28723; peroxiredoxin 3 (PRDX3) was also downregulated in response to myostatin. The finding that PRDX3 was down-regulated in response to myostatin treatment is interesting because PRDX3 probes are printed as frequent control, "house keeping" probes on the A and B slide of the microarray. Expression data in the GeneCards database suggest a high expression in skeletal muscle compared to other tissues (Rebhan *et al.*, 1998). PRDX3 is a mitochondrial thioredoxin peroxidase that uses mitochondrial thioredoxin-2 to scavenge hydrogen peroxide (H_2O_2). The basic reaction is:



Low concentrations of H_2O_2 produced by the mitochondria regulate physiological processes, including cell proliferation, whilst high levels of H_2O_2 are toxic to the cell and cause apoptosis. PRDX3 was shown to be required for myc-mediated proliferation, transformation, and apoptosis after glucose withdrawal in non-muscle cells (Wonsey *et al.*, 2002). A down-regulation of PRDX3 by myostatin would thus suggest higher levels of H_2O_2 and make cells more susceptible to apoptosis (Nonn *et al.*, 2003). However, the present data are not in agreement with the results of another study which indicated that there does not exist any myostatin-induced apoptosis in proliferating cells as shown by TUNEL assay and flow cytometric analysis (Thomas *et al.*, 2000). Conversely, in support of the current data, a recent study identified that the bcl-2 related anti-apoptotic protein (Nr-13) gene was upregulated ~10- fold following recombinant myostatin treatment, and these authors also noted the occurrence of a DNA ladder in C2C12 cells treated with the recombinant myostatin for 72 h in differentiation medium (Yang *et al.*, 2005). Therefore, a hypothesis could be that myostatin induces apoptosis through a down-regulation of the anti-apoptotic factor Nr-13 and PRDX3.

Another gene downregulated by myostatin is Mm_NIA_H3004A02; nitrogen fixation cluster-like (NFU). Expression of this gene in skeletal muscle and heart is highest compared to other tissues according to GeneCard information obtained with Affymetrix microarrays in human tissues (Rebhan *et al.*, 1998). NFU is involved in the metabolism of iron-sulfur clusters that are co-factors in proteins with redox, catalytic, and regulatory functions. NFU mRNA splicing results in a mitochondrial and cytoplasmic isoform (Tong *et al.*, 2003). A link to myostatin-dependent growth control can not be established at current, although this is a novel finding.

The next gene downregulated by myostatin was Mm_NIA_H3081A02; eukaryotic translation factor 4A2 (eIF4A2). Expression of this gene in skeletal muscle is highest compared to other tissues according to GeneCard information obtained with Affymetrix microarrays in human tissues (Rebhan *et al.*, 1998). Eukaryotic initiation factor 4A2 unwinds secondary and tertiary structures in the 5'-untranslated region of mRNA, permitting translation initiation (Zakowicz *et al.*, 2005). Therefore downregulation of this gene suggests that myostatin inhibits translation through negative regulation of eIF4A2 transcription.

Another gene was Mm_NIA_H3138F12; syndecan-4, a positive regulator of satellite cell proliferation. Syndecan-4 null muscle, explanted satellite cells are deficient in activation, proliferation, MyoD expression, myotube fusion, and differentiation. Furthermore, syndecan-4 (-/-) satellite cells fail to reconstitute damaged muscle, suggesting a unique requirement for syndecan-4 in satellite cell function (Cornelison *et al.*, 2004). Therefore, downregulation of eIF4A2 suggests impaired proliferation of satellite cells, thus potentially inhibiting growth responses and tissue repair.

3.3.4 Conclusion

Reduced MPS in the presence of myostatin is mediated through diminished phosphorylation of growth signalling proteins. Microarrays showed that myostatin inhibits transcription of muscle regulating genes. However, verification of these data by sensitive molecular techniques (RT-PCR, Northerns) and more extensive studies in to the other genes identified to be downregulated in this study (p53; apoptosis, CDK4; cell cycle; EST's) are both necessary, but outside the scope of this thesis.

Chapter 4

Phenotypic differences in signal transduction protein concentrations, and the effect of chronic stimulation: regulable or 'housekeeping' proteins?

4.1 Introduction

Chapter 3 examined the role of myostatin in controlling muscle mass, and it was suggested that myostatin mediates some of these effects through signalling pathways to alter both transcription and translation. Given the critical importance of signalling proteins in detecting stimuli and mediating many cellular responses, it followed on to dedicate a chapter to signal transduction proteins and their regulation. Signal transduction proteins are central in mediating physiological responses to environmental stimuli. Furthermore, their specific concentrations have important effects upon the magnitude of cellular responses to many stimuli, as laid out by metabolic control theory (Lion *et al.*, 2004). Many groups have previously reported differences in levels of certain proteins and enzymes between different muscles and individual fibre types, and many contractile proteins and metabolic enzymes vary between 'fast' and 'slow' muscle fibres (Pette & Staron, 1990), and this can have much functional significance. Furthermore, muscle enzymes are under regulation by exercise and subject to change following chronic contractile activity.

It was therefore decided to examine the concentrations of these proteins in order to link them with adative capabilities, and furthermore examine the regulation of signalling proteins, in order to better characterise their plasticity. For these studies, numerous proteins that have been implicated in mediating adaptive responses to exercise were assessed. Many signal transduction proteins have been previously shown to be contraction-responsive (Wackerhage & Woods, 2002) and to mediate a fast-to-slow phenotype conversion, mitochondrial biogenesis or skeletal muscle size regulation. The chosen proteins could be grouped in to the following categories:

Fibre type regulation:

Calcineurin, the Ca^{2+}/calmodulin activated protein phosphatase that dephosphorylates and activates the nuclear factor of activated T cells (NFAT), which is a transcription factor. Inhibition of calcineurin signalling with cyclosporine A induces a faster phenotype in slow Soleus muscle (Chin *et al.*, 1998) and also prevents hypertrophy in response to increased muscle loading (Dunn *et al.*, 1999). Extracellular signal regulated kinase 1/2 (ERK1/2) are signalling proteins within a mitogen activated protein kinase (MAPK) pathway that are activated by contraction (Wretman *et al.*, 2000). Activation of the ERK1/2 pathway in regenerating rat muscle has been shown to induce a slower phenotype (Murgia *et al.*, 2000).

Mitochondrial biogenesis:

The AMP-activated kinase (AMPK) (Winder, 2001) which is activated by exercise and p38α,β which is a MAPK pathway protein that is activated by contraction (Wretman *et al.*, 2000). Both of which are implicated in signalling to induce nuclear/mitochondrial DNA (Puigserver *et al.*, 2001). The transcriptional co-factor PGC-1α, which is known to be induced by AMPK (Terada *et al.*, 2002; Zong *et al.*, 2002) is also implicated in mitochondrial biogenesis and slow fibre regulation.

Exercise responsive, yet precise function unknown:

p38γ/ERK6 is expressed at high levels in skeletal muscle (Lechner *et al.*, 1996) and has been linked to exercise activation, increasing 4- fold following a marathon run, and also having a role in glucose uptake (Ho *et al.*, 2004). c-Jun NH2 kinase (SAPK/JNK) has been shown to be activated by mechanical stretch and so could have a role in adaptive responses to mechanical stress (Aronson *et al.*, 1997).

Growth:

PKB is activated by IGF-1 and insulin and one of its main functions is the regulation of translation and muscle size (Brozinick, Jr. & Birnbaum, 1998). The p70 ribosomal protein S6 kinase (p70 S6K) which is the kinase that is a downstream substrate for PKB, (as is 4E-BP1 through mTOR) and mediates the increased translation of mRNAs with a 5' tract of pyrimidine sequences (TOP) (Proud *et al.*, 2001). Glycogen synthase kinase 3β (GSK3β) has been shown to negatively regulate skeletal muscle hypertrophy through phosphorylation of eIF2B (Rommel *et al.*, 2001).

Atrophy:

NFκB signalling has been implicated in muscle atrophy due to cachexia (von Haehling *et al.*, 2002). Myostatin is a potent inhibitor of muscle growth, signalling through SMAD proteins (McPherron *et al.*, 1997). Increases in systemic myostatin cause cachexia (Zimmers *et al.*, 2002) whilst myostatin knockout causes hypertrophy (McPherron *et al.*, 1997). Myostatin mRNA decreases in response to strength training (Roth *et al.*, 2003). NFκB is activated in response to exercise (Hollander *et al.*, 2000) and by pro-inflammatory cytokines (Li & Reid, 2000).

Therefore the first specific aim of this study was to systematically determine the relative signalling protein concentrations between fast rat EDL and slow rat Soleus muscles using Western blotting, in order to ascertain if any differences exist which may specifically affect cellular responses in different muscle types. The second aim of this study was to examine whether signalling enzymes are in the class of regulable proteins, such as muscular enzymes which are extremely responsive to training, and which an adaptive change in concentrations leads to a change in metabolic capacity.

In order to do this, chronic *in vivo* electrical stimulation (CMNS), which leads to an incomplete fast-to-slow phenotype conversion (Salmons & Henriksson, 1981), was applied for 6 wk to rat EDL. This was done in order to see whether a phenotype altering stimuli could modulate signalling protein concentrations, and to see if they were in the category of 'housekeeping' genes or are in fact regulable enzymes. If these signalling proteins were under regulation by contraction, then it would be interesting to examine whether CMNS, producing a fast-to-slow shift in the predominantly fast EDL, could bring the cellular concentrations of a control EDL compared with stimulated EDL to comparable ratios of a control EDL and control Soleus, as measured in the first study.

The specific hypotheses of these studies being:

1. Signalling proteins have different concentrations between muscles and that these differences would be predominantly dependent upon fibre type.

2. CMNS of EDL muscle promotes a fast-to-slow phenotype shift which in turn will lead to changes in signal transduction protein concentrations between control EDL/Stimulated EDL to mirror those those measured in control EDL/Soleus muscles, as in the first study.

Methods

As described in chapter 2.

4.2 Results

4.2.1 EDL and Soleus ATPase staining

Adenosine triphosphatase (ATPase) staining was performed in order to further verify muscle identification and show the higher proportion of type I fibres in EDL (figure 4.1a) whilst the converse in slow Soleus (figure 4.1b). Dark staining indicates type IIa fibres, slight staining exposes type IIb fibres, and the lightest staining type I fibres.

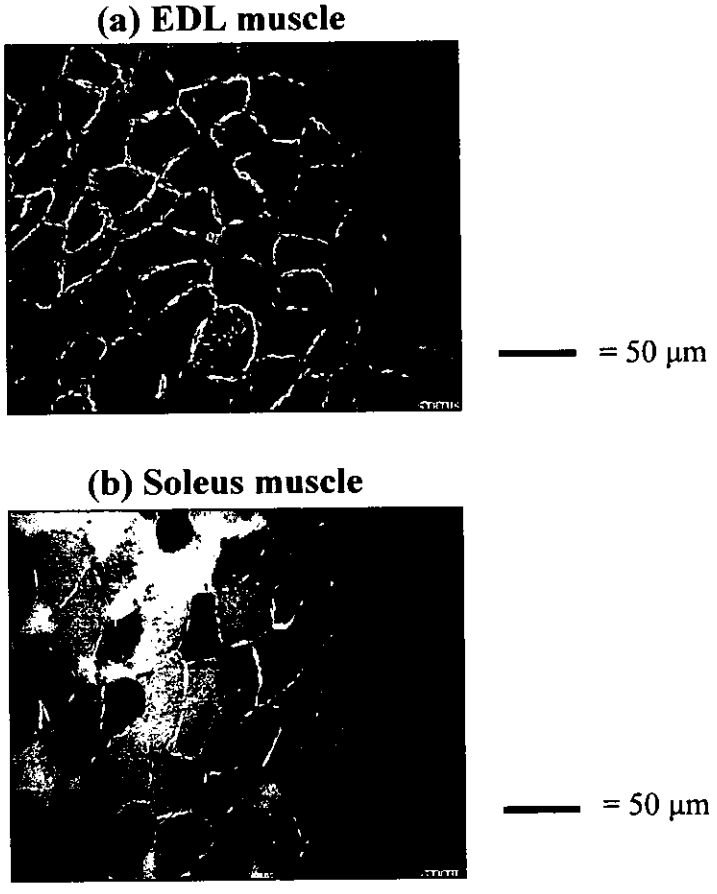


Figure 4.1. Cross-sectional images for ATPase activity using histochemical staining of the typically fast Extensor Digitorum Longus (a), and the predominantly slow Soleus (b). Histochemistry results were typical of 4 such experiments; calibrataion bars show 50 micrometres (μm).

4.2.2 CMNS induces phenotypic change in EDL

During the 6 week period of chronic electrical stimulation (CMNS), visible and palpable oscillatory dorsiflexion was evident in all animals up to the point of harvest of the tissue. Stimulated EDLs had taken on a 'redder' appearance (figure 4.2) indicating increased myoglobin and angiogenesis, and had significantly lost ~50 % in weight, stimulated EDLs weighing 94 ± 9 mg and the control EDLs which weighed 181 ± 12 mg (n= 4, p= 0.001). Whilst no evidence for a change in myosin heavy chain was assessed with more sensitive assays such as MHC immunoblotting, these factors demonstrate the efficacy of CMNS in producing a profound phenotypic change.

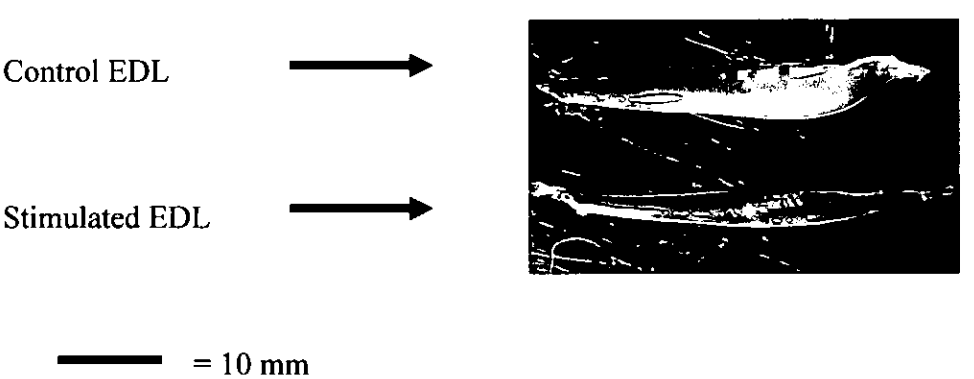
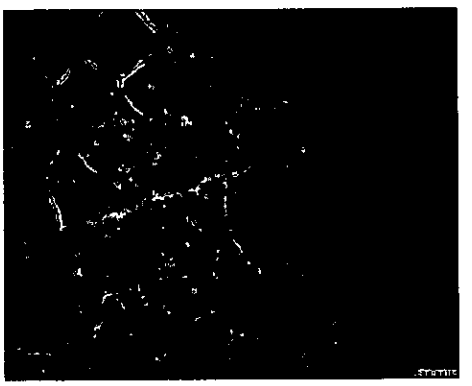


Figure 4.2. Control EDL (upper) and CMNS stimulated contralateral EDL (lower). Note how the stimulated EDL had a deeper red colouring and displayed a reduction in weight when compared to contralateral controls. This information provides evidence demonstrating profound phenotypic following CMNS. These results are typical of 4 such experiments. Calibration bar indicates 10 millimetres (mm); meaurement excludes tendons.

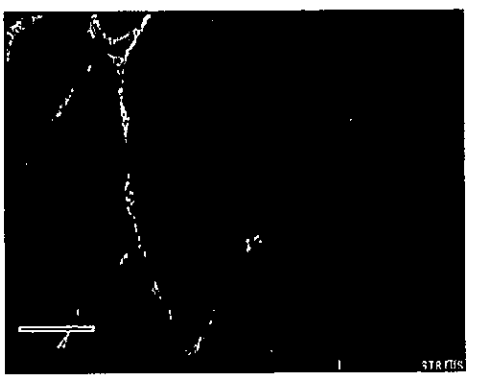
Following CMNS, mitochondrial density, as indicated by staining for NADH tetrazolium reductase activity (NADH-Tr) was assessed. Staining is more pronounced in the stimulated EDL (figure 4.3b) than in control EDL (figure 4.3a), suggesting a greater mitochondrial density and oxidative capacity.

(a) Control EDL



= 50 μm

(b) Stimulated EDL



= 50 μm

Figure 4.3. Magnified images of NADH-Tr mitochondrial stains of unstimulated EDL (a) and stimulated EDL (b). The darker staining is a correlative indicator of denser mitochondria and oxidative enzymes. Histochemistry results were typical of 4 such experiments; calibrataion bars show 50 micrometres (μm).

4.2.3 Concentrations of signalling proteins in rat EDL and Soleus and following 6 weeks of CMNS of EDL

These results show that the concentrations of signal transduction proteins in Soleus and EDL are systematically different between the 4 animals studied (figures 4.4 - 4.14). The mitogen activated protein kinases (MAPK) pathways (JNK, p38, ERK1/2) are present in different concentrations between the phenotypes and do not follow an orderly pattern. Growth and control of translation pathway proteins are all higher in EDL (except GSK3β), as is the negative regulator of skeletal muscle mass, myostatin. However, the cachexia- mediating NFκB is higher in slow Soleus.

Chronic electrical stimulation of EDL muscle, a stimulus promoting a fast-slow transformation, did not consistently bring the concentration levels of signalling proteins measured to parallel that of slow Soleus muscle. Those proteins in EDL/stimulated EDL that significantly changed concentrations to reflect expression levels equivalent to control EDL/Soleus consisted of: p70 S6K, 4E-BP1, PGC-1α, calcineurin, ERK1/2 and NFκB. Conversely, the following proteins when stimulated reflected a concentration shift not of EDL/stimulated EDL being similar to EDL/soleus, but in fact moving away from the expected direction: calcineurin, PKB, GSK3β, AMPK, p54 JNK, myostatin and SMAD2/3. Other proteins such as p38γ/ERK6, p38αβ and p46 JNK were not significantly altered by stimulation. Selective protein turnover as denoted by ubiquitination patterns of proteins was also apparently under altered regulation following the 6 weeks of CMNS. Therefore changes in protein concentrations do not follow a set programme, even in the case of sub-groups such as growth or phenotype controlling proteins.

Proteins implicated in fibre type regulation: The relative calcineurin intensity was 2.6-fold higher in the EDL than in the Soleus, whereas it was decreased to 0.7 of control in response to stimulation. ERK1 and ERK2 were both higher in all Soleus compared to EDL 1.8 and 1.6-fold, respectively. Stimulation increased ERK1 and ERK2 protein concentrations by 1.7 and 3.6-fold respectively, moving them in the direction of slow Soleus (figures 4.4 & 4.5).

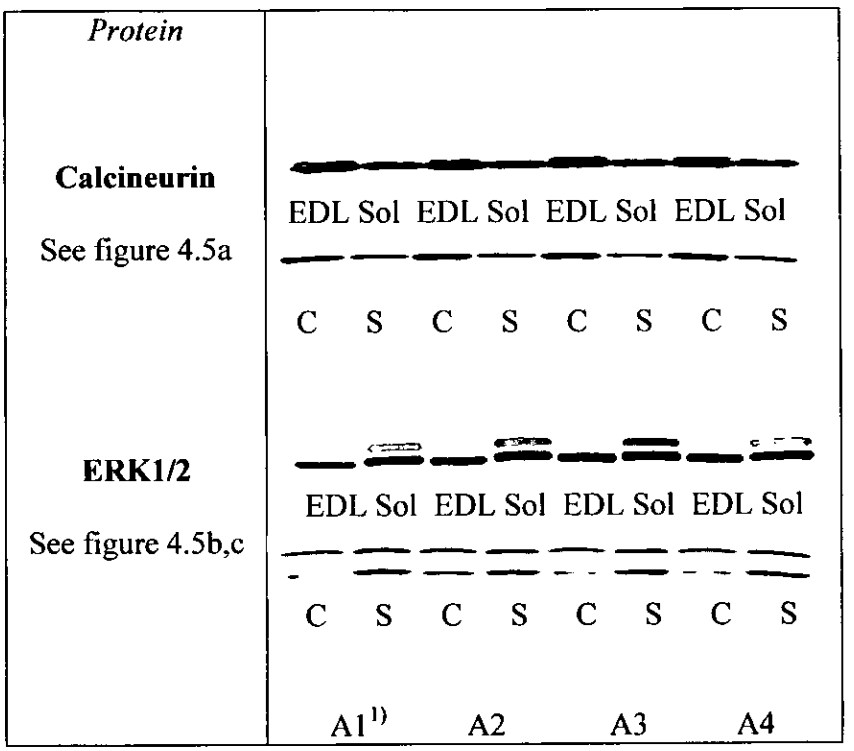


Figure 4.4 Immunoblots showing protein levels of Calcineurin and ERK1/2 in control EDL and Soleus muscles and chronically stimulated rat EDL (S) and control EDL (C) in four animals. Lanes 1-2, 3-4, 5-6, 7-8 correspond to one animal each. [1)] A1-A4 Animals 1-4 (n=4). See figure 4.5 for representative bar charts.

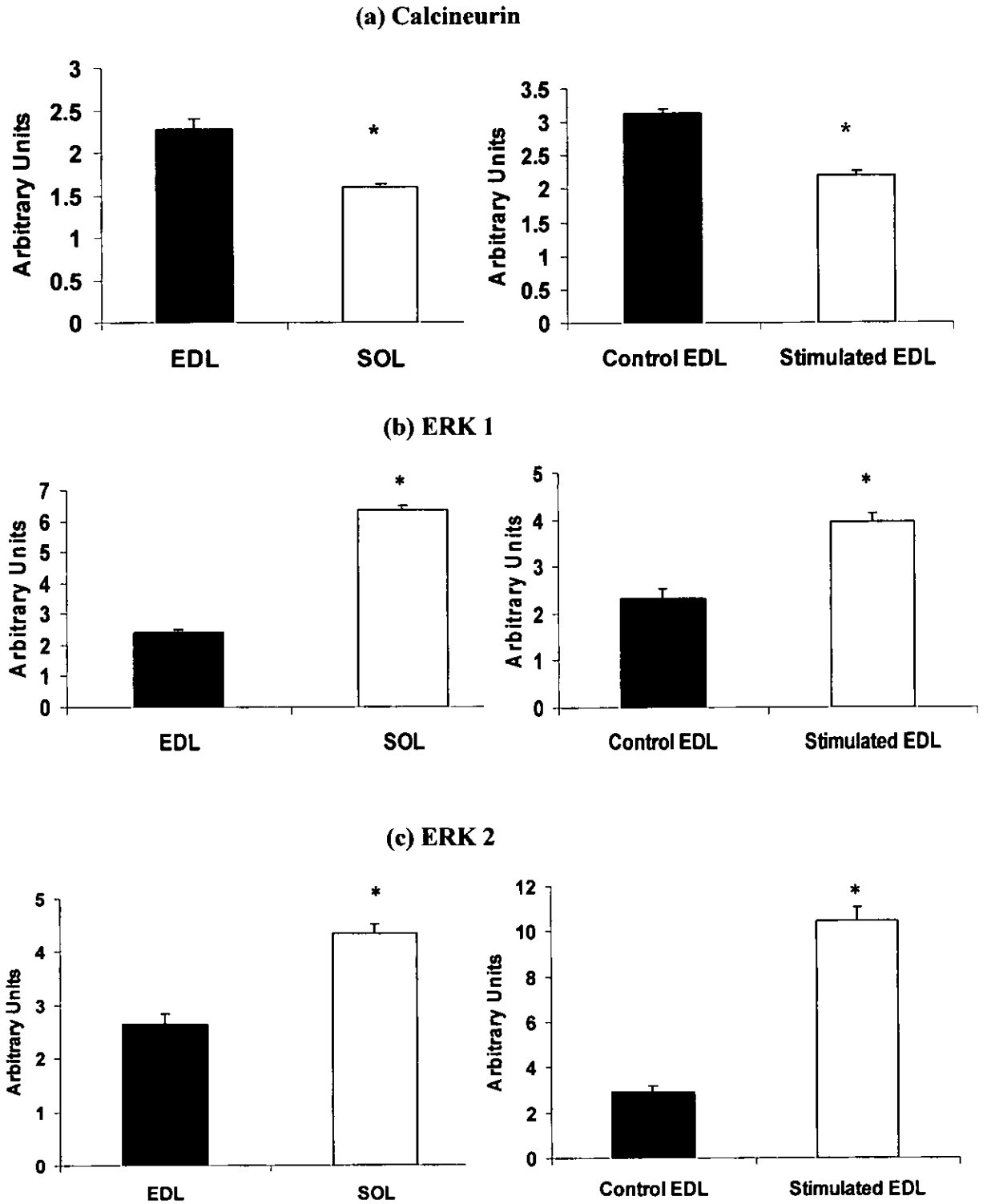


Figure 4.5. Bar charts showing the change in relative concentrations of (a) Calcineurin, (b) ERK1 and (c) ERK2 in rat EDL and Soleus muscles and in response to CMNS. Results are means ±SE arbitrary densitometric units (n=4, * $p<0.05$).

Proteins implicated in mitochondrial biogenesis: The Soleus has a 1.2-fold higher AMPK concentration, whereas there was a decrease in AMPK to 0.7 of control EDL in response to stimulation. Whereas, p38α,β protein levels are 1.4-fold higher in EDL, and when stimulated protein levels reduce non-significantly to 0.9 of control EDL. PGC-1α has a 1.4-fold higher concentration in Soleus, and in stimulated EDL increases 2.3-fold (figures 4.6 & 4.7).

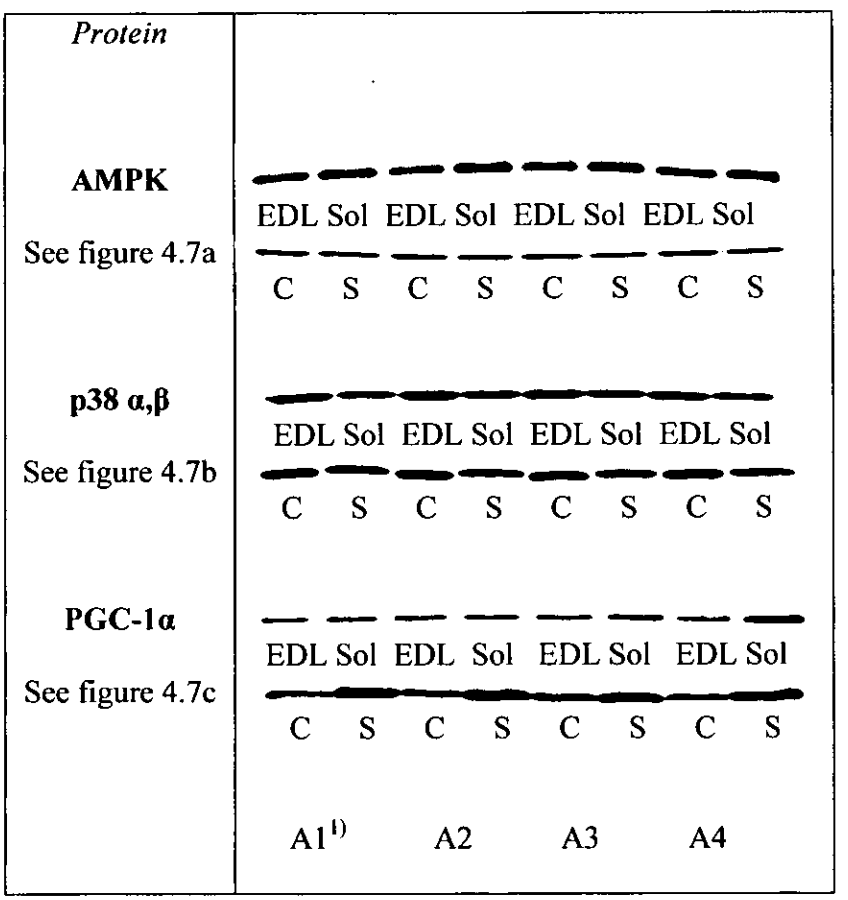


Figure 4.6 Immunoblots showing protein levels of AMPK, p38 α,β and PGC-1α in control EDL and Soleus muscles and chronically stimulated rat EDL (S) and control EDL (C) in four animals. Lanes 1-2, 3-4, 5-6, 7-8 correspond to one animal each. [1)] A1-A4 Animals 1-4 (n=4). See figure 4.7 for representative bar charts.

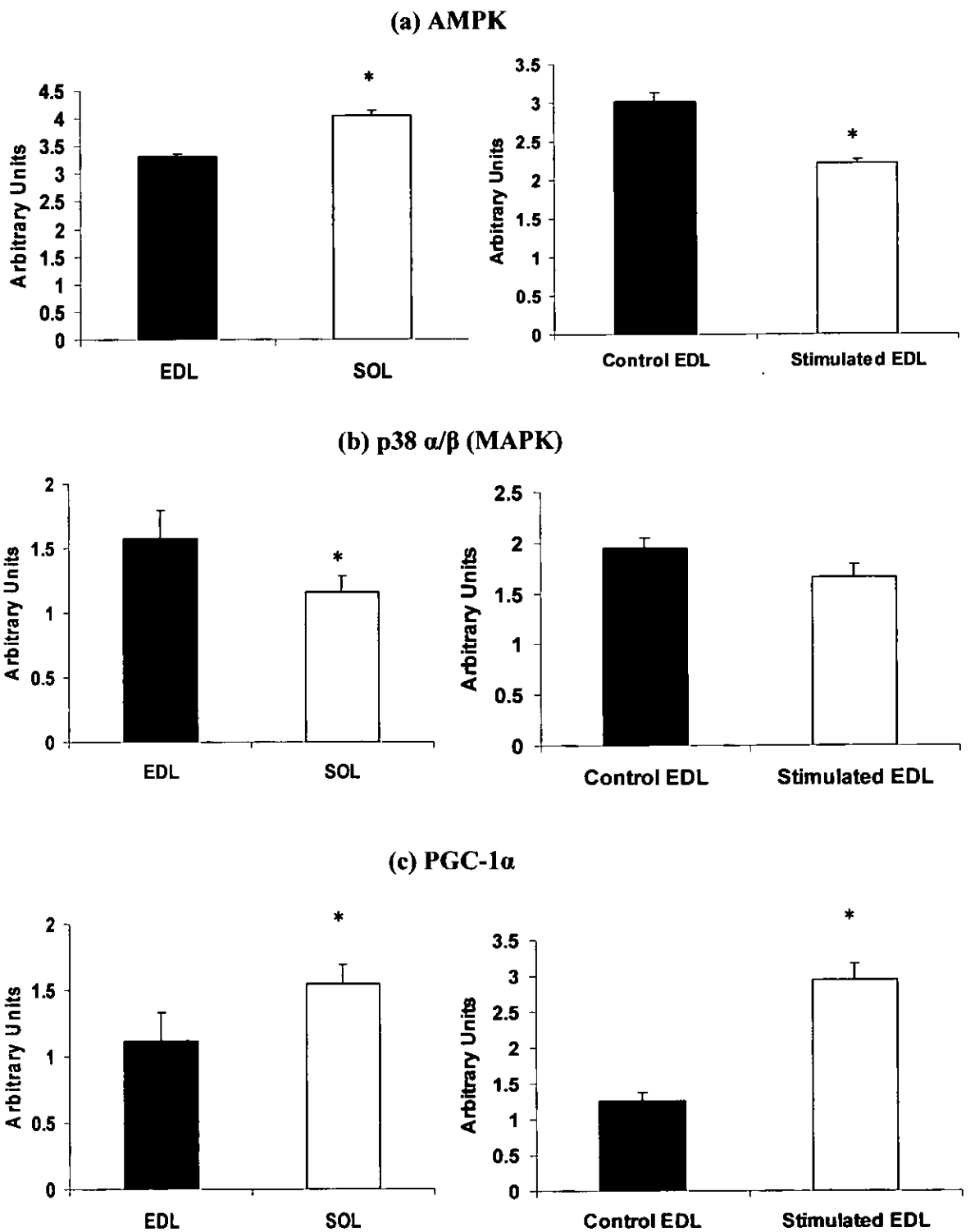


Figure 4.7. Bar charts showing the change in relative concentrations of (a) AMPK, (b) p38 α,β and (c) PGC-1α in rat EDL and Soleus muscles and in response to CMNS. Results are means ±SE arbitrary densitometric units (n=4) * $p<0.05$.

Proteins implicated in adaptation to contractile activity and expressed at high levels in skeletal muscle (GenCard information), yet precise function unknown: There is a lesser concentration of p38γ/ERK6 in EDL and this is further reduced in response to stimulation, although there are no significant changes (figure 4.8 & 4.9). In contrast, p54 JNK is 3-1-fold higher in EDL, but then increases 2.8-fold in response to stimulation; whereas p46 JNK is 5-fold higher in Soleus and not significantly changed following stimulation.

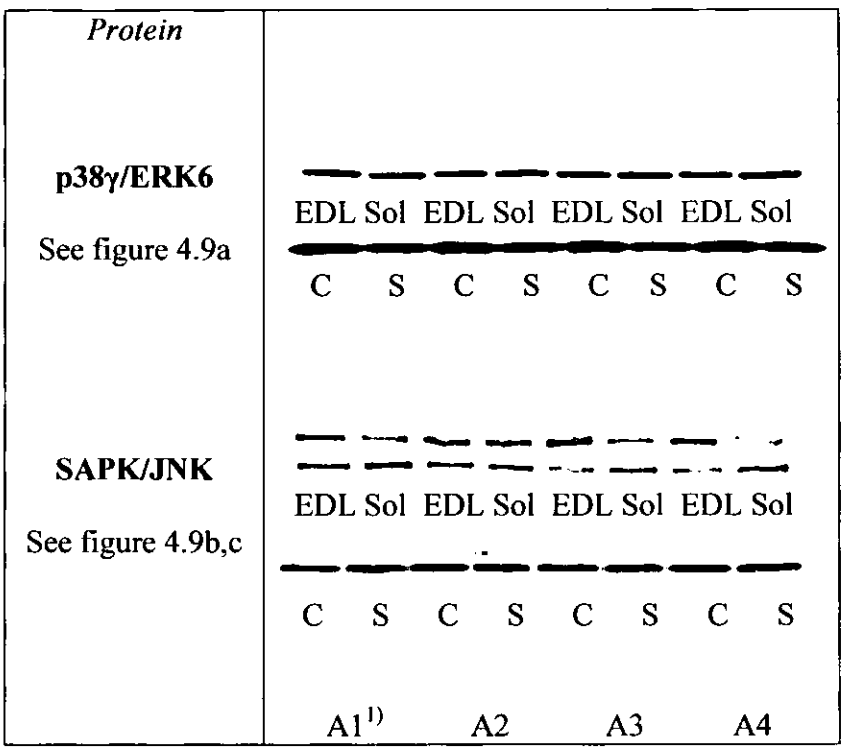


Figure 4.8 Immunoblots showing protein levels of p38γ/ERK6, p54 JNK (upper band) and p46 JNK (lower band) in control EDL and Soleus muscles and chronically stimulated rat EDL (S) and control EDL (C) in four animals. Lanes 1-2, 3-4, 5-6, 7-8 correspond to one animal each. [1)] A1-A4 Animals 1-4 (n=4). See figure 4.9 for representative bar charts.

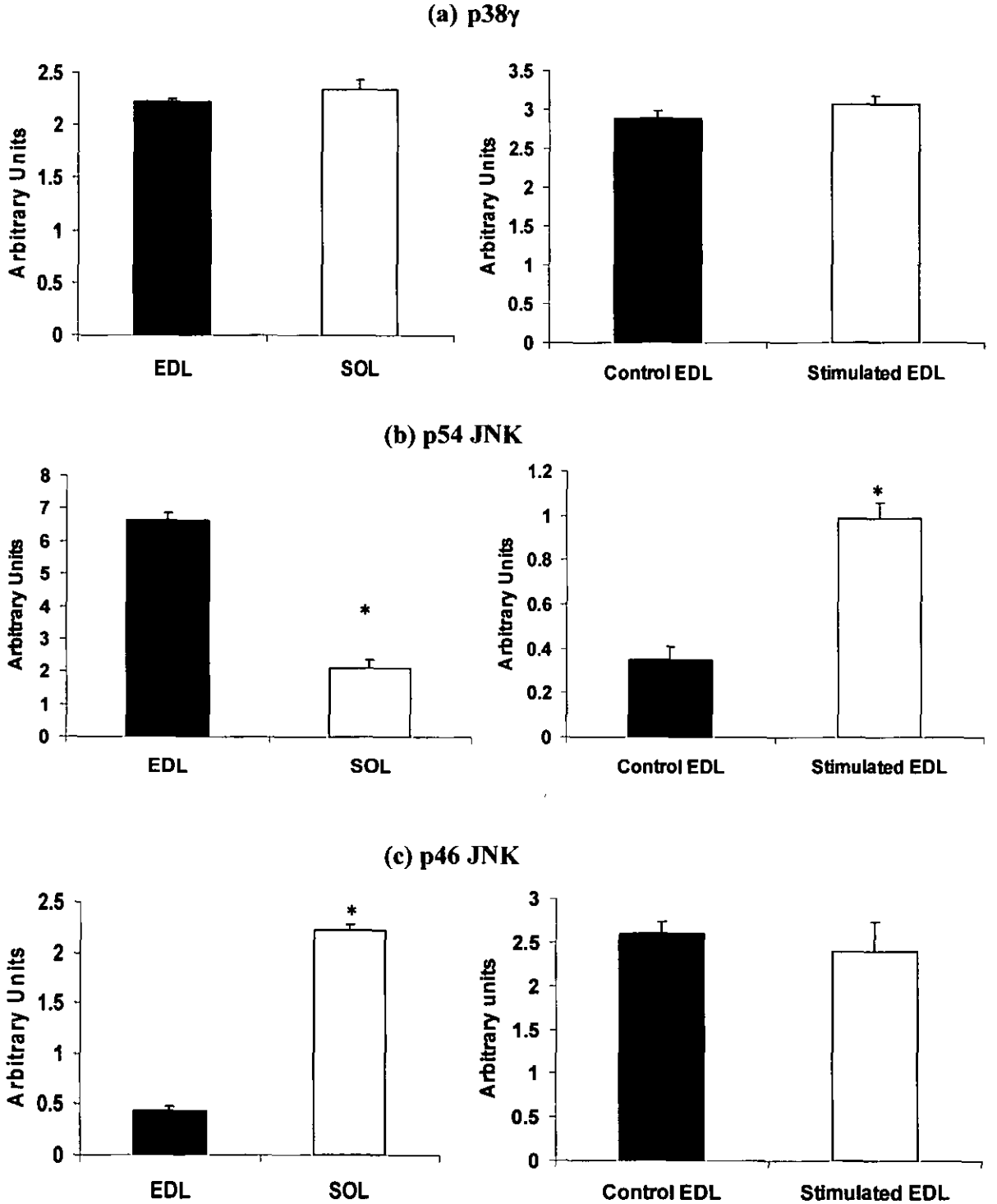


Figure 4.9. Bar charts showing the change in relative concentrations of (a) p38γ, (b) p54 JNK and (c) p46 JNK in rat EDL and Soleus muscles and in response to CMNS. Results are means ±SE arbitrary densitometric units (n=4) * $p<0.05$.

IGF-1 and insulin-dependent translational and carbohydrate metabolism regulation: Growth mediating pathways do not respond uniformly. The EDL has a 1.4-fold higher concentration of PKB than Soleus, and there is a 1.6-fold increase in PKB protein level with stimulation of the EDL. 4E-BP1 and p70 S6K are 5.4 and 6.9-fold higher in EDL, and stimulation leads to a decrease in concentrations to 0.12 and 0.4 of control EDL, respectively. GSK3β, originally 1.4-fold higher in Soleus, is reduced in this direction in response to stimulation, although not significantly (figures 4.10 & 4.11).

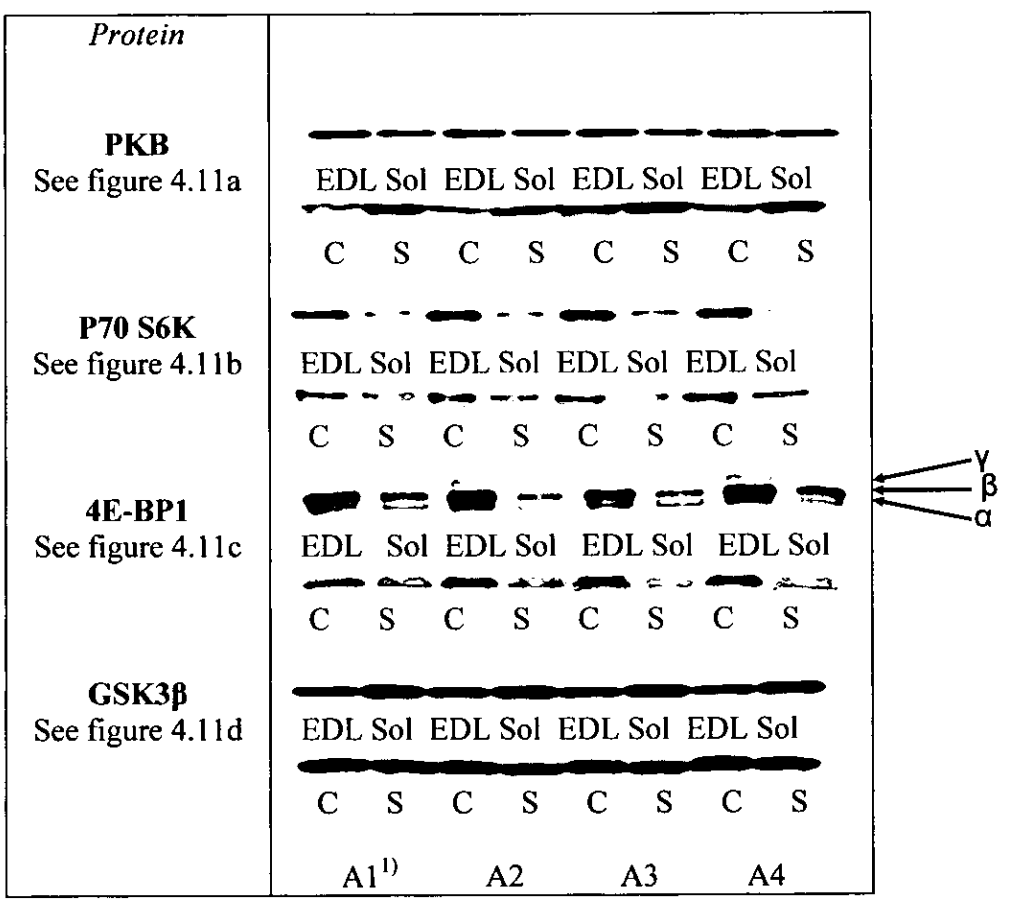
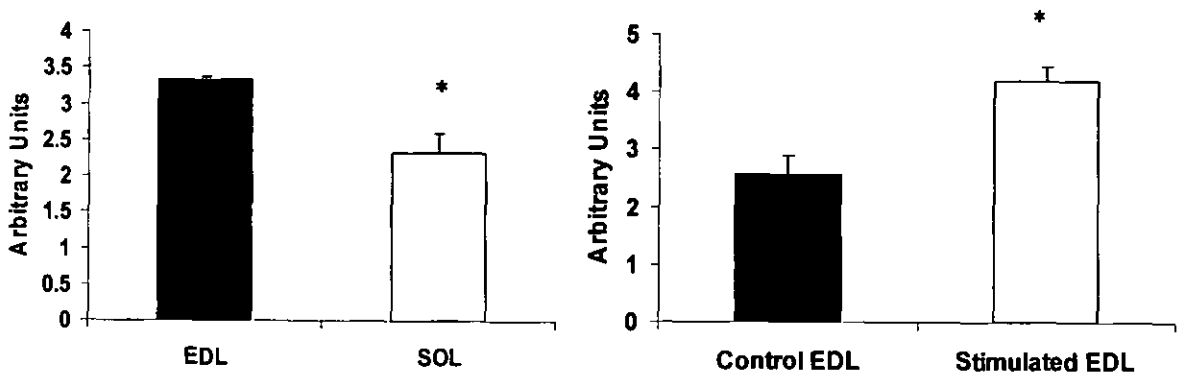
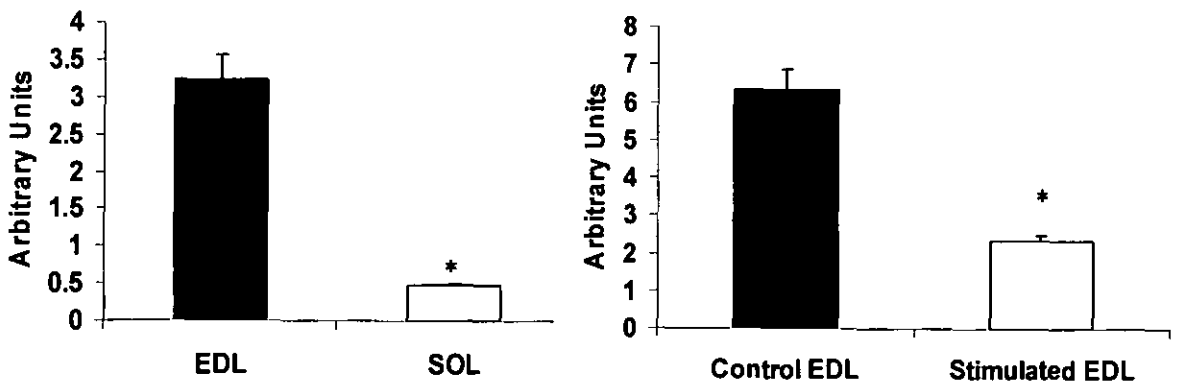


Figure 4.10. Immunoblots showing protein levels of PKB, p70 S6K, 4E-BP1 and GSK3β in control EDL and Soleus muscles and chronically stimulated rat EDL (S) and control EDL (C) in four animals. Lanes 1-2, 3-4, 5-6, 7-8 correspond to one animal each. [1)]A1-A4 Animals 1-4 (n=4). See figure 4.11 for representative bar charts.

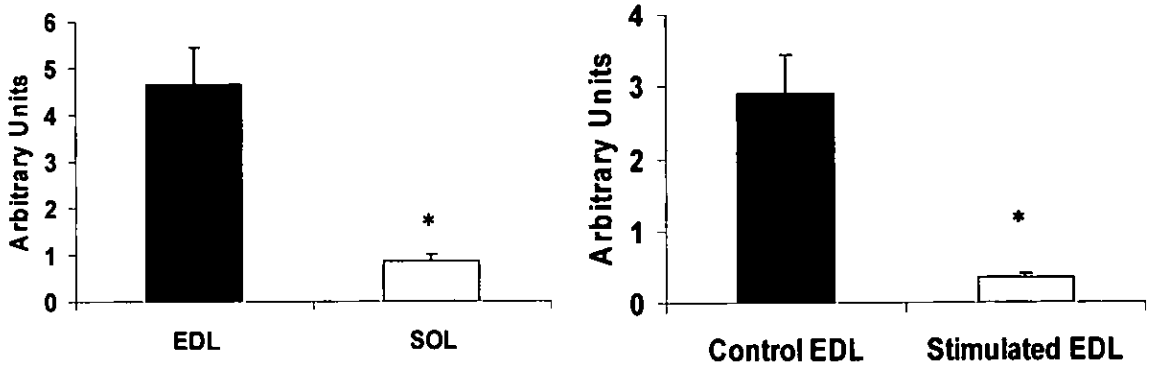
(a) PKB



(b) p70 S6K



(c) 4E-BP1



(d) GSK3β

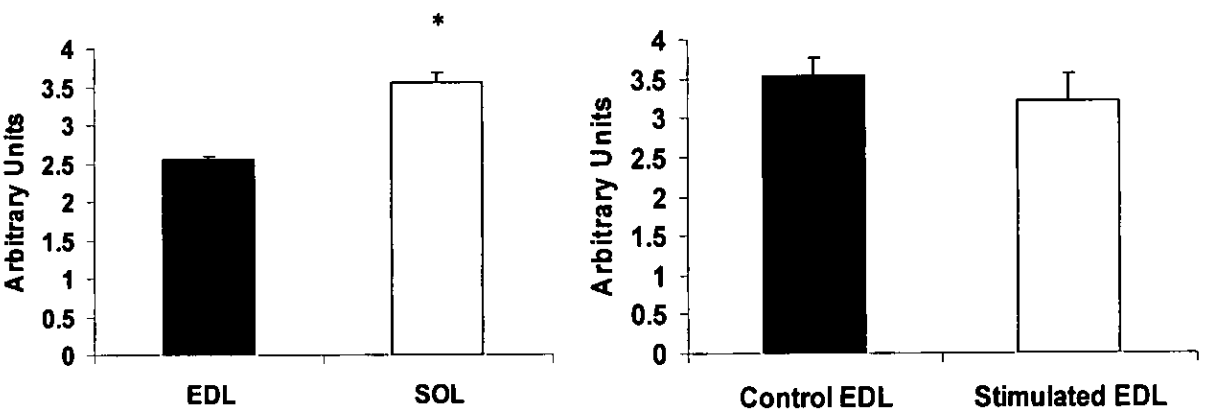


Figure 4.11. Bar charts showing the change in relative concentrations of (a) PKB, (b) p70 S6K, (c) multiple isoforms of 4E-BP1 (α,β,γ) and (d) GSK3β in rat EDL and Soleus muscles and in response to CMNS. Results are means ±SE arbitrary densitometric units (n=4) * $p<0.05$.

Proteins implicated in mediating atrophy: The NFκB concentration is 3-fold higher in Soleus, and stimulation leads to a shift in this direction, increasing 5.1-fold in stimulated EDL. Myostatin is 2-fold higher in EDL and stimulation leads to a 2.9-fold increase in myostatin concentration. SMAD2 is 1.6-fold higher in EDL and stimulation increases SMAD2/3 concentration by 2.7-fold (figures 4.12 & 4.13).

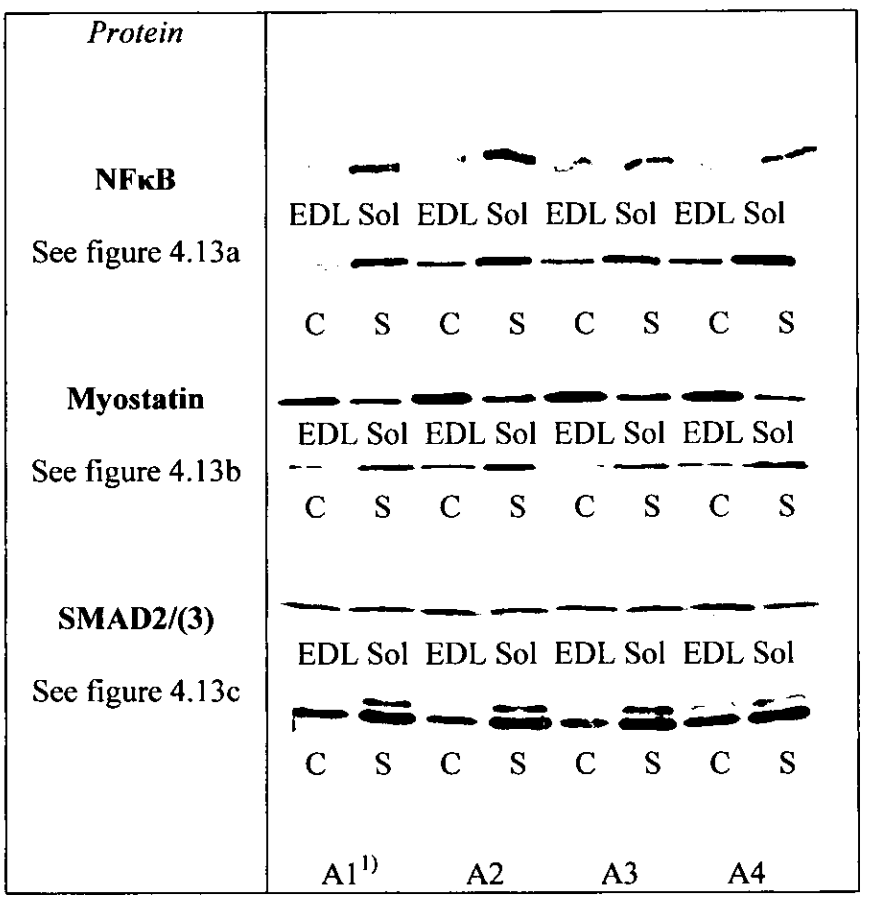


Figure 4.12 Immunoblots showing protein levels of NFκB, Myostatin and SMAD2/3 in control EDL and Soleus muscles and chronically stimulated rat EDL (S) and control EDL (C) in four animals. Lanes 1-2, 3-4, 5-6, 7-8 correspond to one animal each. [1)] A1-A4 Animals 1-4 (n=4). See figure 4.13 for representative bar charts.

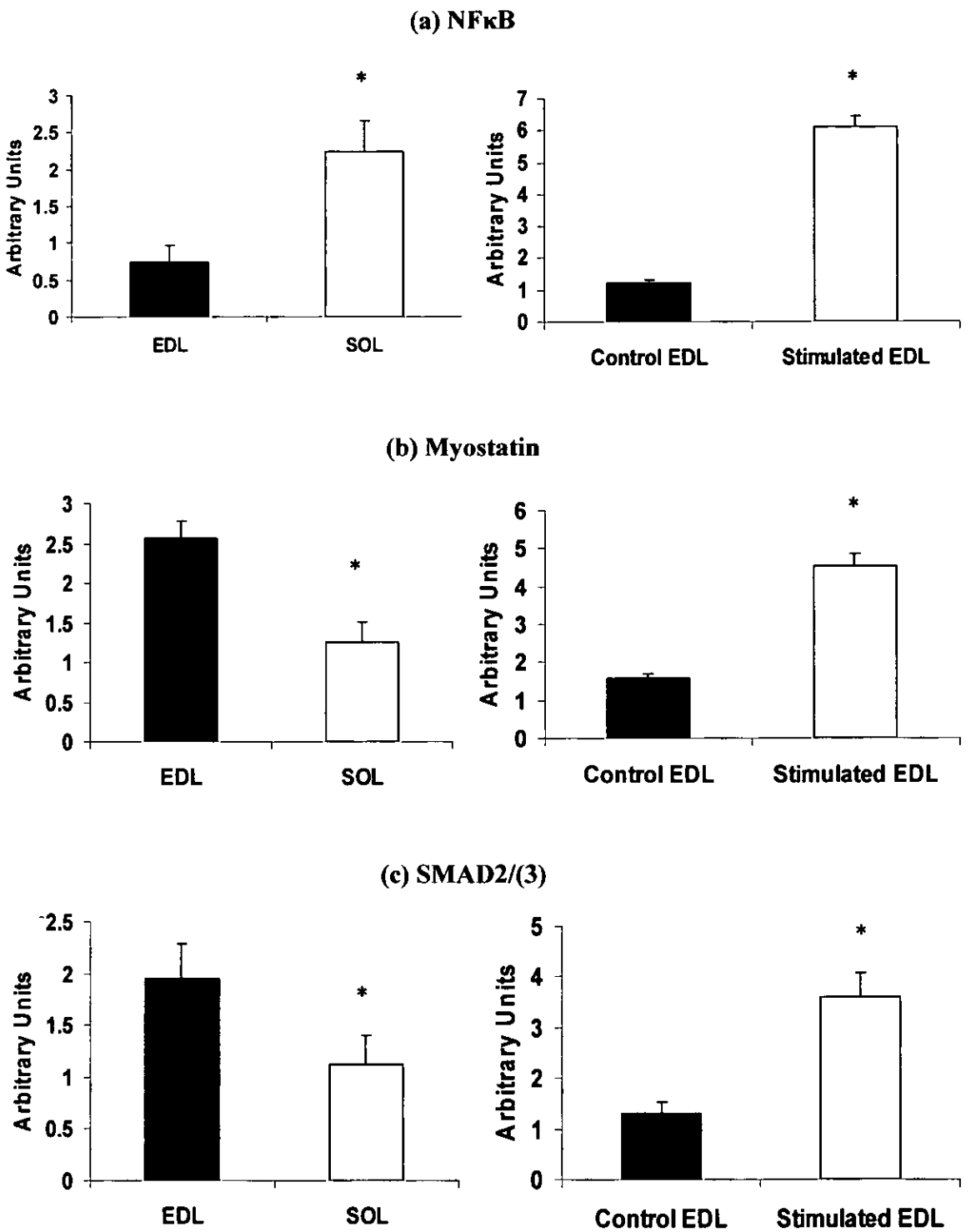


Figure 4.13. Bar charts showing the change in relative concentrations of (a) NFκB, (b) myostatin and (c) SMAD2/3 in rat EDL and Soleus muscles and in response to CMNS. Results are means ±SE arbitrary densitometric units (n=4) * $p<0.05$.

Proteins involved in mediating protein breakdown: Note the different pattern of ubiquitination of proteins following 6 weeks of chronic electrical stimulation. Ubiquitintation indicates the 'tagging' of protein for subsequent degradation by the proteasome. There is specific increased ubiquitination around the 10-35 kDa and 75 kDa areas (figure 4.14). This is not a quantitative assay and would require proteomic analysis to pinpoint the nature of these findings. Furthermore this form of electrophoresis is limited to proteins of < 200 KDa in mass, and therefore cannot divulge the total range of ubiquitination following CMNS of EDL.

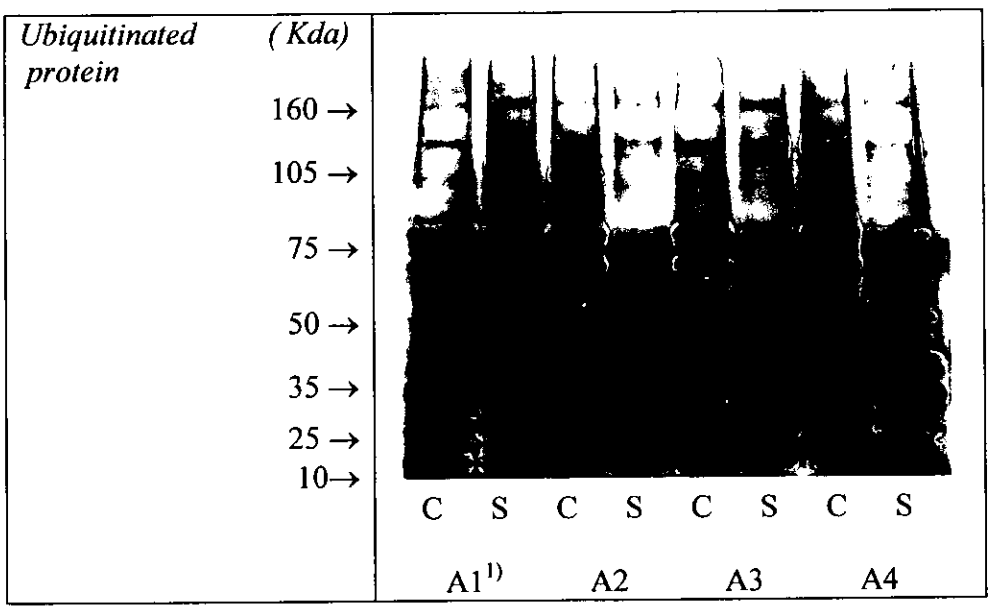


Figure 4.14. Immunoblot of ubiquitinated protein in chronically stimulated rat EDL (S) and control EDL (C) in four animals. Approximate protein sizes in KDa are marked in red around ~75 KDa and ~10-35 KDa and appear to show areas of altered protein turnover following CMNS, as depicted by increased ubiquitination. Lanes 1-2, 3-4, 5-6, 7-8 correspond to one animal each. [1)] A1-A4 Animals 1-4, (n=4).

4.3 Discussion

Following CMNS, the EDL changed in both size and phenotype as is evident from the greater NADH-Tr activity in stimulated EDL, and the ~50 % reduction in weight of the stimulated muscle. This phenotypic change is further evident from the redder appearance of the stimulated EDL, indicating increased myoglobin content (figure 4.2). However, since no biochemical enzyme or MHC characteristics were assessed, it cannot be confirmed as to the level of fast-to-slow transition. Nevertheless, after 6 weeks of CMNS, the fast-to-slow transformation is reportedly well advanced (Jarvis *et al.*, 1996). The current results show differences between the fast EDL and slow Soleus muscles in the relative protein concentrations of most signal transduction proteins measured. This is likely to reflect different signal transduction protein concentrations in type I and type II fibres given the relative predominance of fibre types in both EDL and Soleus. Furthermore, chronic electrical stimulation changed signalling enzyme concentrations. It should be noted here that although total protein change may be significant to the output of a pathway, the specific activity of these kinases have not been measured. Therefore, it remains unknown as to the ultimate significance of these data, although amplification of a set of signals is theoretically limited by the total amount of protein present as laid down in metabolic control theory (Lion *et al.*, 2004). The discussion therefore assumes that the change in signalling protein levels will have an effect upon the output of a pathway although the limitations of this assumption not incorporating specific activities are understood. Nevertheless, it is suggested that the changes in protein concentrations will result in a different sensitivity of the fast-to-slow transformed muscle for signals that occur during exercise but also to insulin and growth signalling.

4.3.1 Fibre type regulating MAPK proteins, p38γ and JNK

Of the two pathways that have been suggested to promote a slow phenotype, calcineurin is expressed at higher levels in fast EDL whereas ERK1 and ERK2 are both expressed at higher levels in the slow Soleus muscle (figures 4.4 & 4.5). These results are supported by a paper reporting higher levels of calcineurin in Vastus Lateralis (intermediate) and plantaris (fast) versus Soleus (slow) rat muscle (Spangenburg *et al.*, 2001), suggesting that a high calcineurin concentration is related to a high percentage of fast fibres. In contrast to this study, higher ERK1 and ERK2 concentrations were reported in rat EDL compared to Soleus (Wretman *et al.*, 2000). The fact that ERK1/2 increases in response to stimulation of EDL suggests a necessity for increased signalling through this pathway, and this makes sense since ERK1/2 is implicated in control of phenotype and transcriptional responses (Higginson *et al.*, 2002). Interestingly, there is a reduction in calcineurin following stimulation which may be a negative feedback effect to the changed programme of gene expression. Systematic differences were not observed in the p38γ/ERK6 concentration between EDL and Soleus muscle, and there was only a non-significant effect of stimulation on relative concentrations. Also, whilst p54 JNK is higher in EDL, p46 JNK is higher in Soleus. In addition, only p54 JNK responded to stimulation and increased in concentration (figures 4.8 & 4.9). The reasons for these discrepancies are not known.

4.3.2 Proteins implicated in mitochondrial biogenesis

Exercise has been shown to activate p38α/β in skeletal muscle (Somwar *et al.*, 2000) and TNFα-regulated p38 has been shown to induce mitochondrial biogenesis

(Puigserver *et al.*, 2001). Therefore, contraction-induced p38 activation may have a greater magnification of response in fast EDL rather than slow Soleus muscle due to the 1.4-fold higher concentration. AMPK was present at a reduced level in EDL, perhaps suggesting that there is a reduced sensitivity to AMPK-induced mitochondrial biogenesis in fast EDL. Chronic electrical stimulation significantly ($p<0.05$) decreased the AMPK level to 0.7 of control EDL and the p38α/β level 0.9 (not significant) of the one measured in the control EDL, respectively. Both AMPK and p38 α/β are activated by contraction and mediate mitochondrial biogenesis probably via induction and phosphorylation of PGC-1α (Zong *et al.*, 2002; Puigserver *et al.*, 2001). The decreases in levels of AMPK and p38 α/β (figures 4.6 & 4.7) are unanticipated because CMNS leads to a large increase in mitochondrial biogenesis and content, as is evident by large increases in mitochondrial enzyme activities (Chi *et al.*, 1986; Henriksson *et al.*, 1986). The decrease in AMPK and p38 can possibly be interpreted as negative feedback effect, meaning that once the cell has adapted to the stimulus, the level of proteins mediating this effect are downregulated. Calcineurin, which decreased 0.7 in response to chronic electrical stimulation, has also been related to PGC-1α-induced mitochondrial biogenesis (Lin *et al.*, 2002). Therefore, this might be another example of negative feedback, or possibly exhibits that calcineurin is not as important in mediating progression to slower fibre typing with chronic contractile activity.

4.3.3 IGF-1 signalling and regulation of growth

The PI-3K-PKB-AKT pathway has recently been shown to mediate IGF-1 induced hypertrophy through activation of translational regulators (Bodine *et al.*, 2001;

Pallafacchina *et al.*, 2002). In this pathway, GSK3β has been shown to be a negative regulator skeletal muscle hypertrophy (Rommel *et al.*, 2001; Vyas *et al.*, 2002). The finding that, PKB/AKT and p70 S6K are 1.4-fold and 6.9-fold higher, respectively, and GSK3β 1.4-fold lower in fast EDL compared to slow Soleus suggests that fibres with a faster phenotype should respond more robustly to IGF-1 (figures 4.10 & 4.11). Therefore, individuals with a higher percentage of fast fibres might therefore respond more to given IGF-1 concentrations than those with a low percentage of fast fibres (Costill *et al.*, 1976). In these experiments proteins belonging to the PKB pathway were expressed at higher levels in EDL than in Soleus with the exception of GSK3β, which is an inhibitor of muscle hypertrophy (Vyas *et al.*, 2002). Thus, a given IGF-1 or insulin concentration would activate translation and protein synthesis more in fast than in slow fibres. It is therefore hypothesised that the expression pattern of the proteins belonging to this pathway might partially explain the larger diameter of type II fibres compared to type I fibres. In addition, the higher expression of PKB and lower expression of GSK3β in rat EDL might partially explain the increased insulin-stimulated glucose uptake compared to rat Soleus (Shoji, 1988). Chronic electrical stimulation led to a decrease in EDL mass and this is consistent with the decrease in p70 S6K and 4E-BP1 to 0.4 and 0.12 respectively, of control EDL that has also been measured in this study. However, PKB increased and GSK3β decreased which is surprising because a change in the other direction would be expected judging from the Soleus and EDL levels of these proteins. The significant ($p<0.05$) 1.6-fold increase in PKB and slight decrease of GSK3β to 0.9 of the control EDL might be related to the role of these proteins in insulin signalling (Cross *et al.*, 1995) and could be a partial explanation for the increased insulin-stimulated glucose uptake that is observed in trained rat skeletal muscle (James *et al.*, 1985).

4.3.4 Atrophy signalling

Myostatin is a negative muscle growth factor (McPherron *et al.*, 1997) and increases in systemic myostatin induce cachexia (Zimmers *et al.*, 2002). Myostatin mRNA has also been shown to be correlated with myosin heavy chain isoform IIb expression in normal muscle (Carlson *et al.*, 1999), and the 2-fold higher myostatin protein levels in EDL compared to Soleus support this hypothesis. Myostatin levels also increase 2.9-fold in response to stimulation (figures 4.12 & 4.13). This is indicative of its role as a negative regulator of muscle mass, since increased myostatin levels lead to muscle wasting (McPherron *et al.*, 1997; Zimmers *et al.*, 2002). Myostatin signals via SMAD2 and SMAD3 (Langley *et al.*, 2002; Bogdanovich *et al.*, 2002). Electrical stimulation caused an increase of 2.7-fold for SMAD2/3, making the muscle more sensitive to myostatin-induced atrophy. Since chronic stimulation leads to a ~50 % loss of muscle weight, perhaps myostatin increases might at least partially explain this phenomenon. Myostatin-signalling could also be responsible for increasing the rate of proteolysis, although this hypothesis awaits testing.

NFκB has been shown to be activated by exercise (Hollander *et al.*, 2001) and is also implicated in mediating TNFα-induced cachexia (Li & Reid, 2000). The 3-fold higher NFκB in slow Soleus than EDL might therefore mean that a slow muscle is more susceptible to NFκB-dependent cachexia. Similarly, NFκB which is activated by contraction (Hollander *et al.*, 2001) and has been shown to induce muscle atrophy due to cachexia (Guttridge *et al.*, 1999; Guttridge *et al.*, 2000), was increased 5.1-fold in stimulated EDL compared to control EDL. The strong induction of these atrophy-signalling proteins in stimulated muscle could be a partial explanation for the drop in

muscle mass from 181 ± 12 mg in the control EDL to 94 ± 9 mg in the stimulated EDL. Similarly, the higher levels of these proteins in Soleus compared to EDL could be an explanation for the smaller size of type I fibres compared to type II fibre subtypes.

Finally, chronic electrical stimulation led to a different ubiquitination of proteins in stimulated EDL (figure 4.14), therefore showing a change in patterns of protein breakdown following CMNS. It is not possible to comment upon the nature of this change without further proteomic analysis, although it provides some evidence that selective protein turnover is present following CMNS.

4.3.5 Conclusion

In conclusion, signal transduction proteins do belong to the class of proteins whose concentrations vary between slow and fast skeletal muscles, and this is likely to reflect differences on a fibre level. In addition, signal transduction proteins that promote particular functions such as muscle growth or slow phenotype formation are not necessarily expressed at higher levels in fibre phenotypes that have that particular trait. When chronic stimulation is applied, the levels of signal transduction proteins in EDL do not always parallel those seen in the Soleus. The fact that most signal transduction pathway proteins were shown to be regulable in muscle implies that contraction-activated pathways set the levels of these signal transduction proteins. Because of this, animals and possibly humans with differences in their fibre type composition are likely to vary in their response to training or insulin because of differences in muscular signal transduction protein concentrations.

Chapter 5

The effect of CMNS upon chronic signalling and the effects of endurance and resistance-like electrical stimulation on acute signalling in skeletal muscle: a molecular explanation for the specificity of physiological responses to training?

5.1 Introduction

Chapter 4 identified the regulation of signal transduction proteins in distinct muscle types and in response to CMNS. This chapter focuses upon the specificity of the activation of these proteins in response to different modes of contractile activity.

5.1.1 Signalling involved in responses to CMNS

As discussed in chapter 1, the mechanisms of adaptation to exercise involve the modulation of cellular signalling in response to signals produced by physical activity. Thus a major question in exercise physiology is: how does muscle adapt to chronic exercise training in the long-term and maintain this state of phenotype? To answer this question, the CMNS model was adopted once more, but this time the primary intent was to characterise the signal transduction pathways that are activated after 6 wk CMNS in rat skeletal muscle, through looking at the phosphorylation status of contraction responsive proteins. After 6 wk of CMNS, the fast-to-slow transformation is well advanced (Jarvis *et al.*, 1996) and thus the signalling is largely 'maintenance'. The aim was thus to characterise the signal transduction pathways that are activated by the chronic 10 Hz electrical stimulation in order to identify those pathways whose ongoing signalling is likely to be responsible for maintaining the changed phenotype, and moreover, can explain the characterised physiological adaptations. The signalling proteins chosen were examined by Western blotting with phospho-specific antibodies. Each protein has been implicated in fast-to-slow phenotype shift or implicated in atrophy (since the muscle loses ~ 50 % mass following 6 weeks of CMNS). They consisted of the AMP-activated kinase (AMPK) (Winder, 2001), calcineurin (Kubis *et*

al., 2002), extracellular regulated kinase 1 and 2 (ERK1/2) (Wretman *et al.*, 2001), p38 MAP kinase (p38α/β) (Lee *et al.*, 2002), c-Jun NH2 kinase (JNK) (Aronson *et al.*, 1997), myostatin-"mothers against decapentaplegic homologue" (SMAD) signalling (Roth *et al.*, 2003) and upstream binding factor (UBF). The role of all these proteins, have been discussed previously, apart from UBF which is a protein controlling transcription of ribosomal RNA (rRNA), and was examined since it had been suggested there is increased RNA content following CMNS. Examination of the phosphorylation of these proteins would give an indication of the chronic signalling which leads to the phenotypic change in response to endurance type exercise, and expand the total protein concentration data in chapter 3 to give a full picture of the cellular responses of signalling proteins to chronic contractile activity with CMNS.

5.1.2 Signalling controlling response to growth or phenotype changes

Once the chronic signalling involved in changing muscle phenotype is established, a second major question in the field of molecular exercise physiology is: what are the acute signals controlling these long-term adaptations, and how do they differ between endurance and resistance training to produce such distinct physiological adaptations?

Endurance training and chronic electrical low-frequency stimulation promote mitochondrial biogenesis, a fast-to-slow muscle fibre phenotype transformation and weight loss (Salmons & Henriksson, 1981; Kubukeli *et al.*, 2002). In contrast strength training promotes hypertrophy due to the stimulation of protein synthesis (Tipton & Wolfe, 2001). These differences between resistance and endurance training adaptations suggest that different forms of contractile activity can induce unique

signalling responses. For example, since skeletal muscle contraction and the set of signals associated with it (i.e. increased tension, [Ca^{2+}], energy turnover, nutrient usage, energy stress, free radicals) are similar, the adaptations are clearly not. Therefore, the aim of this study was to examine whether critical signals activate signal transduction pathways to different extents and with specificity in response to resistance training-like electrical stimulation and endurance training-like stimulation. In order to identify the mechanisms which lead to contrasting physiological adaptations to growth versus fast-to-slow phenotype shift, an *in vitro* isolated muscle system was used. Protocols of either 3 h of continuous 10 Hz-frequency electrical stimulation to mimic endurance training (LFS), or an intermittent 100 Hz protocol (HFS) to mimic resistance training were adopted. Such stimulation patterns were shown to induce the known endurance (fast-to-slow phenotype shift) and resistance training (hypertrophy) like adaptations at least in fast skeletal muscles *in vivo* (Nader & Esser, 2001). This system had the added benefit of being free of any systemic and nutrient effects (especially amino acids), which may have been seen if this was performed *in vivo*. In order to add a further dimension to this study, stimulation protocols were applied to both 'fast' extensor digitorum longus, (EDL) and 'slow' Soleus isolated rat muscles (Ariano *et al.*, 1973) in order to also see whether the response to specific LFS and HFS stimulations depends on the muscle phenotype.

The list of signalling proteins examined by Western blotting in this second study was extended from those examined following CMNS in order to incorporate hypertrophic proteins and to provide a comprehensive set of contrasting signals to both HFS and LFS protocols. The additional proteins examined for their individual phosphorylation status were: protein kinase B (PKB), which is a key mediator of growth responses

thorough the P-I3K pathway; tuberin (TSC2) which is a protein downstream of PKB involved in control of growth through modification of mTOR activity; mammalian target of rapamycin (mTOR) the protein responsive to amino acids, PKB and AMPK signalling; p70 ribosomal S6 kinase (p70 S6K) and 4E-binding protein 1 (4E-BP1) both downstream controllers of translational initiation; glycogen synthase kinase 3β (GSK-3β), which is downstream of PKB; eukaryotic initiation factor 2B (eIF2B) phosphorylated by GSK3β and controls translational initiation; eukaryotic elongation factor 2 (eEF2) which controls peptide elongation, and the concentration of the transcriptional co-factor peroxisome proliferator activated receptor gamma coactivator-1α (PGC-1α) implicated in slow fibre phenotype and mitochondrial biogenesis.

The specific hypotheses of these studies being:

1. CMNS can induce chronic signalling of those pathways controlling phenotypic change, such as AMPK, ERK1/2 and will cause increased activation of atrophy related signals such as SMAD2/3. Specific activation of such proteins can explain known physiological adaptations.

2. HFS and LFS models will lead to the selective activation of signalling pathways which can explain the known physiological responses.

Methods

As described in chapter 2. CMNS stimulation parameters were reported in chapter 4.

5.2 Results

5.2.1 CMNS induces chronic signalling of many proteins

Phosphorylated AMPK (Thr172) was ~4-fold higher in the stimulated EDL compared to the contralateral EDL, suggesting a marked activation of the AMPK pathway (figures 5.1 & 5.2). For all MAPK proteins, phosphorylation changed with CMNS. p38α/β (Thr180/Tyr182) phosphorylation was 4.5-fold higher than control thus suggesting mitochondrial biogenesis signalling. ERK 1 and ERK 2 (Thr202/Tyr204) phosphorylation were increased 6.3 and 2.7-fold, respectively in response to CMNS suggesting the importance of this protein in the control of fibre type.

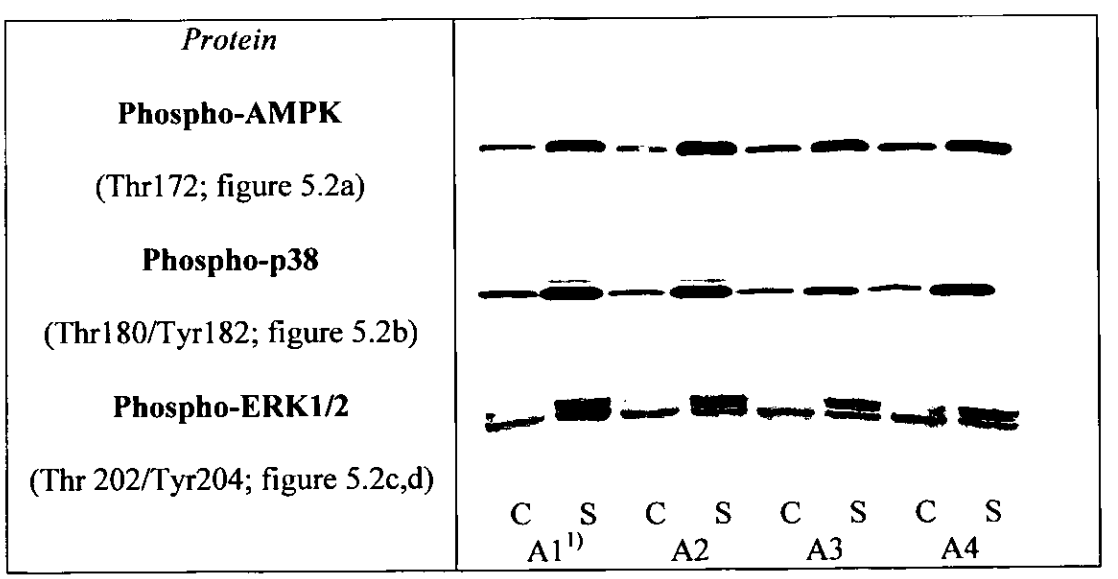


Figure 5.1. Immunoblots of proteins implicated in control of fibre type and mitochondrial biogenesis. Phospho-AMPK Thr172, phospho-p38 Thr180/Tyr182, phospho-ERK1/2 Thr202/Tyr204 in rat EDL. C= Contralateral control EDL, S= After chronic electrical stimulation with 10 Hz for 6 wk. Lanes 1-2, 3-4, 5-6, 7-8 correspond to one animal each. [1)] A1-A4 Animals 1-4 (n=4).

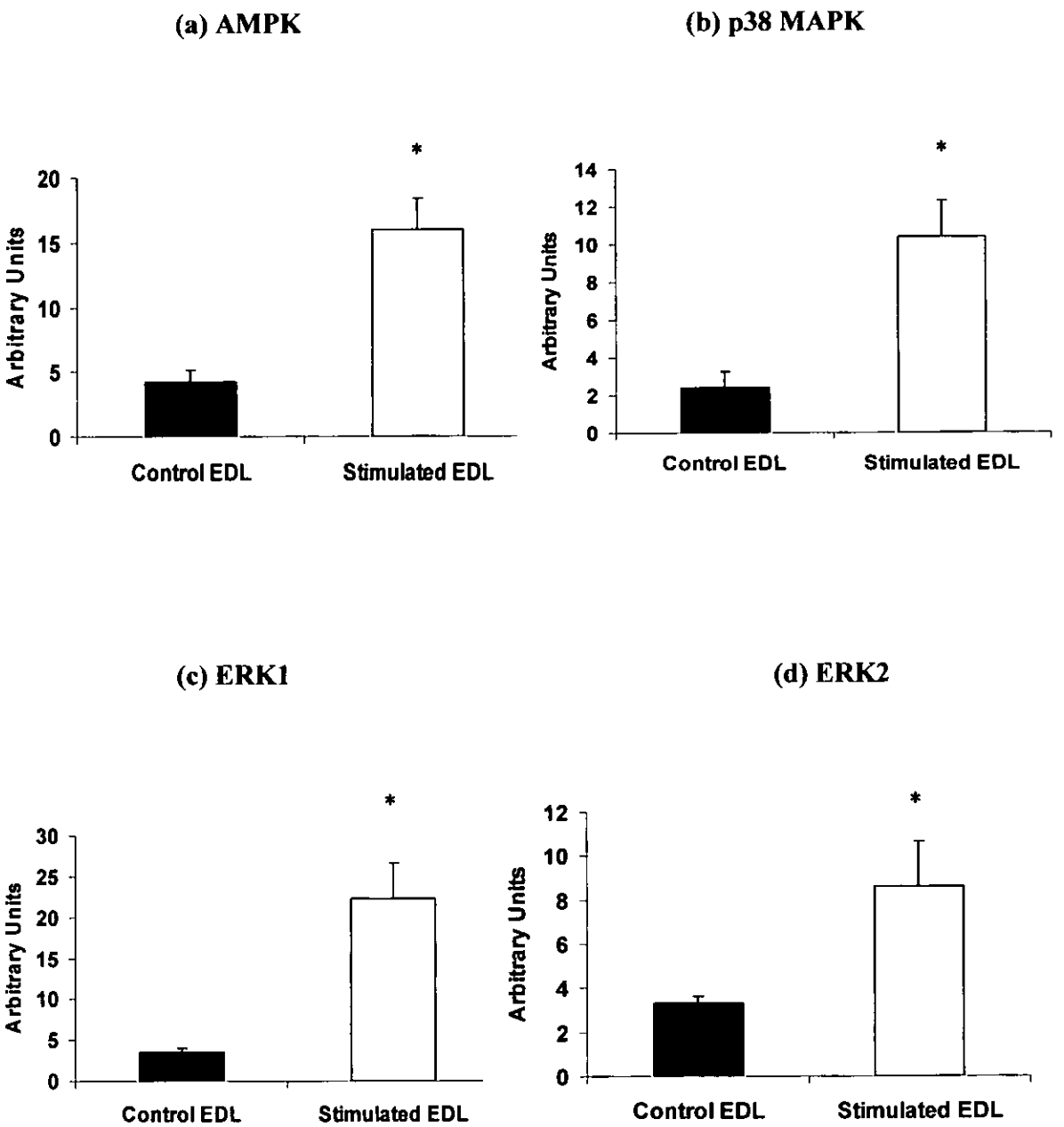


Figure 5.2. Bar charts showing the phosphorylation of fibre type and mitochondrial biogenesis regulating proteins in response to 6 weeks CMNS of rat EDL. (a) AMPK Thr172, (b) p38 MAPK Thr180/Tyr182, (c) ERK1 Thr202/Tyr204 and (d) ERK2 Thr202/Tyr204 in control EDL and chronically stimulated rat EDL. Results are means ±SE arbitrary densitometric units (n=4) * $p<0.05$.

The phosphorylation of p54 JNK (Thr183/Tyr185) was ~2-fold higher following CMNS, suggesting that this protein has a role in adaptation. The phosphorylation of the signalling intermediaries of myostatin, SMAD2/3 (Ser433/435) increased by ~2.5-fold in response to stimulation compared to control. In attempt to find an explanation for the increased ribosome biogenesis that takes place during CMNS (Cummins & Salmons, 1999) the phosphorylation of UBF, which is known to regulate the transcription of ribosomal RNA, was also measured. UBF (Ser637) phosphorylation was reduced to 0.7 of control EDL in the stimulated EDL.

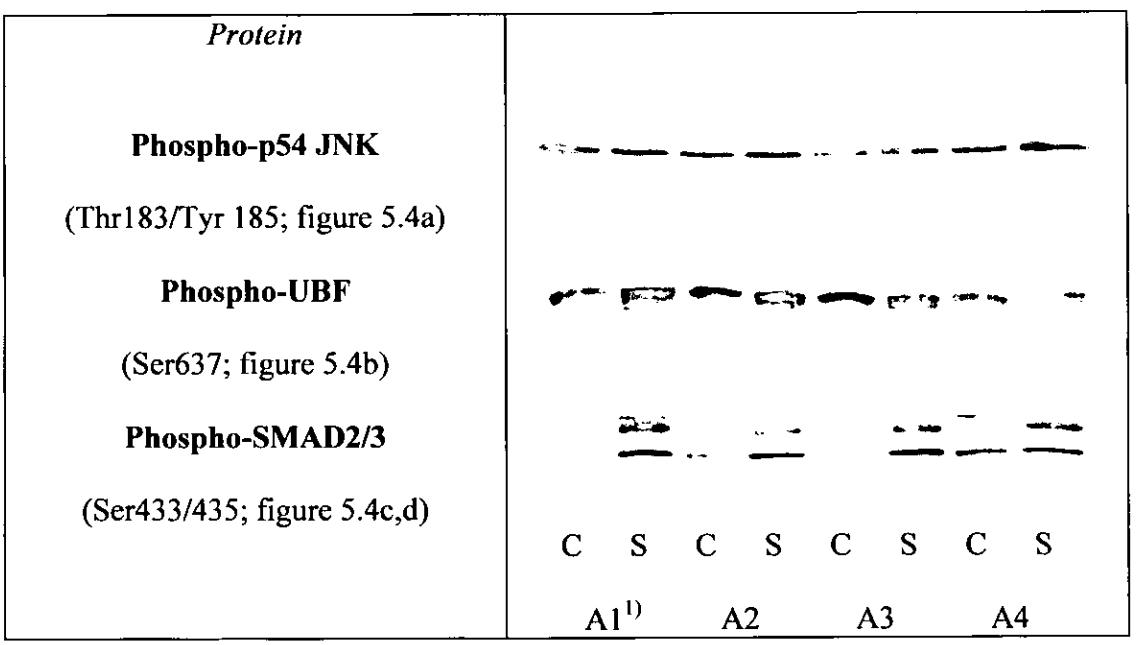


Figure 5.3. Immunoblots of atrophy, growth and mechanotransduction-linked proteins in response to 6 weeks of CMNS in EDL. Phospho-JNK p54 Thr183/Tyr185, phospho-SMAD2/3 Ser433/435 and phospho-UBF Ser637 in rat EDL. C= Contralateral control EDL, S= After chronic electrical stimulation with 10 Hz for 6 weeks. Lanes 1-2, 3-4, 5-6, 7-8 correspond to one animal each. [1)] A1-A4 Animals 1-4 (n=4).

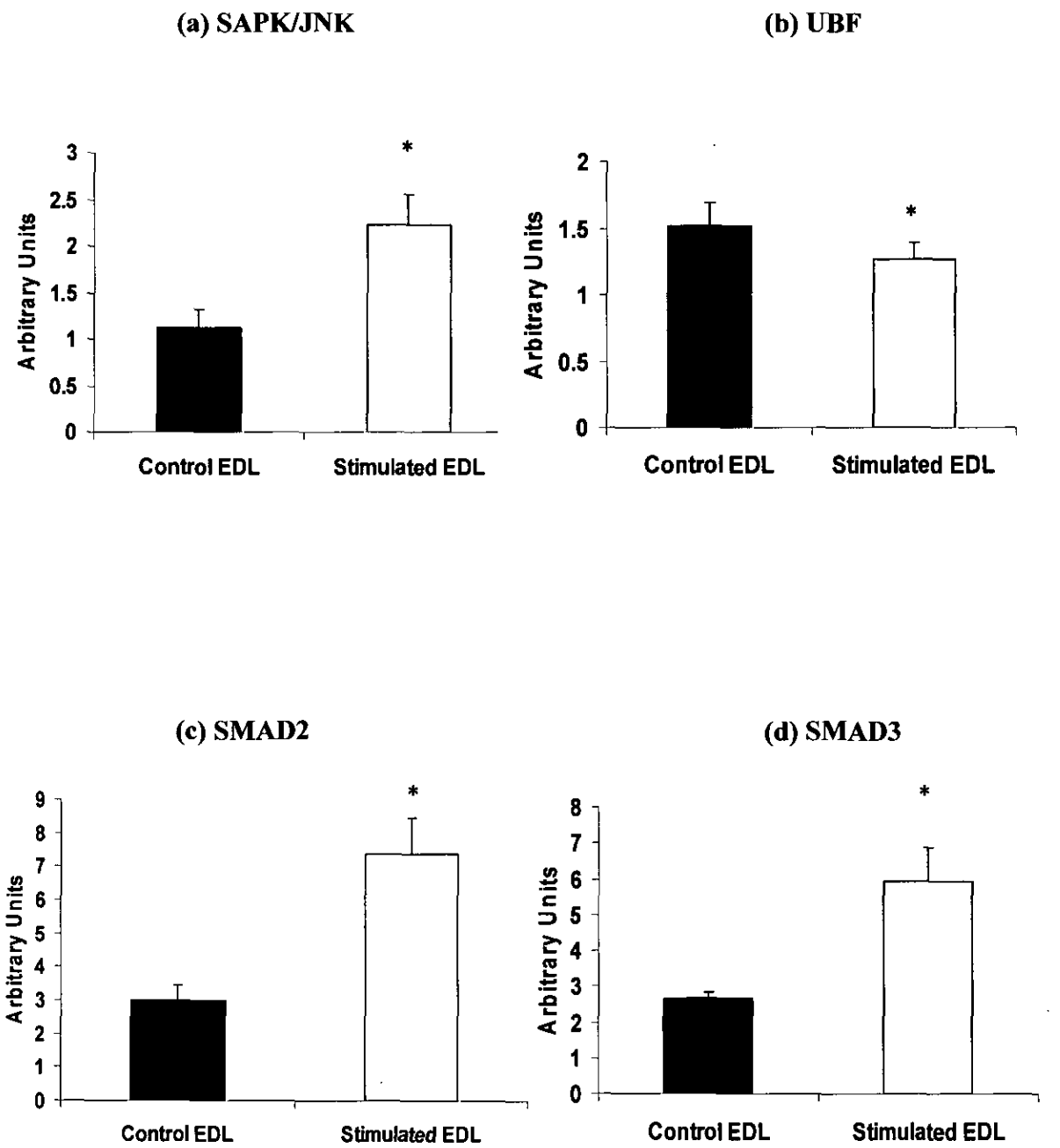


Figure 5.4. Bar charts showing phosphorylation of atrophy, growth and mechanotransduction-linked proteins in response to 6 weeks of CMNS in EDL. (a) p54 JNK Thr183/Tyr185, (b) UBF Ser637, (c) SMAD2 Ser433/435 and (d) SMAD3 Ser 433/435 and in control EDL and chronically stimulated rat EDL. Results are means ±SE arbitrary densitometric units (n=4) * $p<0.05$.

5.2.2 In vitro HFS and LFS stimulation induces selective signalling

5.2.2.1 Tension developed during *in vitro* LFS and HFS stimulation

The stimulation protocols differed significantly ($p<0.01$) in tension generated in response to the two types of stimulation. EDL and Soleus generated a tension of 5.3 ± 0.4 mN and 4.6 ± 0.4 mN, respectively, during the first 3 s-burst of the HFS protocol which significantly decreased to 3.7 ± 0.3 mN and 4.0 ± 0.3 mN, respectively, during the last (60^{th}) burst. During LFS, force stabilised in the EDL and Soleus at 2.3 ± 0.2 mN and 2.2 ± 0.2 mN after 30 s of stimulation and the force decreased significantly to 1.6 ± 0.2 mN and 2.0 ± 0.2 after 3 h of stimulation (figure 5.5). The tension generated during the LFS protocol was significantly lower than in the HFS (all $n=8$, $p<0.05$).

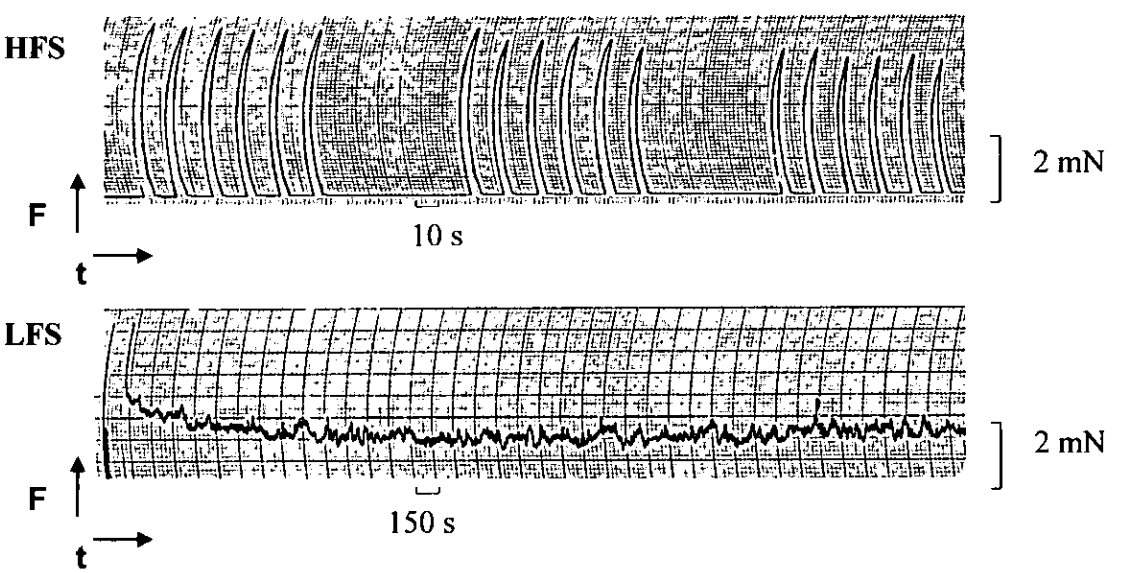


Figure 5.5. Chart recordings of stimulation traces in EDL. HFS showing 3 sets of the 6 repetitions (top) and LFS showing 90 min of the 3 h protocol (lower). The tension generated was >2-fold higher in the HFS protocol. Calibration bars show (F) force in mN; (t) time is correct for 10 mm of graph paper ($n=8$, $p<0.05$).

5.2.2.2 Validation of LFS and HFS models by measuring protein synthesis and UCP3 expression

Myofibrillar and sarcoplasmic protein synthesis was measured and UCP3 mRNA in control and 3 h after LFS and HFS, respectively, to see whether known adaptive responses to endurance and resistance training could be induced (n=6 EDL per group). Under these conditions, myofibrillar and sarcoplasmic protein synthesis were 5.32 and 2.65 times higher 3 h after HFS compared to control, respectively ($p<0.05$). In contrast in LFS, protein synthesis was only stimulated to a small degree; this, however, did not reach significance (figure 5.6a). UCP3 mRNA increased significantly ($p<0.001$) to (figure 5.6b) 11.70 ± 0.96 of control 3 h after LFS but remained at 1.25 ± 0.12 of control 3 h after HFS.

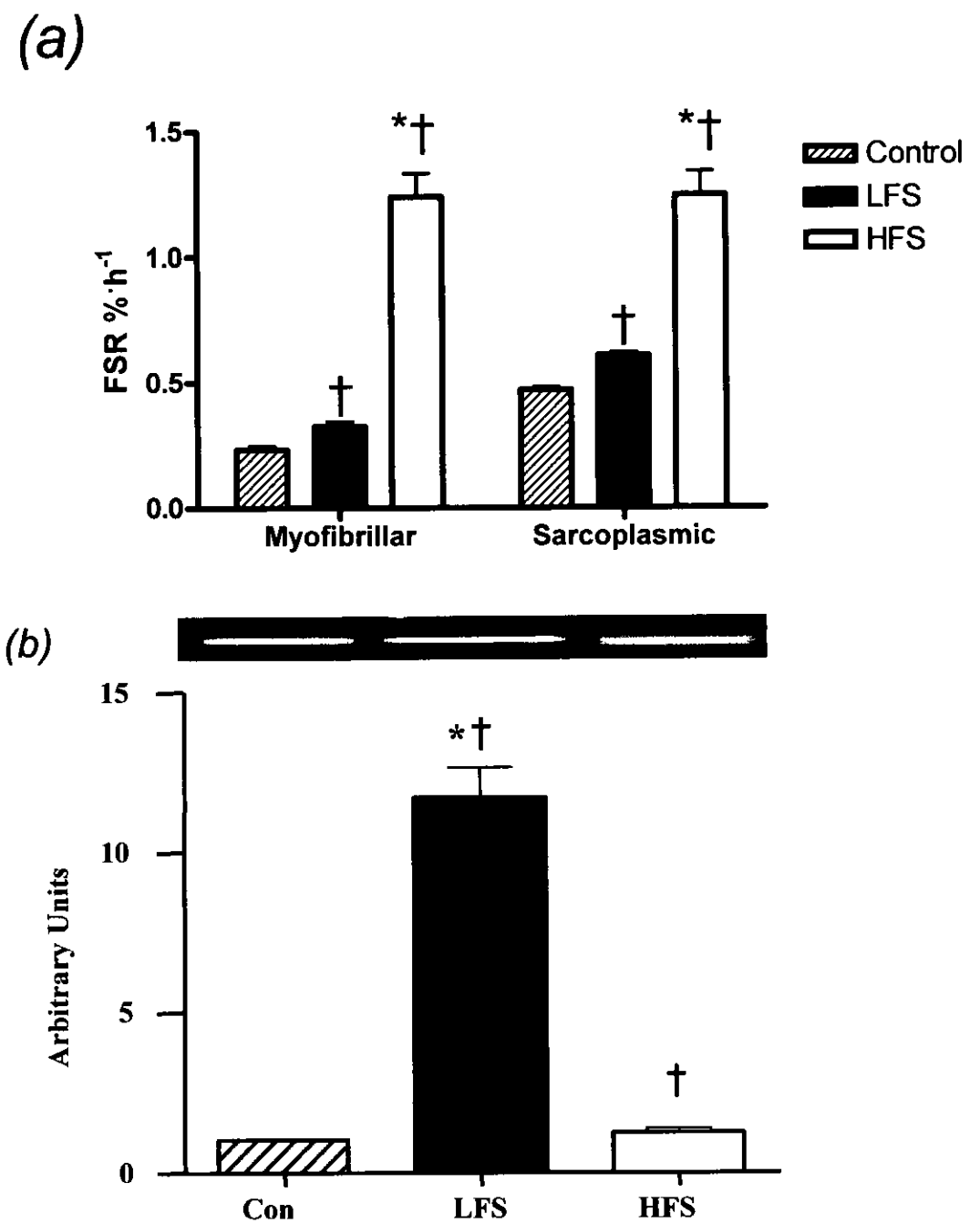


Figure 5.6. (a) Myofibrillar and sarcoplasmic protein synthesis in rat EDL muscles incubated without stimulation and 3 h after LFS or HFS, respectively (n=6 EDL per bar; mean ± SEM). (b) UCP3 mRNA relative to control by RT-PCR (n=6 EDL per bar; mean ± SEM). All values were normalised to the relative intensity of the control bands. *Significantly different from control; †Significant difference between LFS and HFS stimulation protocols (ANOVA, Tukey's post hoc, $P<0.05$).

Western blots were carried out for both total and phosphorylated froms of all proteins mentioned in the introduction. Examples of such immunobots are shown in table 5.1. The phosphorylation status of many proteins was changed, and many of these changes were highly specific to LFS or HFS (figures 5.7-5.11).

Table 5.1. Examples of Western blot results. All results are from Western blots on EDL protein extracts; adjacent bands are copied from one blot (n=8).

Target (phosphorylation site)	*Stimulation*	*Control*	*Post stimulation*	*3 h post stimulation*
AMPK (Thr172)	LFS (10 Hz)			
	HFS (100 Hz)			
Total PGC-1α	LFS (10 Hz)			
	HFS (100 Hz)			
PKB (Ser473)	LFS (10 Hz)			
	HFS (100 Hz)			
TSC2 (Thr1462)	LFS (10 Hz)			
	HFS (100 Hz)			
Total TSC2	LFS (10 Hz)			
	HFS (100 Hz)			
mTOR (Ser2448)	LFS (10 Hz)			
	HFS (100 Hz)			
P70 S6K (Thr389)	LFS (10 Hz)			
	HFS (100 Hz)			

Table 5.1 continued.....

Target (phosphorylation site)	*Stimulation*	*Control*	*Post stimulation*	*3 h post stimulation*
4E-BP1 (Thr37/46)[1)]	LFS (10 Hz)			
	HFS (100 Hz)			
GSK-3β (Ser9)	LFS (10 Hz)			
	HFS (100 Hz)			
eIF2B (Ser535)	LFS (10 Hz)			
	HFS (100 Hz)			
eEF2 (Thr56)	LFS (10 Hz)			
	HFS (100 Hz)			
ERK1/2 (Thr180/Tyr182)	LFS (10 Hz)			
	HFS (100 Hz)			
p38 (Thr180/Tyr182)	LFS (10 Hz)			
	HFS (100 Hz)			
JNK (Thr180/Tyr182)[2)]	LFS (10 Hz)			
	HFS (100 Hz)			

[1)] The density of all bands corresponding to the various 4E-BP1 isoforms were quantified together due to their close proximity making them indistinguishable by densitometry. Isoforms detected were: upper band α, middle band β, lower band γ. [2)] Only JNK2 (p54) was quantified since this had been specifically linked to mechanotransduction (Martineau & Gardiner, 2001). For each protein, 4 Soleus and 4 EDL were examined, and thus n=8.

5.2.2.3 AMPK-PGC-1α signalling

The phosphorylation of AMPK at Thr172 increased significantly in response to LFS but not HFS (figure 5.7a). LFS significantly increased AMPK phosphorylation 2.02 ± 0.11-fold directly after stimulation and 1.90 ± 0.06-fold 3 h later. In contrast to LFS, AMPK phosphorylation was significantly decreased to 0.69 ± 0.08 of control three h after HFS. The AMPK-activation pattern was matched directly after stimulation by a concentration change of PGC-1α: PGC-1α increased significantly by 1.30 ± 0.04-fold directly after LFS and fell to 0.82 ± 0.03 of control directly after HFS (figure 5.7b) (all $n=8$, $p<0.05$). No significant effects were observed 3 h post stimulation.

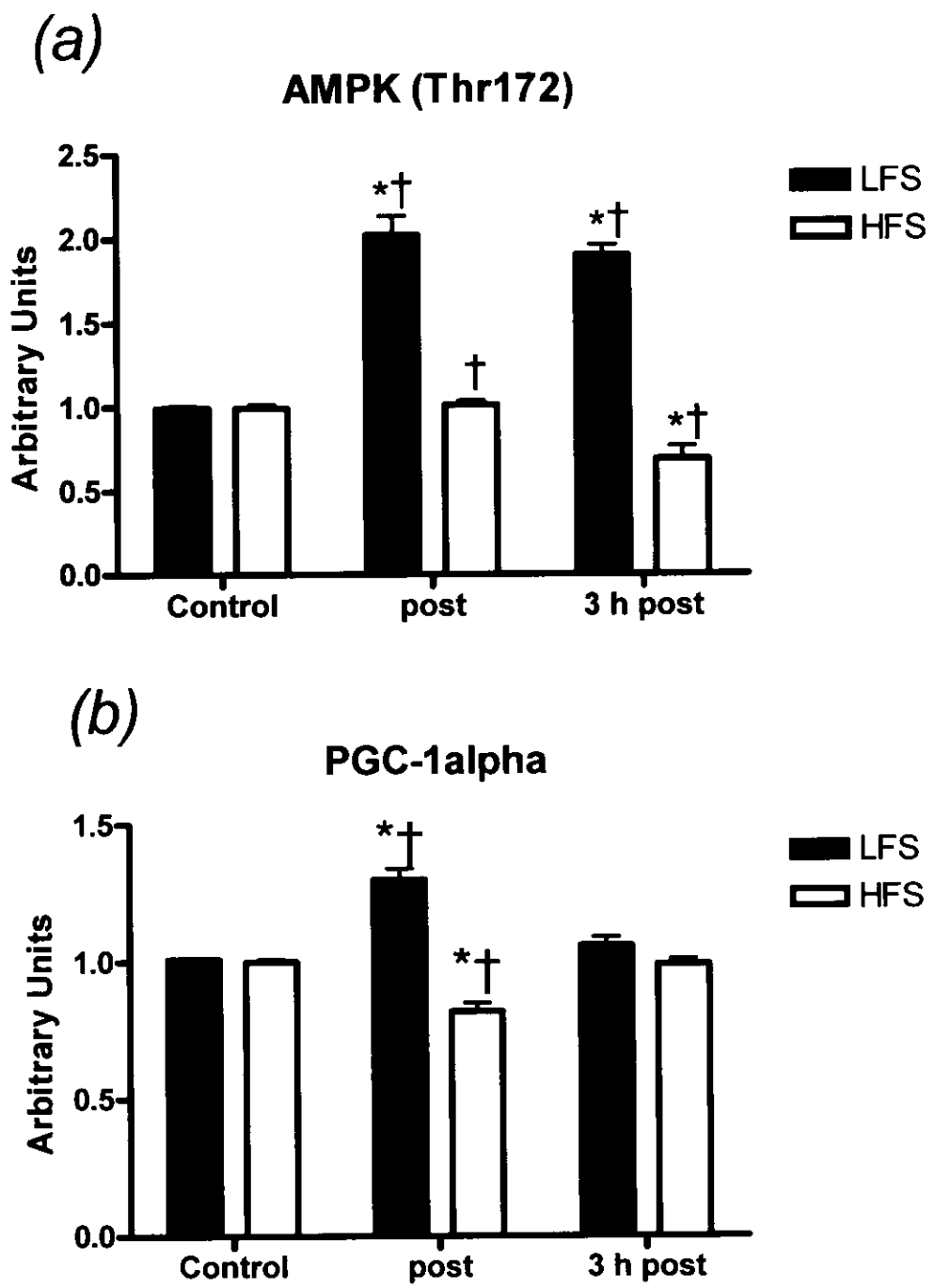


Figure 5.7. (a) AMPK Thr172 phosphorylation relative to total AMPK; (b) total PGC-1α (n=8; 4 EDL and 4 Soleus per bar; mean ± SEM) of resting muscle (control), directly after (post) and 3 h after (3 h post) LFS and HFS. All values were normalised to the relative intensity of the control bands. *Significantly different from control; †Significant difference between LFS and HFS stimulation protocols (ANOVA, Tukey's post hoc, $p<0.05$).

5.2.2.4 PKB-TSC2-mTOR signal transduction pathway

The signalling response of the PKB-TSC-2-mTOR-related signalling cascade was a mirror image of the AMPK response: HFS activated the signalling through this cascade, the translation initiation regulators p70 S6K, 4E-BP1 and eIF2B and the translation elongation regulator eEF2. A second finding is that PKB, TSC2 and mTOR phosphorylation only increased directly after HFS whereas translational regulators were activated both directly and three h after HFS, with the exception of 4E-BP1. Thirdly, LFS markedly inhibited the phosphorylation of TSC2 and downstream translational regulators. Finally, the phosphorylation at the Ser2448 site on mTOR was not affected by LFS.

5.2.2.5 The effect of HFS and LFS on PKB, TSC2, mTOR and GSK-3β phosphorylation

HFS increased PKB Ser473 (figure 5.8a) and TSC2 Thr1462 (figure 5.8b) phosphorylation significantly more in the EDL than in the Soleus. HFS increased PKB phosphorylation by 8.36 ± 1.52-fold and by 2.09 ± 1.04-fold and TSC2 phosphorylation by 3.66 ± 0.20-fold and to 2.6 ± 1.3-fold in the EDL and Soleus directly after HFS, respectively. Neither PKB nor TSC2 phosphorylation were significantly changed three h after HFS. The concentration of total TSC2 was also measured because of the possibility of ubiquitin-dependent TSC2 degradation (see discussion). Total TSC2 significantly decreased to 0.82 ± 0.02 of control after HFS and was not different from baseline 3 h later (figure 5.8c). Finally, the

phosphorylation of mTOR at Ser2448 was significantly increased 1.75 ± 0.08-fold directly after HFS and was 1.23 ± 0.04 of control 3 h later (figure 5.8d), (all $p<0.05$). To summarise, PKB Ser473, TSC2 Thr1462 and mTOR Ser2448 are acutely phosphorylated in response to HFS and the phosphorylation is not significantly different from control 3 h later.

In opposition to these findings of HFS responses, from TSC2 downstream the signalling proteins and translational regulators related to the PKB-mTOR-signalling cascade were deactivated in response to LFS. The only exception was mTOR phosphorylation at Ser2448 which was not affected by LFS. TSC2 Thr1462 phosphorylation significantly decreased to 0.24 ± 0.02 directly and 0.55 ± 0.06 of control 3 h after LFS (phosphorylation was significantly lower in Soleus than EDL 3 h after LFS).

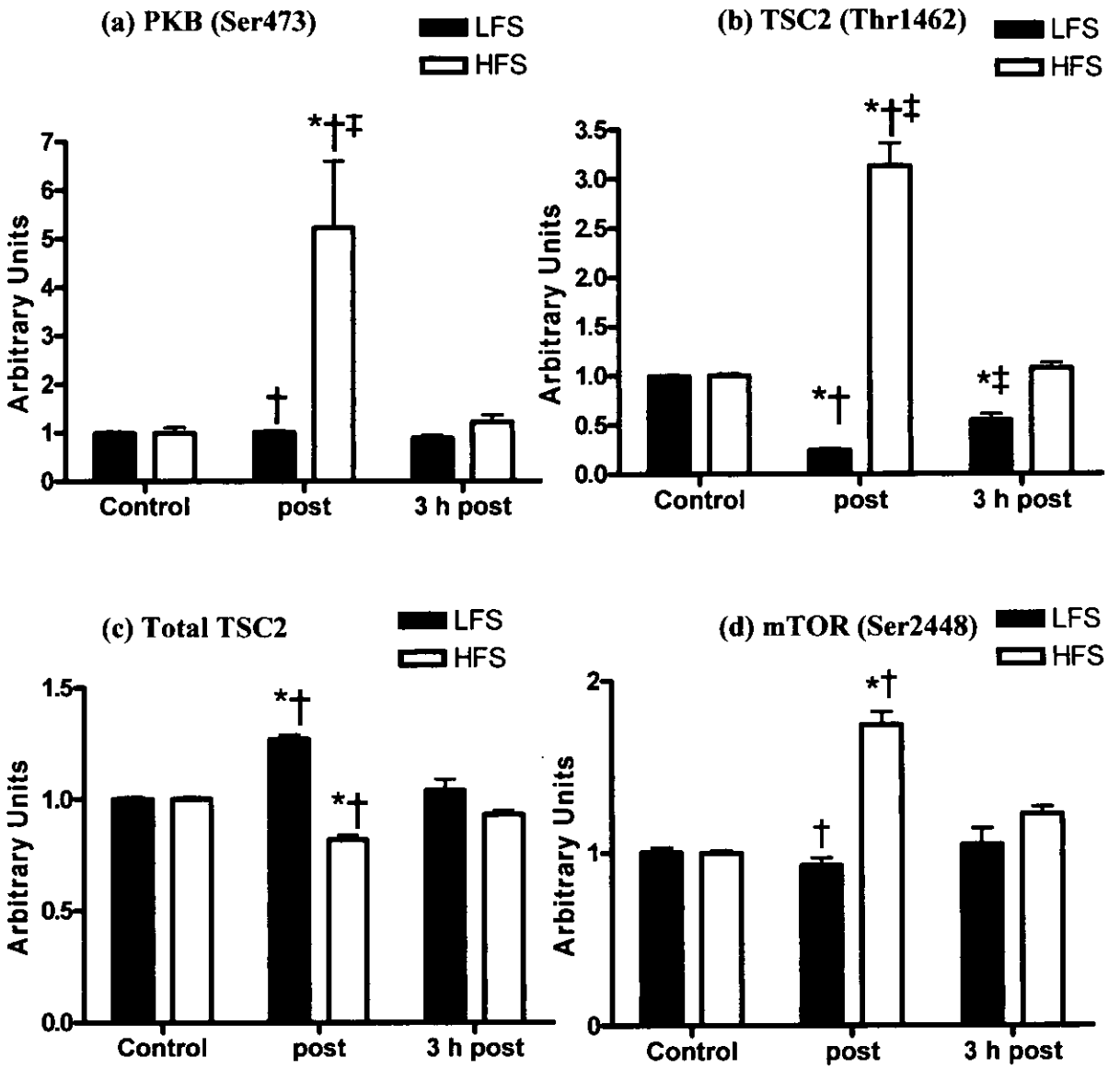


Figure 5.8. (a) PKB Ser473 phosphorylation relative to total PKB; (b) TSC2 Thr1462 phosphorylation relative to total TSC2; (c) total TSC2; (d) mTOR Ser2448 phosphorylation relative to total mTOR. All n=8; 4 EDL and 4 Soleus per bar; mean ± SEM) of resting muscle (control), directly after (post) and 3 h after (3 h post) LFS and HFS. All values were normalised to the relative intensity of the control bands. *Significantly different from control; †Significant difference between LFS and HFS stimulation protocols; ‡Significant difference between EDL and Soleus muscles (ANOVA, Tukey's post hoc, $p<0.05$).

5.2.2.6 The effect of HFS and LFS on translational regulators

All translational regulators were significantly activated directly and 3 h after HFS with the exception of 4E-BP1, which was only activated directly after stimulation. The phosphorylation of p70 S6K at Thr389 significantly increased by 6.85 ± 0.94-fold and by 9.76 ± 0.60-fold directly and three h after HFS, respectively (figure 5.9a). The phosphorylation of 4E-BP1 at Thr37/46 significantly increased by 3.20 ± 0.13-fold directly after HFS but was not significantly different from control 3 h later (figure 5.9b). The phosphorylation of eIF2B at Ser535 (Figure 5.9d) was 0.51 ± 0.05 and 0.76 ± 0.02 of control directly and 3 h after HFS, respectively (eIF2B is activated by dephosphorylation). Similar to PKB, the phosphorylation of GSK-3β at Ser9 increased 3.08 ± 0.12-fold directly after HFS and returned to 1.13±0.04 of control three h post stimulation (figure 5.9c), (all n=8, $p<0.05$).

In contrast to this, LFS significantly decreased the phosphorylation of p70 S6K to 0.22 ± 0.03 and 0.17 ± 0.03 of control and of 4E-BP1 to 0.36 ± 0.02 and 0.10 ± 0.03 of control directly and three h after LFS, respectively. The phosphorylation of eIF2B was significantly increased 1.19 ± 0.05-fold of control 3 h after LFS stimulation, (all n=8, $p<0.05$).

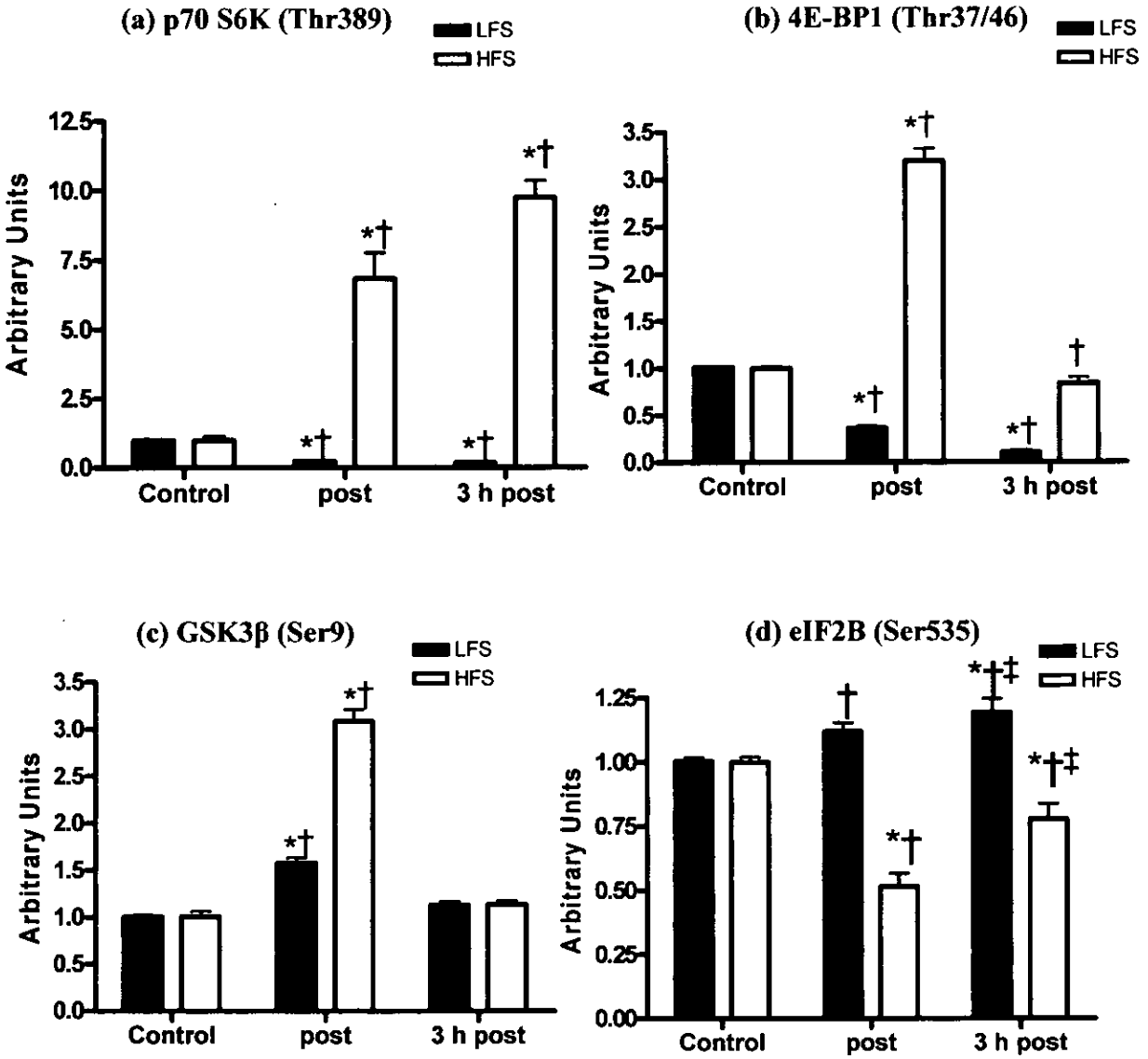


Figure 5.9. (a) p70 S6K Thr389 phosphorylation relative to total p70 S6K; (b) 4E-BP1 Thr37/46 phosphorylation relative to total 4E-BP1; (c) GSK-3β Ser9 phosphorylation relative to total GSK-3β; (d) eIF2B Ser535 phosphorylation relative to total eIF2B. All n=8; 4 EDL and 4 Soleus per bar; mean ± SEM of resting muscle (control), directly after (post) and 3 h after (3 h post) LFS and HFS. All values were normalised to the relative intensity of the control bands. *Significantly different from control; †Significant difference between LFS and HFS protocols; ‡Significant difference between EDL and Soleus muscles (ANOVA, Tukey's post hoc, $p<0.05$).

The elongation factor eEF2 at Thr56 (figure 5.10), is reduced from 0.78 ± 0.06 and 0.38 ± 0.03 of control in HFS whereas phosphorylation is increased in response to LFS at both times points (eEF2 is activated by dephosphorylation).

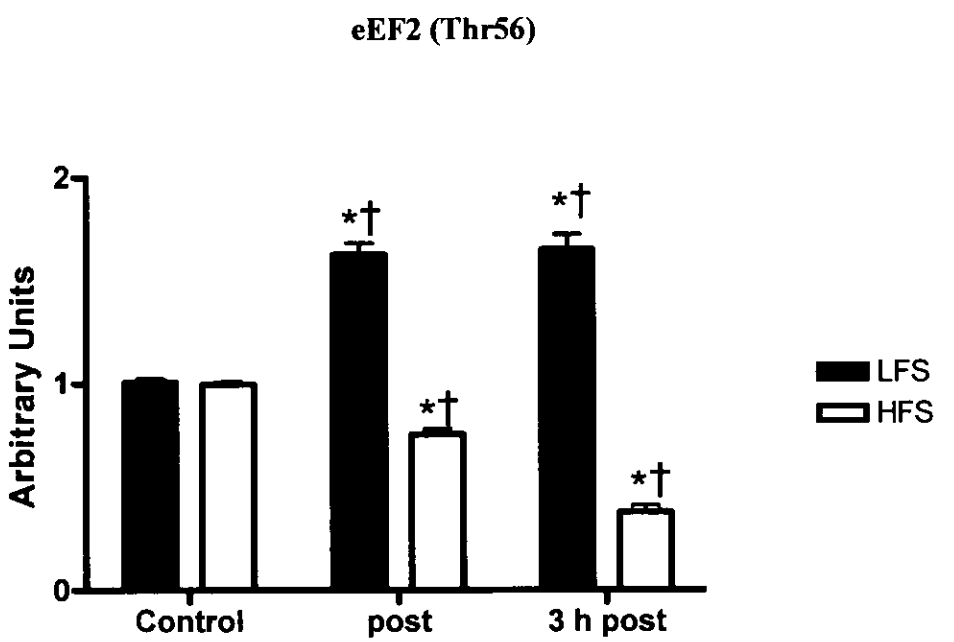


Figure 5.10. eEF2 phosphorylation in response to stimulation. n=8; 4 EDL and 4 Soleus per bar; mean ± SEM) of resting muscle (control), directly after (post) and 3 h after (3 h post) LFS and HFS. All values were normalised to the relative intensity of the control bands. *Significantly different from control; †Significant difference between LFS and HFS stimulation protocols (ANOVA, Tukey's post hoc, $p<0.05$).

5.2.2.7 MAPK pathways: HFS and LFS effects on ERK1/2, p38 and JNK

In contrast to all other proteins, phosphorylation of the three MAPK studied here was not significantly different between the stimulation protocols (figure 5.11a-c). The only exception was ERK2 Thr180/Tyr182 phosphorylation which increased in response to both protocols directly and 3 h after stimulation but significantly more so in response to HFS than LFS (figure 5.11a). ERK1 Thr180/Tyr182 phosphorylation increased significantly after both protocols to ~10 fold of control. In contrast, neither stimulation protocol had a significant effect on p38 Thr180/Tyr182 phosphorylation (figure 5.11b). JNK phosphorylation at Thr183/Tyr185 increased significantly by 1.16 ± 0.03-fold after HFS and then fell both 3 h after LFS and HFS to ~0.77 ± 0.05 (figure 5.11c), (all significant changes $p<0.05$).

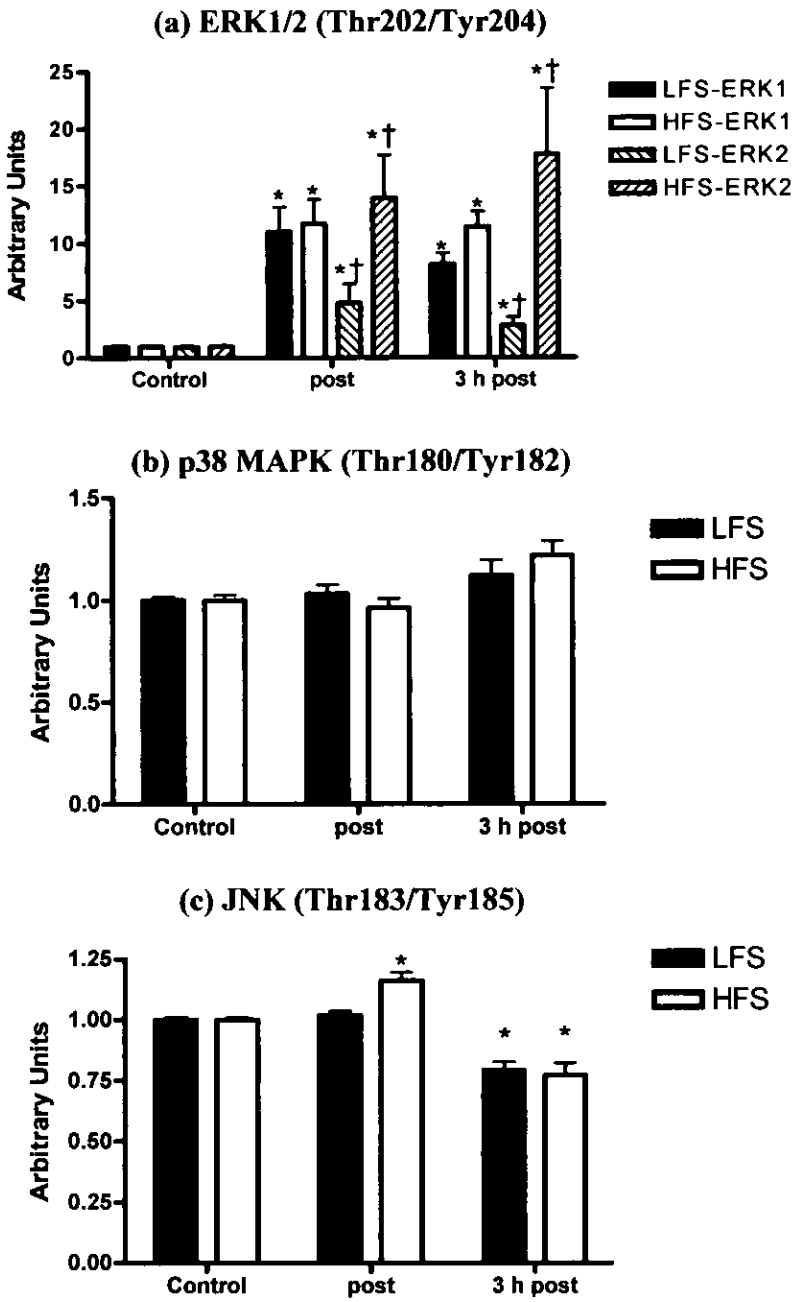


Figure 5.11. (a) ERK1/2 phosphorylation at Thr202/Tyr204 (b) Phosphorylation of p38 at Thr180/Tyr182 (c) Phosphorylation of SAPK/JNK at Thr183/Tyr185. All n=8; 4 EDL and 4 Soleus per bar; mean ± SEM of resting muscle (control), directly after (post) and 3 h after (3 h post) LFS and HFS. All values were normalised to the relative intensity of the control bands. *Significantly different from control; †Significant difference between protocols; (ANOVA, Tukey's post hoc, $p<0.05$).

5.3 Discussion

The results of the *in vivo* study have demonstrated the activation of multiple signal transduction pathways in response to CMNS which can partially explain the physiological changes following CMNS and endurance training. Furthermore, the *in vitro* studies showed that AMPK-PGC-1α signalling can be specifically induced by LFS and that the PKB-TSC2-mTOR cascade and MPS is specifically induced by HFS, which can explain the specificity of growth or phenotype changing responses. In the following section, the activity (phosphorylation) changes in signalling with respect to functions of these pathways is discussed in order to attempt to explain the known adaptations to CMNS and furthermore, to explain the contrasting responses to resistance-like HFS and endurance–like LFS electrical stimulation.

5.3.1 Myostatin signalling

CMNS led to an increased signalling through SMAD2/3 (figure 5.3) which was evident from the 2.5-fold increase in SMAD2/3 Ser465/467 phosphorylation. The cachectic effect of the increase in myostatin (Chapter 4) and signalling through SMAD2/3 in the stimulated EDL compared to control, could be a partial explanation for the ~50 % drop in EDL mass, since increases in myostatin concentrations have been linked with atrophy (Zimmers *et al.*, 2002). Neither myostatin, nor SMAD2/3, were examined in response to isolated muscle stimulation since antibodies were not available at that time. However, this would be worthwhile examining in order to see if there was a diverse signalling response in HFS and LFS. For example, perhaps if HFS could reduce myostatin, then this might explain some hypertrophic responses.

5.3.2 Regulation of ribosome biogenesis

About 80 % of cellular RNA is ribosomal (rRNA), and it is the ribosomes that are the machinery of protein translation (Nader *et al.*, 2002). It has been reported that there was an increase in RNA synthesis during CMNS (Cummins & Salmons, 1999) and this is likely to be largely a result of increased rRNA synthesis. rRNA is encoded in multiple copies in chromosomes and is mainly transcribed by RNA polymerase I in response to DNA binding of both the upstream binding factor UBF and promoter selectivity factor 1 (SL-1) transcription factors. Both increased UBF phosphorylation (Stefanovsky *et al.*, 2001) and overexpression of UBF (Hannan *et al.*, 1996) increases the transcription of rRNA in tissues other than skeletal muscle.

Surprisingly, in this study a significant ($p<0.05$) decrease in UBF Ser637 phosphorylation to 0.7 of control was noted (figures 5.3 & 5.4), which does not explain the reported increased rRNA synthesis following CMNS (Williams *et al.*, 1987). Phosphorylation of UBF was not measured in response to *in vitro* stimulation, although it may have been interesting in order to see if the HFS protocol could specifically induce phosphorylation of UBF. This may have provided insights into the capacity for protein synthesis following HFS.

5.3.3 Mitochondrial biogenesis signalling – activated through CMNS, and selectively following LFS

The observed 4-fold increase in AMPK Thr172 and 4.5-fold increase in p38α/β Thr180/Tyr182 phosphorylation can partially explain the increase in

mitochondrial content and oxidative and fat catabolism enzyme activities that occurs in response to CMNS (Henriksson *et al.*, 1986). AMPK has been shown to stimulate mitochondrial biogenesis via increasing the expression of the co-activator peroxisome proliferator activated receptor γ coactivator-1 (PGC-1α) (Terada *et al.*, 2002; Zong *et al.*, 2002). In addition, activated AMPK will increase glucose uptake into the contracting muscle (Hayashi *et al.*, 1998), increase the expression of GLUT4 transporters (Ojuka *et al.*, 2002) and regulate fat metabolism (Hardie & Pan, 2001). Mitochondrial biogenesis is also likely to be affected by the increased phosphorylation of p38α/β noticed following CMNS. p38α/β was shown to increase phosphorylation of PGC-1α and this is known to also promote mitochondrial biogenesis (Knutti *et al.*, 2001).

In the *in vitro* experiments, whilst PGC-1α was induced with LFS and AMPK was activated (figure 5.7), surprisingly, p38α/β was not activated by the LFS protocol. This was in agreement with Wretman *et al.*, (2001) who showed that only high mechanical stress could activate p38 signalling, which would not be elicited by isometric contractions specific to LFS. The activity of Ca^{2+}/calmodulin-dependent kinase (CamK) isoforms were not measured but it would be expected that the $[Ca^{2+}]_i$ rise induced by stimulation was sufficient to increase Ca^{2+}/calmodulin-activation of skeletal muscle CamK isoforms. If this was the case, then CamK was also likely to contribute to the stimulation of PGC-1α induction and eventual mitochondrial biogenesis (Wu *et al.*, 2002). This, however, remains speculative since the isoform reputed to be controlling this process (CAMKIV) is reportedly not expressed in skeletal muscle as discussed in chapter 1 of this thesis. Thus, AMPK, p38 and CamK were likely to affect the expression and phosphorylation of the transcriptional co-

activator PGC-1α, which in turn together with other mitochondrial transcription factors such as NRF1,2 (Scarpulla, 2002) and TFAM (Larsson *et al.*, 1997) will have affected the expression of mitochondrial genes encoded in both nuclear and mitochondrial DNA.

Only the LFS protocol increased AMPK Thr172 phosphorylation, whereas the HFS protocol had no effect. A possible explanation for the lack of AMPK activation by HFS is that AMP does not sufficiently change during the HFS protocol. For example, contractions at high intensity cause a steady decrease of phosphocreatine (PCr) (Miller *et al.*, 1987). PCr is linked to ADP via the creatine kinase reaction and ADP to AMP via the myokinase. It is likely that the 3 s stimulation bursts were probably too short to markedly increase AMP which could recover during the 10 s rests (or 1 min between sets that followed) in between. The fact that AMPK has been reportedly activated in response to high-intensity exercise does not disagree with the present study and can be explained since longer stimulations at 100 Hz will eventually deplete PCr and markedly increase AMP which explains the increase of AMPK activity after longer, more sustained 100 Hz stimulations (Sakamoto *et al.*, 2004).

Conversely, the continuous LFS was likely to have led to a steady-state with a constant, moderate decrease of PCr and increase of AMP which was sufficient for AMPK activation. Furthermore, AMPK has a glycogen binding domain (Hudson *et al.*, 2003) and AMPK is more activated when the concentration of muscle glycogen is low (Wojtaszewski *et al.*, 2002). Therefore, the glycogen depletion associated with LFS was likely to have activated AMPK further, especially since the protocol was 3 h long. The increase in AMPK phosphorylation by LFS is consistent with a transient

increase in the transcriptional co-factor PGC-1α, which is known to be induced by AMPK (Terada *et al.*, 2002). An increased expression of PGC-1α occurs after endurance training (Baar *et al.*, 2002) and can explain an increase in mitochondrial biogenesis and an upregulation of slow motor proteins (Wu *et al.*, 1999; Lin *et al.*, 2002), which are other known adaptations to endurance training. The inhibition of PGC-1α directly after HFS can be explained by the finding that PKB suppresses PGC-1α expression (Daitoku *et al.*, 2003).

5.3.4 Mitogen activated protein kinases in response to CMNS and *in vitro* stimulation

CMNS also led to an increase in ERK1/2 signalling of 6.3 and 2.7-fold, respectively (figures 5.1 & 5.2) which is expected since ERK1/2 is implicated in inducing a slower muscle phenotype (Higginson *et al.*, 2002). p54 JNK Thr183/Tyr 185 phosphorylation was also increased 2-fold in response to CMNS (figures 5.1 & 5.2). The precise reason for this is not known, and activation is perhaps surprising since JNK is implicated in response to increases in stretch, but the CMNS model elicits isometric contractions. In the *in vitro* model, the results are in agreement with some studies (Nader & Esser, 2001) and in contrast to others (Wretman *et al.*, 2001; Martineau & Gardiner, 2001) as no stimulation pattern-specific effects on ERK1, p38 and JNK phosphorylation at the activity-related Thr/Tyr sites were found (figure 5.11). A stimulation-specific effect was found for ERK2 which increased more, up to 17.75 ± 5.77-fold of control 3 h after HFS whereas the maximal response after LFS was a 4.81 ± 1.69-fold increase directly after LFS. However, phosphorylation did not change in opposite directions. Moreover, this study did not detect any increase in p38

phosphorylation in response to either LFS or HFS. As previously mentioned, the ERK1/2 pathway has been shown to promote the formation of slow fibres and to induce slow and inhibit expression of fast motor proteins (Murgia *et al.*, 2000; Higginson *et al.*, 2002), suggesting that ERK1/2 is involved in the fast-to-slow exchange of MHC isoforms in response to exercise and electrical stimulation. This does not contradict the finding that ERK1/2 phosphorylation also increases in response to HFS. Limited fast-to-slow changes in the expression of myosin heavy chain isoforms from IIx to IIa have been reported in response to resistance training (Williamson *et al.*, 2001). The JNK response to HFS was small and not significantly different from the LFS response at any point contrary to the finding that JNK Thr183/Tyr185 phosphorylation is quantitatively related to the tension (Martineau & Gardiner, 2001).

5.3.5 Selective activation of the PKB-mTOR signalling cascade by HFS

HFS specifically increased the phosphorylation of the anabolic PKB-TSC2-mTOR, GSK-3β and significantly activated the translation initiation regulators p70 S6K, 4E-BP1, eIF2B and the translation elongation factor eEF2 (figures 5.8-5.10). This was reflected in the increased protein synthesis in response to HFS. In stark contrast to this, LFS stimulated the opposite response by inhibiting this signalling network from TSC2 downstream and also not increasing MPS. It should be mentioned here, that whilst MPS was not inhibited by LFS, this was due to the physiological amino acid mix which was used in the sarcoplasmic and myofibrillar MPS assays, stimulating mTOR in the fasted state post-LFS state.

The activation of the PKB-TSC2-mTOR signalling cascade specifically by HFS appears to be related to the high intensity of the HFS protocol. It has been recently hypothesised that high intensity contractions activate currently unknown 'upstream' signal transduction events which regulate the activation of specific muscle growth factors such as IGF-1, MGF and myostatin (Rennie *et al.*, 2004), although the HFS-related signal, its sensor and the upstream signalling events that activate PKB, mTOR, translational regulators and protein synthesis are as yet unknown. Passive stretch which is known to activate protein synthesis (Vandenburgh & Kaufman, 1979), PKB (Sakamoto *et al.*, 2003) and p70 S6K (Baar *et al.*, 2000) is unlikely to be the signal because all contractions were isometric in this *in vitro* model. The unknown signal must therefore be related to the higher stimulation frequency, higher intermittent $[Ca^{2+}]_i$, >2-fold higher tension generated or another unknown factor related to the specific nature of the HFS protocol. The hypothesis that high intensity is necessary for PKB activation is supported by Sakamoto *et al* who reported PKB activation in response to 100 Hz stimulation *in situ* (Sakamoto *et al.*, 2002).

5.3.6 HFS, but not LFS activates translational regulators PKB, TSC2 and mTOR

HFS, but not LFS, increased TSC2 Thr1462 and mTOR Ser 2448 phosphorylation. PKB is known to phosphorylate TSC2 at Thr1462 resulting in TSC2 inactivation (Inoki *et al.*, 2002). The results of this study have also found that PKB phosphorylated TSC2 was more highly ubiquitinated, suggesting that PKB-phosphorylation of TSC2 at Thr1462 leads to its degradation by ubiquitin-dependent proteolysis (Inoki *et al.*, 2002). The current study identified a significant 3.13 ± 0.23-fold increase in TSC2

Thr1462 phosphorylation (normalised to total TSC2) and a significant decrease to 0.82 ± 0.02 of control total TSC2 directly after HFS ($p<0.05$). These data further support the hypothesis that TSC2 is subsequently degraded after phosphorylation by PKB. In contrast, LFS significantly decreased phosphorylation of TSC2 at Thr1462 to 0.24 ± 0.02 and 0.55 ± 0.06 directly and 3 h after LFS, respectively. LFS also significantly increased the TSC2 concentration to 1.27 ± 0.02 of control directly after LFS, suggesting that TSC2 Thr1462 phosphorylation and TSC2 concentration are linked. Taken together, these findings suggest that TSC2 mediates the anabolic response to HFS and inhibits protein synthesis during LFS-induced catabolism. TSC2 partially controls activity of mTOR via the GTPase Rheb (Inoki *et al.*, 2002; Inoki *et al.*, 2003a) which is likely to regulate mTOR phosphorylation via a yet unknown Rheb-dependent kinase. PKB can also directly phosphorylate mTOR at Ser2448 which is the site investigated in the present study (Nave *et al.*, 1999). It was found there was a 1.75 ± 0.08-fold increase in mTOR Ser2448 phosphorylation directly after HFS but no effect of LFS confirming that this site is not dependent on TSC2-Rheb signalling which was affected by LFS.

5.3.7 HFS, but not LFS activates translational regulators p70 S6K, 4E-BP1, eIF2B, GSK3β and eEF2

HFS significantly activated and LFS significantly inhibited the translational regulators p70 S6K, 4E-BP1, eIF2B and eEF2. In contrast to their upstream regulators, translational regulators were activated also 3 h after HFS with the exception of 4E-BP1. These data suggest that HFS can activate a prolonged translation initiation and elongation signalling response even in fasted muscles. The origin of this long-term

translation activation effect is unknown and cannot be related to the short term upstream signalling not present 3 h post HFS.

The significant 6.85 ± 0.94-fold and 9.76 ± 0.60-fold ($p<0.05$) increases of p70 S6K phosphorylation directly and 3 h after HFS can explain the increased protein synthesis 3 h after HFS. The long-term increase in p70 S6K phosphorylation is consistent with the observation that the phosphorylation of p70 S6K and protein synthesis in skeletal are activated long-term after resistance exercise (Baar & Esser, 1999; Hernandez *et al.*, 2000). It is unclear why p70 S6K Thr389 phosphorylation is elevated 3 h after HFS since phosphorylation of PKB at Ser473, TSC2 at Thr1462 and mTOR at Ser2448 are back to normal. Therefore, the prolonged p70 S6K activation must be related to some yet unknown upstream factor. An increase in 4E-BP1 phosphorylation was observed in response to resistance exercise in rats (Bolster *et al.*, 2003) which is in line with these observations of a significant 3.20 ± 0.13-fold ($p<0.05$) increase in 4E-BP1 phosphorylation directly after HFS. However, unlike the p70 S6K response, 3 h after HFS 4E-BP1 phosphorylation at Thr37/46 was not significantly different from control. In contrast to this, LFS caused a significant ($p<0.05$) decrease in 4E-BP1 phosphorylation suggesting reduced protein synthesis.

Another regulatory pathway affecting translation initiation is the PKB-GSK-3β-eIF2B cascade. Activated PKB phosphorylates GSK3β at Ser9 which inhibits the activity of GSK-3β (Cross *et al.*, 1995). An inhibition of phosphorylated GSK-3β leads to a reduced phosphorylation of eIF2Bε at Ser535 which promotes translation initiation (Welsh *et al.*, 1998) and inhibition of GSK-3β by phosphorylation promoted hypertrophy in cultured muscle cells (Vyas *et al.*, 2002). The 3.08 ± 0.12 fold increase

in GSK-3β Ser9 phosphorylation directly after HFS and decrease of eIF2Bε Ser535 phosphorylation to 0.51 ± 0.05 and 0.78±0.06 of control directly and 3 h after HFS supports the hypothesis that this pathway is involved in stimulating an increase in protein synthesis in response to HFS. It is unclear why GSK-3β Ser9 phosphorylation only changed acutely whereas eIF2B Ser535 was decreased directly and 3 h after HFS. eEF2 catalyses the translocation of peptidyl-tRNA during translation elongation and is regulated by mTOR via p70 S6K and p90 RSK1 (Wang *et al.*, 2001). Phosphorylation of eEF2 at Thr56 by its upstream kinase inactivates eEF2 (Proud, 2004b). These data show a significant, prolonged decrease of eEF2 Thr56 to 0.76 ± 0.02 and 0.38 ± 0.03 directly and 3 h after HFS, respectively, suggesting an activation and increased stimulus for peptide elongation.

In direct contrast to the HFS response, LFS affected the phosphorylation of p70 S6K, 4E-BP1, GSK3β, eIF2B and eEF2 at various sites suggesting a pronounced inhibition of translation initiation and elongation that was still present 3 h after LFS (aside from GSK3β). Recent studies have shown that AMPK can directly phosphorylate TSC2 at Thr1227 and Ser1345 leading to an inhibition of mTOR and regulators of translation initiation (Bolster *et al.*, 2002; Cheng *et al.*, 2004; Dubbelhuis & Meijer, 2002). Therefore it appears that TSC2 is capable of being modified by both PKB and AMPK to have contrasting effects on Rheb, and thus a 'tug of war' between these proteins could exist when both are activated together. In addition, the translation elongation regulator eEF2 is also inhibited by AMPK via its upstream eEF2 kinase (Horman *et al.*, 2002b; Browne *et al.*, 2004). These results suggest that LFS inhibits energy-consuming protein synthesis as long as the muscle is kept in a fasted state. An inhibition of translational regulators by stimuli similar to LFS was noted by one group

who reported a decrease of 4E-BP1 phosphorylation after endurance exercise which was reversed by subsequent feeding (Gautsch *et al.*, 1998). Activation of mTOR via a nutrient-sensitive pathway by the amino acid mix necessary for the measurement of protein synthesis can explain why myofibrillar and sarcoplasmic protein synthesis 3 h after LFS were not significantly lower compared to control as the signalling responses would suggest.

5.3.8 Conclusion

The results of these studies are extremely novel and comprise the largest amount of data culminating our understanding of both how exercise leads to the appropriate adaptations depending upon the nature of contractile activity, and how contrasting training types produce such very different physiological characteristics even though the gross stimulus – muscle contraction – is the same. Furthermore the AMPK-PKB switch, for the first time, provides an explanation for the specificity in activation of each signalling protein depending upon its known genetic or translational effects.

The results show that a signal transduction network rather than one or two pathways regulate adaptive responses to CMNS (Wackerhage & Woods, 2002), and that chronic signalling is maintained even following 6 weeks of CMNS. This is also extremely likely to be the case with chronic endurance acitivity. The CMNS model also shows that 'rest' is surely not as important as is thought in adaptative processes. Furthermore the data provide an explanation for how distinct signalling states are induced by LFS and HFS to explain the critical question of why there is a growth response to resistance-like exercise and a phenotype shift in response to endurance activity.

Chapter 6

The effect of essential amino acids upon anabolic responses in both healthy the elderly and elderly with Type II Diabetes Mellitus: an explanation for the development of Sarcopenia?

6.1 Introduction

Previous chapters of this thesis have been dedicated to many aspects of muscle phenotype and mass regulation with the primary focus being on physical activity and contraction. Therefore, in order to widen the scope of this study, this chapter was included to examine how muscle is affected by ageing (sarcopenia) and disease (type II DM) thus gaining a view of muscle regulation from both exercise induced-phenotype change and hypertrophy; to the contrasting atrophy noticed in sarcopenia and some disease states.

Sarcopenia is the gradual loss of muscle mass associated with ageing, which begins around the fourth decade of life and accelerates after the age of ~75 years (Baumgartner *et al.*, 1993). Sarcopenia is caused by both a loss of fibres (Lexell *et al.*, 1988) and atrophy of fibres, the latter of which must be mediated by net protein breakdown. Over time as sarcopenia progresses, the elderly become more frail since progressive loss of muscle mass leads to a reduction in strength (Roth *et al.*, 2000). The precise aetiologies of sarcopenia appear to be multi-factorial as discussed in chapter 1 (see 1.8.3). Sarcopenia is not restricted to those with disease conditions since it is noticeable in otherwise healthy ageing. At the same time, age-related sarcopenia is also linked to an increase in the risk of metabolic disorders such as adult onset type II DM (Beaufrere & Morio, 2000). Therefore, there remains the question as to whether additional complications in the muscles, such as type II DM, may lead to an increased susceptibility to the progression and severity of sarcopenia.

As discussed in chapter 1, it is unlikely that changes in muscle protein synthesis (MPS) or breakdown (MPB) are the primary cause of sarcopenia as other factors identified do not appear to be able to explain the very gradual loss of muscle mass in the healthy elderly. Therefore, if a decline in MPS or an increase in MPB is not in evidence then there must be another key mechanism of sarcopenia yet to be identified. Since amino acids are powerful stimulators of muscle anabolism (even in the absence of exercise) and are readily available from foodstuffs, it is possible that dysregulation of this process with ageing (and elderly with Type II DM) could affect MPS responses to essential amino acids (EAA) and thus an explanation could lie therein. Therefore the aim of this study was to determine the anabolic responses to EAA in both healthy elderly and those with type II DM. This was done by measuring MPS in response to various EAA doses. Molecular signalling responses were also determined through measuring total protein and phosphorylation of elements of the translational mediating pathways. The specific hypotheses of this study were:

1) The elderly possess a blunted MPS response to EAA, characterised by reduced mTOR signalling and capacity, when compared with young individuals.

2) Elderly individuals with chronic type II DM possess a blunted anabolic response to EAA when compared with otherwise healthy elderly using the same parameters.

Methods

As described in Chapter 2.

6.2 Results

6.2.1 Basal MPS responses elderly/young

In the young and the elderly, basal post-absorptive fractional synthesis rates (FSR) values for myofibrillar and sarcoplasmic proteins were similar (means, 0.04 ± 0.15 and 0.06 ± 0.01 $\%.h^{-1}$, respectively young n=20, elderly n=24). *The protein synthesis data were obtained by members of Professor Michael J Rennie's laboratory, University of Nottingham/University of Dundee, and included within this thesis in order to put the signalling data into context.

6.2.2 MPS response to EAA doses in the healthy elderly and young

In the young, EAA between 2-10 g stimulated myofibrillar protein synthesis in a dose-dependant fashion but 20 g EAA failed to elicit any additional stimulation (figure 6.1a); sarcoplasmic protein synthesis showed a similar but less responsive pattern of stimulation (figure 6.1b). The elderly also showed dose-dependant increases in rates of myofibrillar and sarcoplasmic protein synthesis but, compared to the values in the young, the curves were shifted to the right and depressed. These results indicate both a decreased sensitivity (relative myofibrillar increase, Δ 13.8 % per g EAA vs. Δ 3.6 %; sarcoplasmic: Δ 10.7 % vs. Δ 4.4 % per g EAA; both $P< 0.001$) and also a decreased responsiveness to EAA (maximal rate, V_{max}, myofibrillar: 0.11 vs. 0.07 $\%.h^{-1}$; sarcoplasmic, V_{max}, 0.13 vs. 0.11 $\%.h^{-1}$, both $P< 0.001$). A higher dose of EAA (40 g) given to the elderly failed to restore rates of myofibrillar or sarcoplasmic protein synthesis to those seen in the young with 10 g of EAA. However, 10 g of EAA in the elderly promoted the rates of MPS to those seen with 5 g in the young.

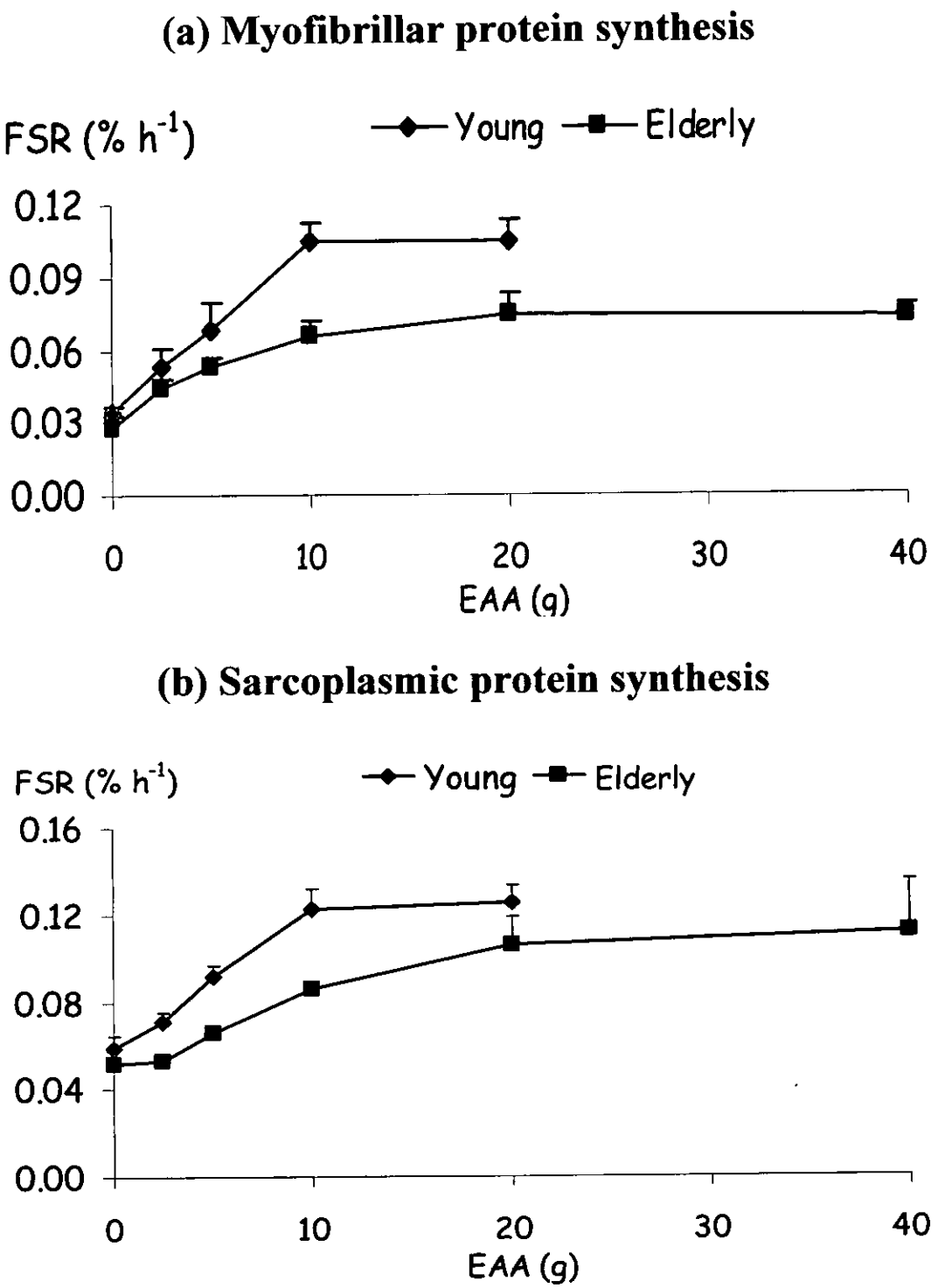


Figure 6.1. Graphs showing the fractional synthesis rate (FSR) dose response of (a) myofibrillar and (b) sarcoplasmic muscle protein fractions in response to 10, 20, 30 or 40 g EAA. Elderly subjects exhibit a reduced muscle protein synthesis (MPS) rate in response to EAA when compared with younger controls for both 10 g and 20 g EAA. Data are means ±SEM, young n=20, elderly n=24 $p<0.05$.

6.2.3 Characterisation of signalling responses in healthy elderly and young

The phosphorylation of mTOR at Ser2448 and p70 S6K at Thr389 increased significantly ($p<0.05$) in both the young and elderly subjects in response to 10 g of EAA but less so in the elderly (~50 % of the response in the young) (figure 6.2a). In the young, mTOR and p70 S6K phosphorylation increased by 5.2 and 8.1-fold, respectively, whereas, in the elderly, mTOR and p70 S6K phosphorylation only increased 2.7 and 3.5-fold, respectively ($p<0.05$) (figure 6.2 a,b). Increased EAA availability also led to increased phosphorylation of 4E-BP1 at Ser 37/46 in both the young 5.9-fold and the elderly 3.2-fold ($p<0.05$) (figure 6.2c). The phosphorylation of eIF2Bε at Ser 544 (eIF2Bε is activated by dephosphorylation; downstream of GSK3β but not of mTOR) fell to ~0.6 of fasted values in response to 10 g EAA in both the young and elderly, so that the actual extent of activation was less in the elderly ($p<0.05$) (figure 6.2d).

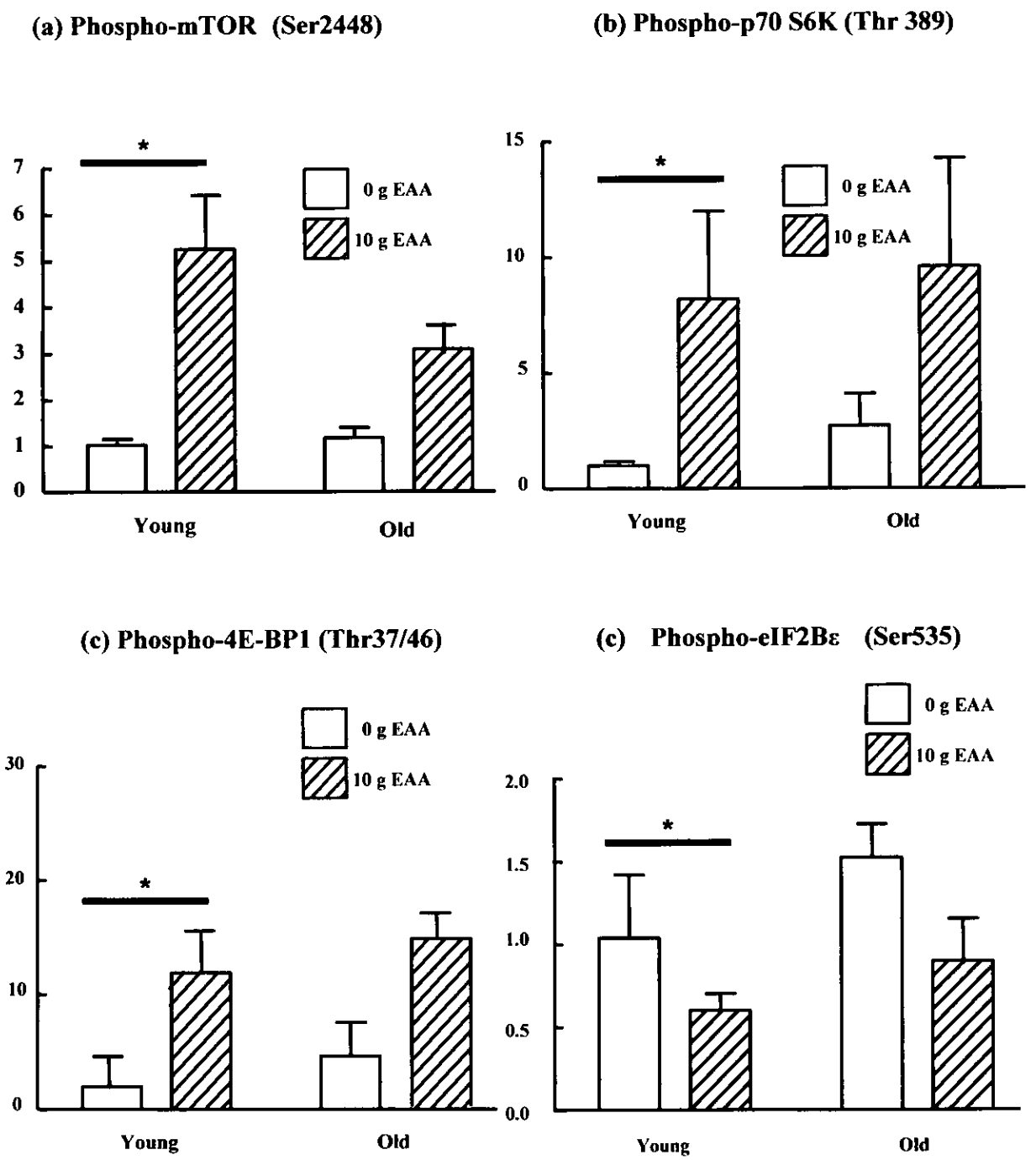


Figure. 6.2. Bar charts showing the phosphorylation of p70 S6K, mTOR, 4E-BP1 and eIF2B. In response to EAA, phosphorylation of each protein was increased significantly more in the young subjects, than in healthy elderly. Data are means ±SE arbitrary densitometric units (n=8) $p<0.05$.

6.2.4 Changes in relative signalling protein levels in healthy elderly individuals compared to young

Levels of total proteins also were different between young and old skeletal muscle. Total levels of mTOR, (~50%) and p70 S6K (~50%) were reduced in the elderly whereas NFκB (~400%) protein were increased in elderly ($p<0.05$; figure 6.3). eIF2B protein concentrations were not significantly different between young and elderly.

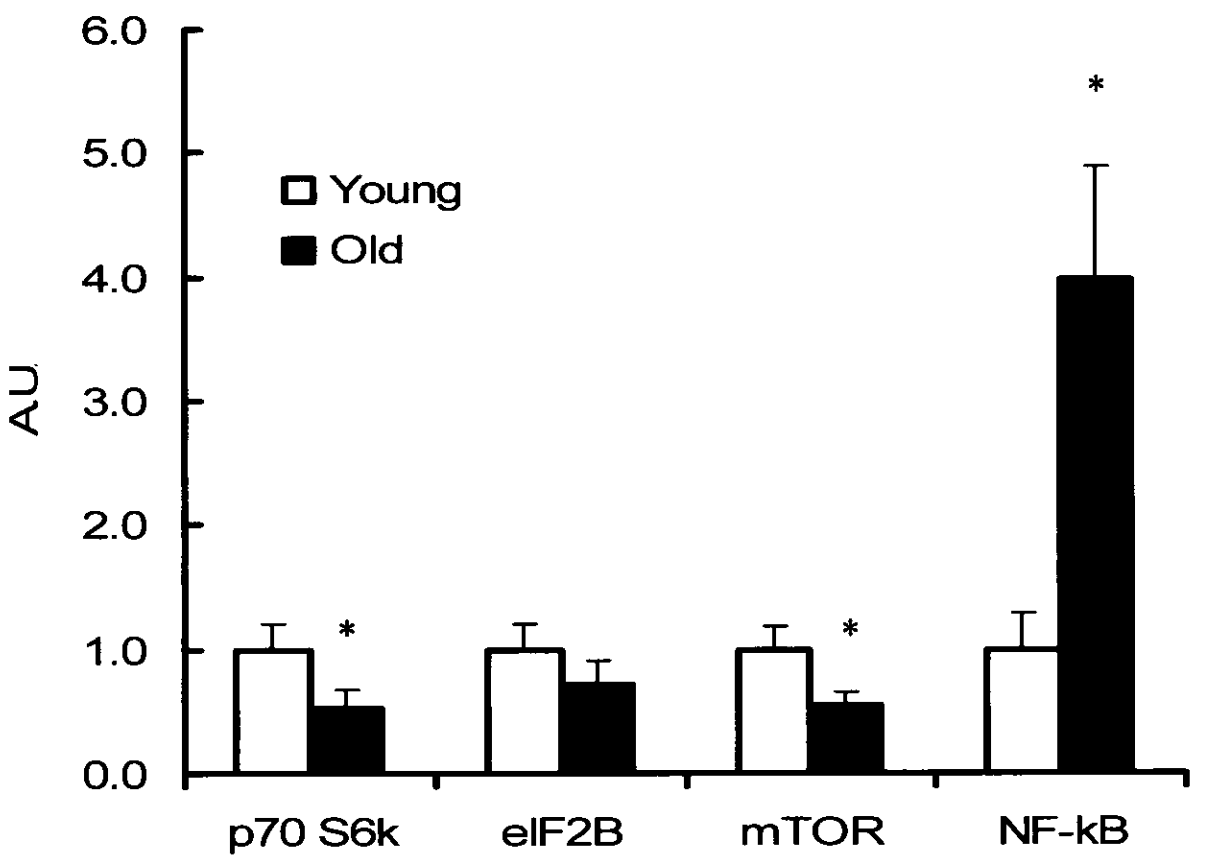


Figure 6.3. Bar chart showing total levels of mTOR, eIF2B, p70 S6K and NFκB relative protein levels in young and old subjects. Data are means ±SE arbitrary densitometric units (A.U), (n=8) $p<0.05$.

6.2.5 Basal MPS responses in Type II DM subjects/healthy elderly

Basal FSR were similar between groups (myofibrillar, 0.027 ± 0.003 $\%.h^{-1}$, sarcoplasmic, 0.051 ± 0.002 $\%.h^{-1}$, grand means ± SD) but the patients with Type II DM showed reduced responsiveness of FSR of both myofibrillar and sarcoplasmic proteins to 20 g EAA (figure 6.4).

6.2.6 MPS response to EAA doses in Type II DM subjects/healthy elderly

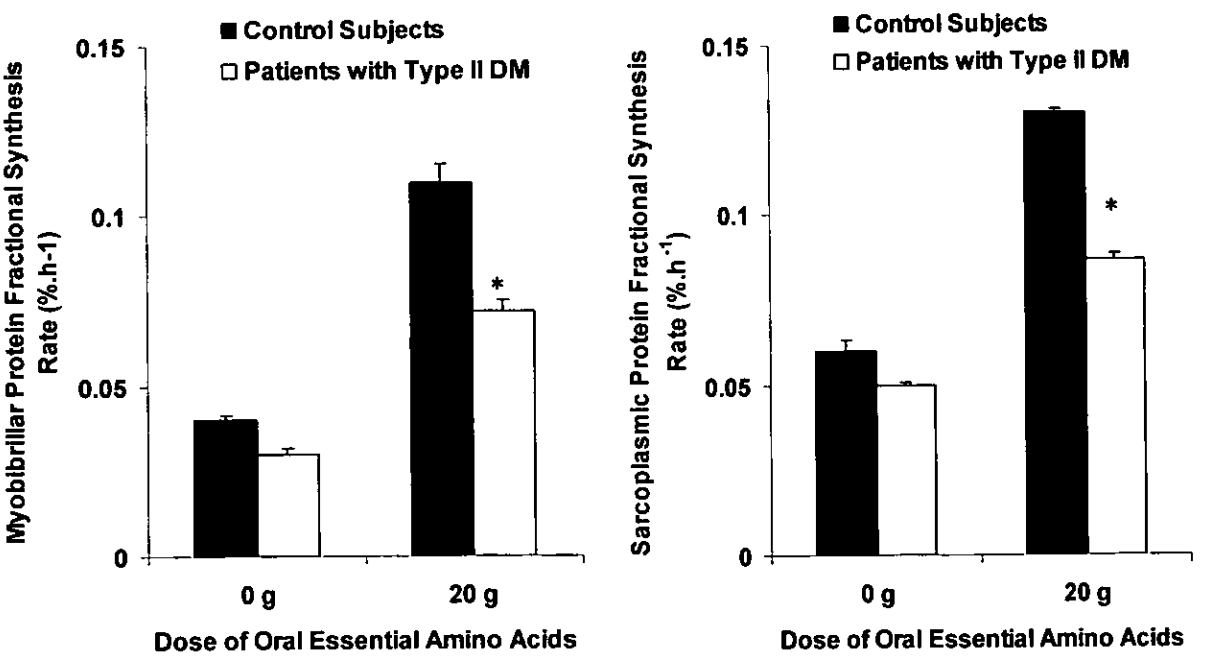


Figure 6.4. Myofibrillar and sarcoplasmic fractional synthesis rates (FSR) in response to 0 g and 20 g EAA. Note that whilst protein synthesis rate are similar with no EAA feeding, an influx of 20 g has a greater effect increasing protein synthesis than in control elderly subjects. Data are means ±SEM, n=8 $p<0.05$.

6.2.7 Characterisation of signalling responses in Type II DM subjects/healthy elderly

Phosphorylation of mTOR at Ser2448, p70 S6K at Thr389 and 4E-BP1 at Thr37/46 only increased significantly ($p<0.05$) in the control but not the diabetic subjects in response to feeding 10 g EAA (figure 6.5).

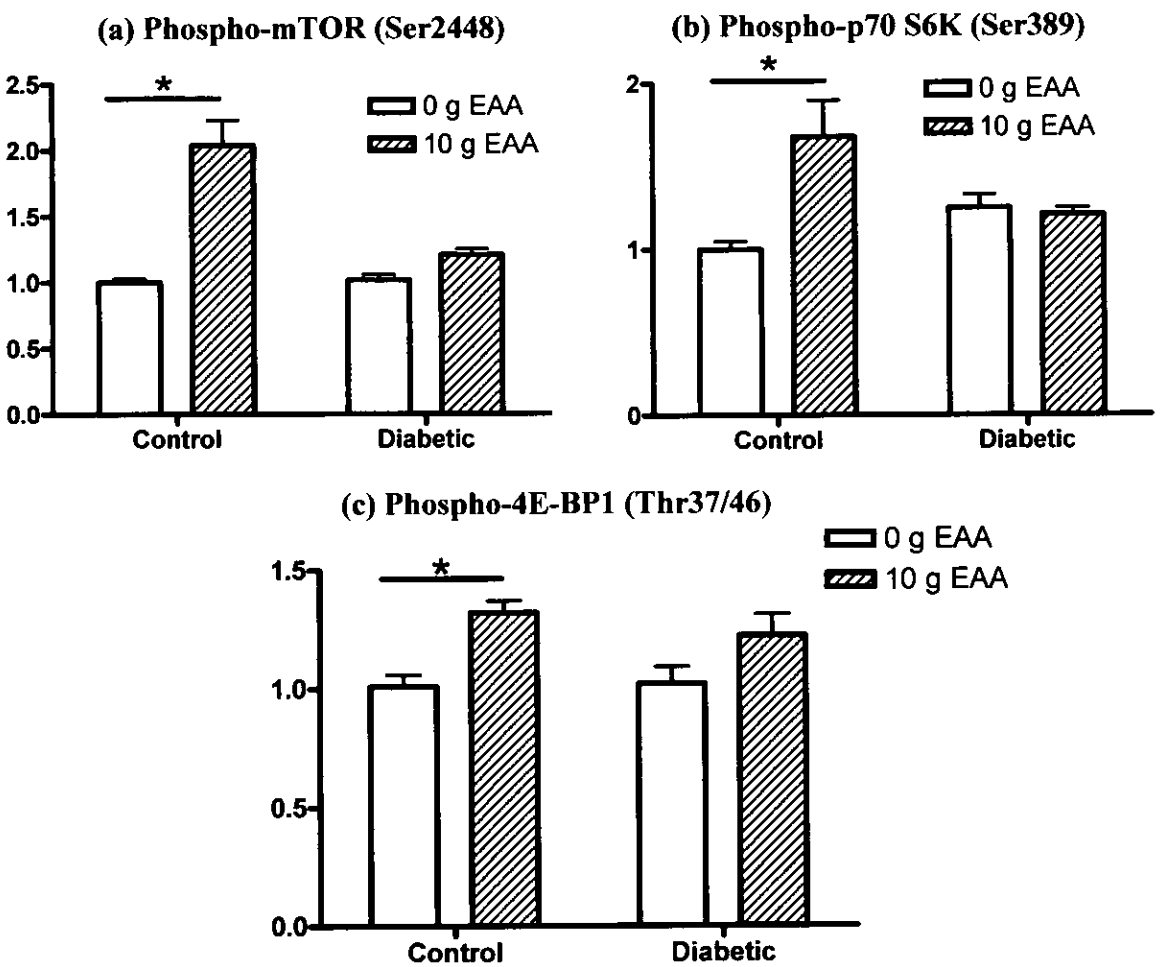


Figure 6.5. Bar charts showing the phosphorylation of mTOR at Ser2448 (a), p70 S6K at Thr389 (b) and 4E-BP1 at Thr37/46 (c) indicating control elderly and elderly with type II DM molecular responses to 20 g EAA. Data are means ±SE arbitrary densitometric units (n=8), *$p<0.05$.

6.2.8 Changes in relative protein levels in Type II DM subjects/healthy elderly

There were no differences in either mTOR or 4E-BP1 levels between groups (n=8), whilst there was a significantly ($p<0.05$) lower level of p70 S6K in the subjects with type II DM. This illustrates that reduced p70 S6K levels could be responsible for some of the blunted response in MPS following EAA.

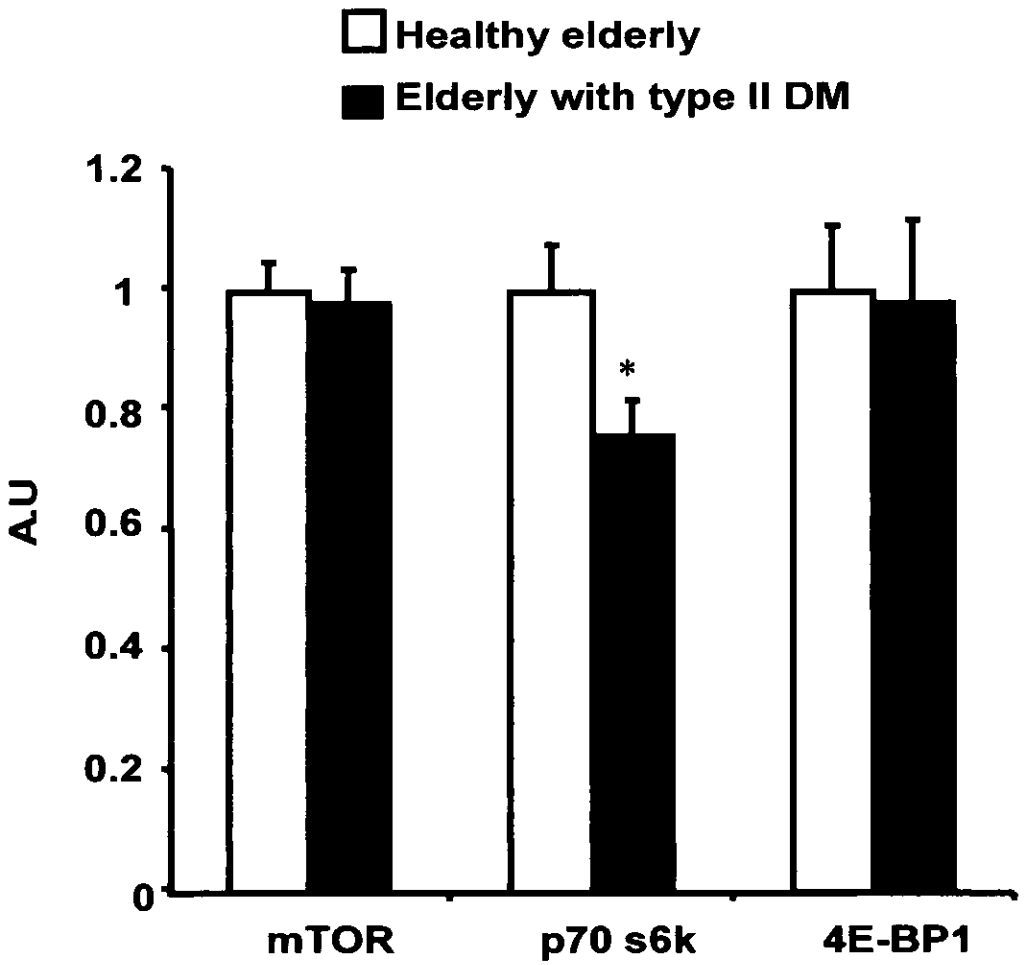


Figure 6.6. Bar chart showing total protein concentrations of mTOR, p70 S6K and 4E-BP1 in the healthy elderly and those with type II DM. Data are means ±SE arbitrary densitometric units (n=8), *$p<0.05$.

6.3 Discussion

These studies highlight perhaps the most likely cause of sarcopenia to date in showing that the elderly have a diminished anabolic response to EAA compared to the young, which might explain the gradual loss of muscle mass with advancing age. Furthermore, those elderly individuals with type II DM are likely to be more susceptible to sarcopenia mediated by blunted EAA responses than the otherwise healthy elderly. There was no difference between young and elderly nor diabetic and aged matched controls in the components of myofibrillar or sarcoplasmic protein synthesis in muscle in the post-absorptive state. Therefore, it can be concluded that the inability of the elderly to respond to the availability of blood EAA should limit their ability to maintain muscle mass and this effect may be magnified in those with muscle disease, and specifically the metabolic state, type II DM.

6.3.1 Molecular responses to EAA – Total protein levels are often at decreased levels in the elderly and those elderly with type II DM

These results have demonstrated that there are marked decrements in the elderly and type II diabetics in the sensitivity and capacity of the muscle protein synthetic apparatus in response to EAA. This leads to a diminished anabolic (protein synthesis) response to EAA. The reduced sensitivity of the system may be caused by the reduction in the amounts of proteins in the mTOR anabolic amino acid sensing and signalling pathway. For example, in the healthy elderly there was significantly diminished mTOR and p70 S6K (~50 %) suggesting this could be true. Interestingly, when compared to the healthy elderly, those with type II DM had a further reduced

level of p70 S6K which is responsible for synthesis of ribosomal proteins and eEF2 phosphorylation. This provides a mechanism by which the capacity of elderly individuals with type II DM to increase MPS in response to EAA is further diminished. Therefore, given that the output of a signalling pathway is in part dependent upon the total amount of a signalling protein (see chapter 3, metabolic control analysis) then the reduced mTOR pathway protein levels may be part explanation for a reduction in the capacity of the elderly system to mount full anabolic responses to protein intake. The protein level of eIF2B was not significantly altered in suggesting that the most important contribution to blunted MPS responses to EAA comes through mTOR.

There was also a marked 4-fold increase in the protein concentration of NFκB in elderly, which may add support to the hypothesis that ageing is associated with a chronic sub-inflammatory process (Chung *et al.*, 2001). The regulatory events responsible for the marked increase in the total NFκB concentration in aged muscles are unclear. NFκB has been shown to mediate TNFα-induced muscle wasting (Li & Reid, 2000) partially by decreasing the expression of myogenic regulatory factor MyoD (Guttridge *et al.*, 2000). The observed higher NFκB levels are likely to amplify the effect of the catabolic cytokines such as TNFα that are often found at higher concentrations in the elderly (Greiwe *et al.*, 2001). It remains to be shown that higher concentrations of NFκB actually have an inhibitory effect on the protein synthetic process. However, evindence for this comes from the fact that direct chronic NFκB activation leads to muscle wasting (Guttridge, 2004) and NFκB targets many genes of the ubiquitin-proteasome pathway (Whitehouse & Tisdale, 2003) and the muscle metalloproteases (Yoo *et al.*, 2002); and so this remains a likely hypothesis.

6.3.2 Molecular responses to EAA – Phosphorylation of translation proteins are often lower in elderly and those elderly with type II DM

There was a significant ($p<0.05$) decrease in the extent of phosphorylation of key elements of protein synthesis pathways in response to EAA, particularly mTOR Ser2448, p70 S6K Thr389 and 4E-BP1 Thr37/46 which might explain the decreased sensitivity to EAA induced MPS (figure 6.2). Since particularly, the cellular amino acid sensor, mTOR Ser2448 phosphorylation (Nave *et al.*, 1999) was reduced in the elderly, there appears some reduction in sensitivity to amino acid stimulation of mTOR activity which therefore blunts translational responses though 4E-BP1 and p70 S6K. eIF2Bε phosphorylation was also activated to a greater extent in the young suggesting that either this protein or upstream kinases, such as GSK3β might also be sensitive to changes in cellular EAA, and that this mechanism is also impaired in ageing independent of mTOR. Further to this, in the diabetic individuals, both MPS, mTOR, p70 S6K and 4E-BP1 phosphorylation were even more suppressed in response to EAA when compared with healthy age-matched elderly (figure 6.5). This suggests that there are additional complications within type II diabetic elderly muscles affecting the ability to mount anabolic signalling responses to EAA. It is very difficult to provide an explanation for this reduced phosphorylation of these proteins, especially since the mechanisms by which amino acids activate mTOR are not established. However, since intracellular amino acid levels within the cells were equivalent to those of the young (Cuthbertson *et al.*, 2004), the blunted effect of MPS by EAA may not be mediated through decreased amino acid import. Therefore, there must be an intracellular mechanism via which amino acids fail to stimulate muscle protein synthesis, and this warrants further attention.

6.3.3 Conclusion

Taken together, these results suggest a process of the blunting of translation mediating signalling and MPS in response to EAA. This indicates impaired capacity for translation of mRNA in response to EAA for the development of sarcopenia. This will be further blunted in elderly with type II DM, and shows that they become 'amino acid resistant' (at least on an intracellular level) as well as insulin resistant which potentially has importance for progress of sarcopenia mediated by reduced EAA-induced anabolism. Furthermore the decreased protein levels of the mTOR pathway suggest that there is a reduced capacity to 'amplify' the EAA-induced anabolic response.

Thus, it appears that it is the defects in the ability of the elderly to make use of the protein in their diet, which would be more consistent with the gradual losses of muscle mass and quality observed. Therefore, given that exercise (particularly resistive type training) reduces the rate of sarcopenia and since there is little decrement in the ability of elderly subjects to respond to resistance exercise (Jozsi *et al.*, 2001); coupled with the fact that EAA stimulates net protein synthesis following exercise even in elderly (Yarasheski *et al.*, 1993) the elderly should eat their main protein-containing meal close to a bout of resistance exercise to maximise maintenance of muscle protein mass. This intervention should allow the elderly to maintain muscle strength, since muscle mass is somewhat proportional to the ability to produce force. Therefore maintenance of muscle mass with protein/exercise combinatorial strategies should reduce frailty, a major cause of morbidity in the elderly.

Chapter 7

General Discussion

7.1 Discussion

The results presented in this thesis provide insights into the molecular control of muscle mass and phenotype in response to both growth stimulating activity and phenotype altering stimuli. Moreover, they identify potential signalling explanations for the decreased EAA-stimulated MPS as a contributory factor to development of sarcopenia, and atrophy in type II diabetics. Overall this makes a substantial contribution to a more thorough understanding of regulation of skeletal muscle mass and phenotype on a molecular level. Below, each chapter of work will be summarised before a detailed summary in order to bring together these findings.

7.2 Myostatin signalling

Myostatin is a major regulator of skeletal muscle size, in that it has the ability to control muscle mass during embryogenesis through cell cycle regulation and also through adult-hood by affecting transcription and translation (Taylor *et al.*, 2001), (figure 7.1). The gene array data presented in chapter 3 provide fascinating insights into the transcriptional regulation under which myostatin exerts its negative effects upon muscle mass, apparently through inhibition of transcription. Although a selection of genes have been identified that were downregulated and the potential effects of this have been discussed, these data have not been verified using RT-PCR or Northern blotting. Furthermore, there are much data that warrant further attention although time restraints of the Ph.D means that the follow-up work will have to be completed following submission of this thesis. Nevertheless, these data open exciting new avenues to further examine the role of myostatin and gene expression.

The current data also provide information on protein synthesis in showing that the reduction in protein synthesis when myostatin is present in cells, could be due to the inhibition of the cellular signalling components controlling initiation of protein synthesis, such as p70 S6K. These data offer potential for targets to curb the negative effects of myostatin on MPS in disease states, where myostatin is elevated. Myostatin would serve to be a much better target for the treatment of muscle wasting diseases and atrophy than IGF-1, and such growth factors, since GeneCards indicate that it is almost uniquely expressed in skeletal muscle.

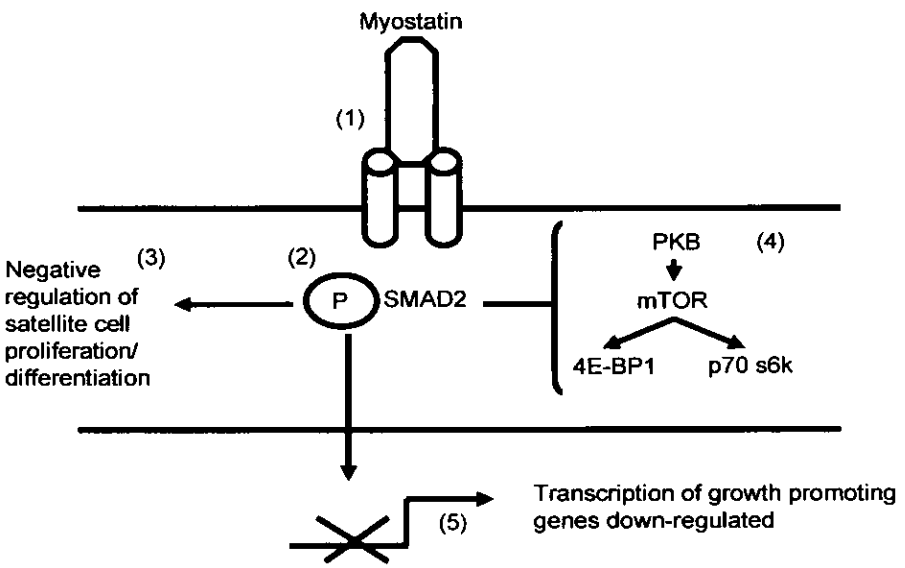


Figure 7.1. Myostatin signalling in adult skeletal muscle as identified by the studies in chapter 3. Myostatin binds to activin type II receptors (1), which leads to the phosphorylation of SMAD2/3 (2). Myostatin will negatively regulate satellite cell proliferation and differentiation through its effects on the cell cycle, and thus inhibit hypertrophy (3). SMAD2 phosphorylation leads to an inhibition of many elements of the insulin/IGF-1 pathway including PKB, mTOR, p70 S6K and possibly 4E-BP1 thus negatively regulating translation and MPS (4). Finally, increased myostatin expression leads to a down-regulation in transcription of many genes which probably promote cell growth such as eIF4A2 and Syndecan-4 (5).

7.3 Concentrations of signalling proteins

Signalling proteins are the cellular mediators that turn a stimulus into either a genetic response, or one that controls translational regulation. Therefore, the regulation of these proteins are of critical importance, especially since varying their levels by genetic knock-out causes a variety of phenotypic effects. The present study identified differences in signalling protein levels between typically fast and slow rat muscle, and then further determined how a phenotypic altering stimuli such as CMNS can render these protein concentrations subject to change. The levels of signalling proteins do differ between muscles and this variation is likely to have an effect upon transmission of a given signal (Atherton *et al.*, 2004) (figure 7.2a). This could mean that if the the levels of signalling proteins also differ between human individuals then this could provide evidence of trainability. For example, if one individual possessed a higher concentration of elements of the PI-3K pathway, such as p70 S6K, then perhaps they would respond more strongly to a growth stimulus such as resistance training; whilst conversely one with higher AMPK levels might respond to endurance type training more effectively. It was further identified that these proteins were subject to change, although not always in a direction one would expect. For example, calcineurin levels (fast-to-slow phenotype) were actually reduced following 6 weeks of chronic electrical stimulation. This may be an example of a negative feedback mechanism where the muscle has adapted to the stimulus and no longer requires the same amplification through this protein, and thus expression is suppressed. Conversely, other proteins were changed in the 'expected' direction thus perhaps the muscle was still adapting. This study showed that signalling proteins are regulable and a change in concentration could change its ability to adapt to future signals (figure 7.2b.)

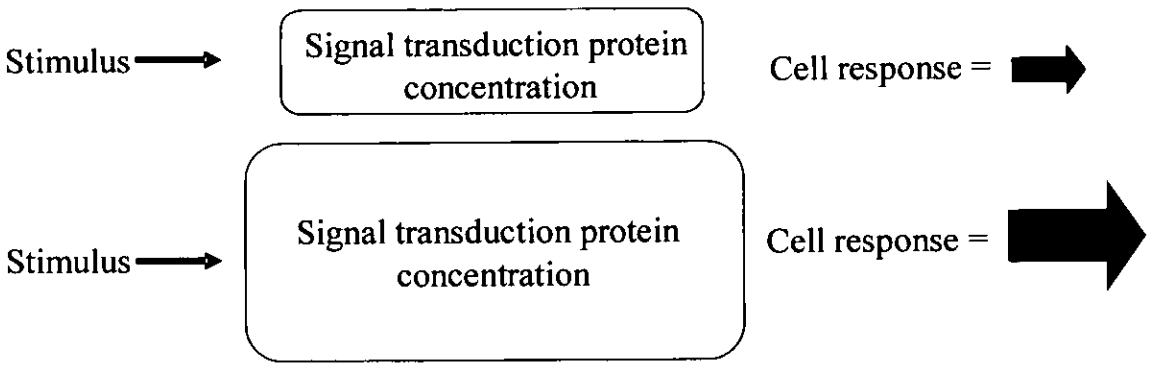


Figure 7.2.a. Diagram showing how the concentration of a signalling protein could affect cellular responses to stimuli. Predominantly fast and slow skeletal muscles contained different concentrations implying that they would display varying levels of cellular responses to exercise and disease stimuli.

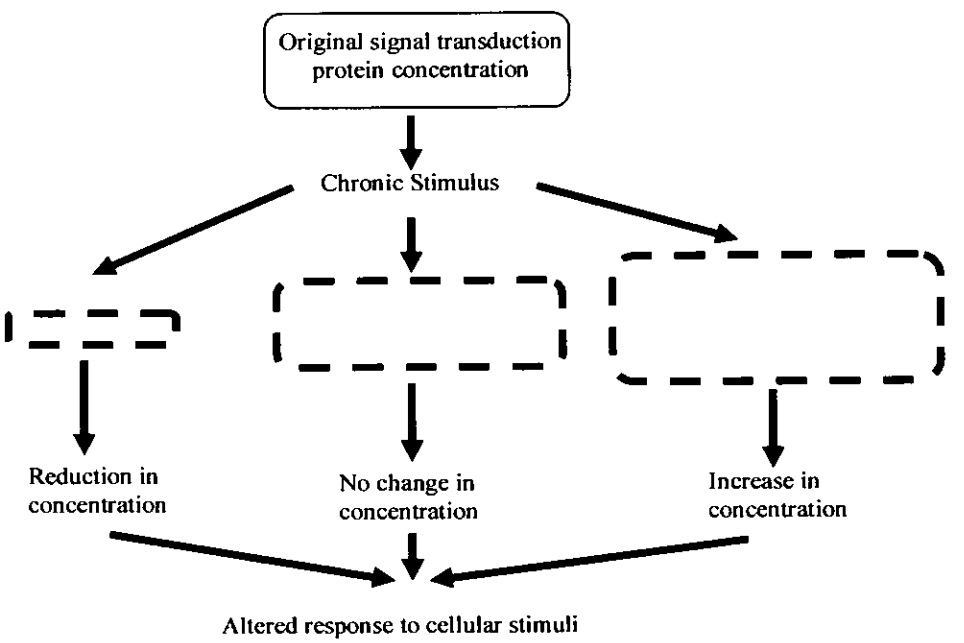


Figure 7.2.b. Diagram showing that signal transduction proteins are under regulation by chronic contractile activity (and may also be by disease), and their concentrations can move in either direction. Any change in signal protein concentrations could have marked effects upon cellular responses to exercise, insulin and/or disease stimuli.

7.4 The AMPK-PKB switch

The data comprising chapter 5 of this thesis are from over 300 experiments and comprise the most novel study to date to contributing to our understanding of the molecular governance of physiological adaptation exercise training. Moreover, they provide fresh information on how critical signals activate pathways to achieve the contrasting responses to resistance and endurance training (figure 7.3).

The ability of skeletal muscles to specifically adapt to variations in physical activity is a phenomenon that had not been previously understood. Shown in chapter 5, there is evidence that this could be controlled by a selective activation of signalling pathways in response to variations in stimuli produced by different modes of contractile activity. This is evident from the selective activation of the AMPK in response to activity which specifically increases AMP and inhibits the PI-3K pathway in response to low frequency chronic exercise stimulation. This leads to an effect of increased signalling producing mitochondrial biogenesis and slow fibre characteristics. Furthermore, whilst under prolonged stress when AMPK is active protein synthesis is inhibited, which limits energy turnover that is not immediately necessary. Conversely, with high frequency intermittent stimulation, metabolic sources and specifically the phosphagen system are allowed to resynthesise ATP before AMP levels are allowed to rise, thus not activating AMPK. At the same time, the PKB pathway is activated by IGF-1 or some other yet unknown factor. Therefore, the net effect is increased protein synthesis. This behaviour has been termed the AMPK-PKB switch and provides a specific mechanism for the selective activation of signalling pathways which lead to the contrasting physiological adaptations to strength and endurance exercise.

These data are not purely for 'molecular satisfaction' but also provide several possible benefits to our knowledge of maximisation of training response. For example, when resistance training and attempting to maximise protein synthesis, individuals should avoid endurance training (even an extended cool down) for the same muscle group afterwards in order to maximise muscle growth. This is evidently important because the activation of AMPK by endurance training is likely to inhibit translation initiation and elongation and thus protein synthesis (Inoki *et al.*, 2003b; Browne *et al.*, 2004). Furthermore, glycogen depletion should be avoided for maximal protein synthesis since AMPK is more activated when glycogen levels are low (Wojtaszewski *et al.*, 2003) as AMPK has a glycogen-binding domain (Hudson *et al.*, 2003), and is therefore presumably incapable of activation when glycogen-bound. Thus, it seems likely that protein synthesis after resistance training is higher in glycogen loaded muscles. Protein synthesis and the signalling in the lower half of the PKB-TSC2-mTOR pathway are activated for up to 48 h after resistance training (Rennie & Tipton, 2000; Hernandez *et al.*, 2000). Therefore, in order to train at a high level, split routine training programmes should be used in order to produce a larger growth stimulus and allow a prolonged protein synthesis response. In addition, knowledge of these mechanisms shows consumption of protein shortly before or after training will maximise positive protein balance. This study may also benefit endurance athletes. Since AMPK activation is maximal when glycogen levels are low, then it could be recommended for people to perform endurance exercise when at some level of glycogen depletion, possibly before breakfast. However, whilst potentially maximising adaptive responses this could of course prove counterproductive given the fact that glycogen depletion correlates with metabolic fatigue and glycogen provides a major substrate during exercise (Hargreaves, 2004).

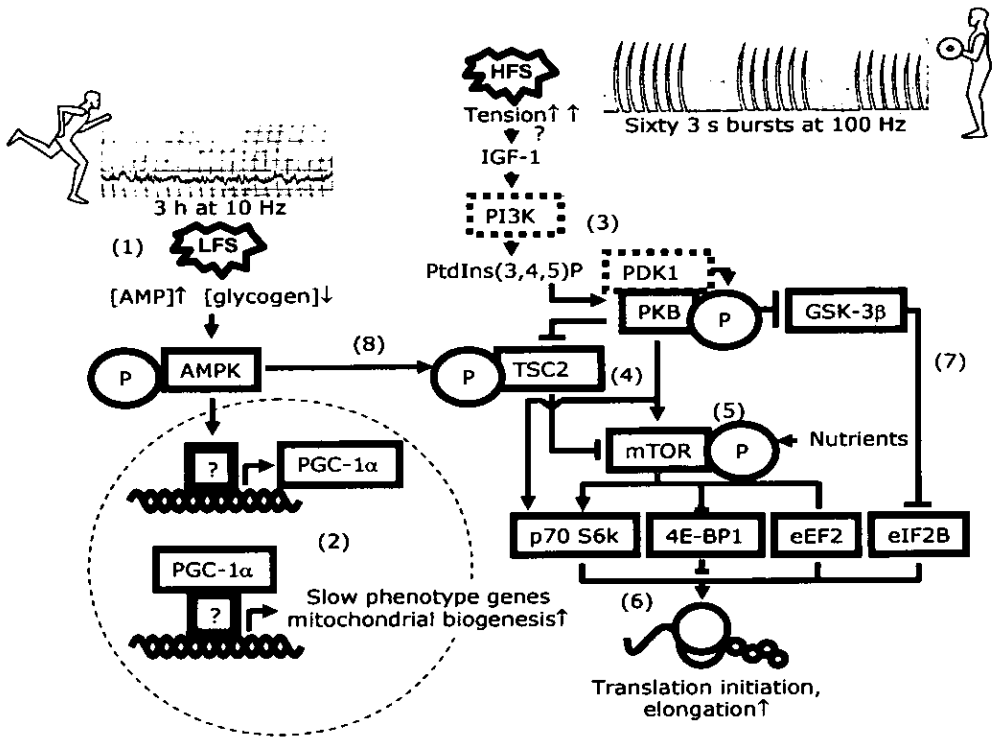


Figure 7.3. An overview of the proposed 'AMPK-PKB switch'. (1) LFS produces increases in AMP and a decline in glycogen which activates AMPK. (2) AMPK induces PGC-1α promoting transcription of slow genes and mitochondrial biogenesis. (3) Some signal, perhaps tension could induce IGF-1 which would activate PKB through PI-3K. (4) PKB will then activate mTOR either directly or though phosphorylation of TSC2 which will activate mTOR through decreasing the intrinsic GTPase activity of Rheb. (5) Nutrients affect mTOR directly, with increased amino acid availability stimulating activity. (6) Combined mTOR activation will direct increases phosphorylation of p70 S6K and 4E-BP1 to promote translational initiation. (7) PKB phosphorylation leads to the inhibition of GSK3β of which a downstream substrate is eIF2B. Once GSK3 inhibitory effect on eIF2B has been removed, the protein becomes more active and its GAP activity increases thus promoting translational initiation. (8) On a different phosphorylation site than PKB, AMPK can also phosphorylate and inhibit TSC2, which will decrease mTOR activity through increasing Rheb GTPase activity (Atherton *et al.*, 2005).

7.5 Sarcopenia in healthy elderly and those with Type II DM

Sarcopenia is a major cause of frailty in the elderly population and it has been identified that the aetiologies of muscle wasting in ageing are both complex and multifactorial. The results presented in chapter 6 provide novel evidence for a reduced anabolic effect to exogenous essential amino acids in the elderly and this is even more clearly pronounced in elderly with type II DM (see schematic model in 7.4). This provides a major mechanism by which a gradual loss of muscle mass may be explained with advancing age and in type II DM, and provides a cogent explanation for the gradual atrophy. Molecular studies showed further evidence of reduced mTOR-downstream signalling suggesting that there is some way in which ageing and type II DM affect cellular components of protein synthesis to reduce the response to EAA through diminished phosphorylation and translational protein concentrations. This in itself is likely to have an effect upon cellular capacity to respond to EAA (figure 7.3). Furthermore, since those elderly with type II DM showed further MPS and cellular diminution of response to EAA, they should be aware that another factor in morbidity of type II DM is likely to be advanced sarcopenia brought about by the EAA resistance, in addition to the characterised insulin resistance.

Therefore, these data have important consequences for the maximisation of the anabolic effects of resistance training and feeding EAA in elderly. Since EAA feeding has an effect upon signalling (Karlsson *et al.*, 2004) and muscle growth (Levenhagen *et al.*, 2001; Esmarck *et al.*, 2001) as does resistance training (even in the elderly), an additive and a timed co-ordination of resistance training and feeding is important in the elderly to make the most of the reduced EAA sensitivity.

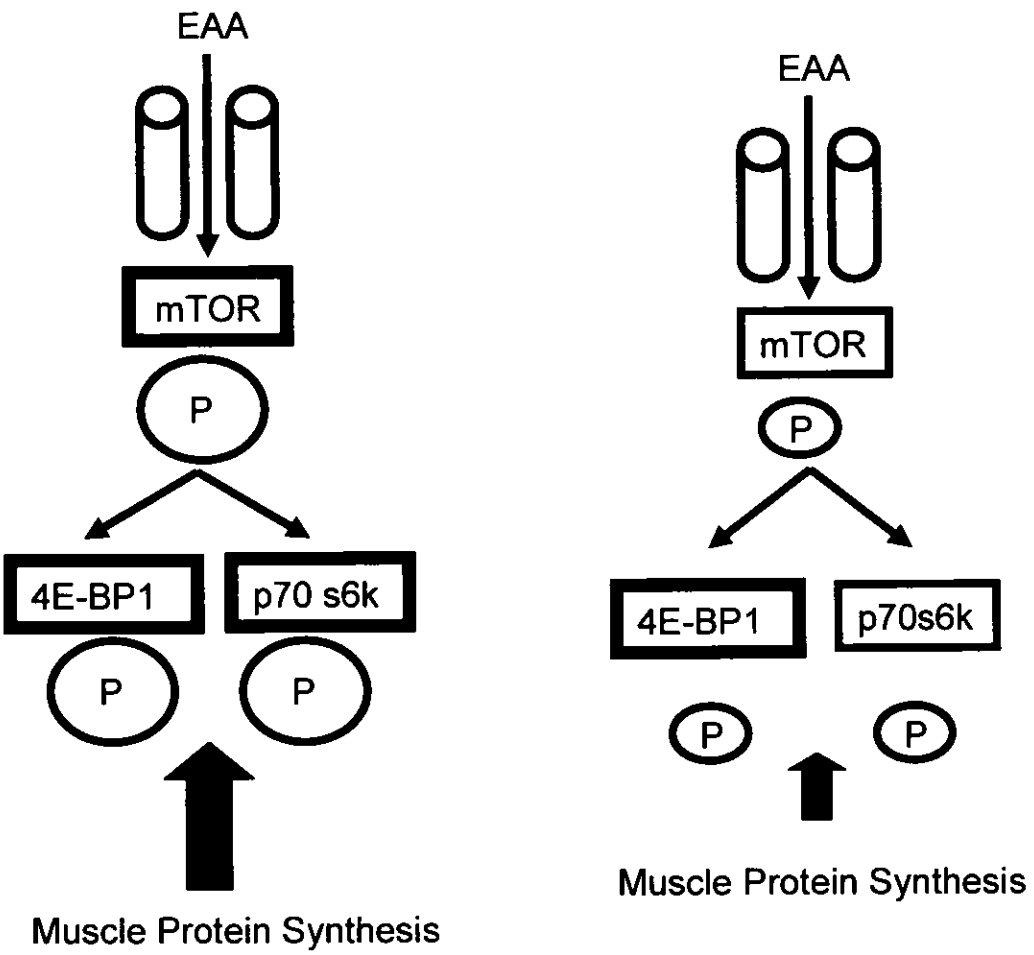


Figure 7.5. Muscle protein synthesis (MPS) responses in the young (left) and elderly (right). In the young an infused dose of EAA leads to a robust stimulation of MPS. However, in the elderly there is a diminished MPS anabolic response to EAA. This could be due to the reduced concentration of components of the IGF-1 pathway, mTOR and p70 S6K as shown in this study (indicated in blue). Furthermore, a blunting in the phosphorylation of mTOR, p70 S6K and 4E-BP1 following EAA administration, shows the blunted response on a molecular level and explains the diminished MPS response (indicated in red). Individuals with type II DM were characterised by an even more striking reduction in concentration of p70 S6K, and phosphorylation of mTOR, p70 S6K and 4E-BP1.

7.6 Thesis summary

The results presented in this thesis are summarised below and have:

1) Identified new insights into the transcriptional effects of myostatin upon muscle mass. Furthermore, they distinguish the mechanisms by which myostatin exerts its inhibitory effect upon skeletal muscle protein synthesis.

2) Characterised differential concentrations of signalling proteins and highlighting potential importance of these upon adaptation and pre-determination to adapt to activity. Furthermore, as muscle enzymes, signalling proteins are regulable and subject to change by CMNS. It is likely these changes are important mechanisms in adaptive responses, and performance.

3) Identified the potential 'switch' that controls whether a muscle hypertrophies in response to resistance training or undergoes gross phenotype change in response to endurance activity, by selectively 'recognising' specific signals produced by disparate contractile activity. This behaviour has been termed the 'AMPK-PKB' switch and provides a mechanism by which muscle knows how to adapt to variations in messages elicited by different contractile activity.

4) Provided new evidence of the importance of EAA in the aetiology of sarcopenia and type II DM. Furthermore, the signalling controlling this diminished sensitivity is characterised and provides an explanation behind the reduced protein synthesis rates in response to exogenous EAA.

7.7 Scope for future studies

1) *The AMPK-PKB switch:* Whilst the 'AMPK-PKB switch' appears to function in rats as shown in chapter 5 of this thesis at least with *in vitro* stimulation, these findings needs to be verified in human beings during exercise and not electrical stimulation. If in agreement with the present data, these studies could then comprehensively demonstrate the reasons for disparate physiological adaptations to different types of training in humans.

2) *Prolonged activation of translational regulators following HFS:* PKB, TSC2 and mTOR are only transiently activated (<3 h) whereas some translational regulators, in particular p70 S6K are activated for longer (>6 h). This activation correlates with MPS in this study, but also in other studies which show p70 S6K phosphorylation and MPS activation for >24h (Hernandez *et al.*, 2000). Therefore, further dissection of these pathways is required in order to characterise the unknown upstream kinases that maintain the activity of these translational regulators, even in the absence of activation of known upstream kinases such as PKB, TSC2 and mTOR.

3) *Identifying the 'upstream' growth signal:* It is still unsure how the HFS model used in this study activates the PKB-TSC2-mTOR cascade. Studies examining mechanotransduction (mechanical stretch), IGF-1 splice variants, and myostatin responses in this *in vitro* model may help to elucidate the unknown upstream signal. This also would need to be verified in the human study, in attempting to correlate a growth signal, such as increased IGF splice variant messages, with activation of the cascade.

4) *Identifying myostatin interactions to control MPS:* Whilst it was clearly identified that myostatin inhibits the PI-3K pathway, the mechanism by which myostatin-SMAD signalling affects MPS is not known. Therefore, immunoprecipitation might be useful to examine PKB pathway-SMAD-FOXO complexes, as might yeast two hybrid screening. Interactions between these SMAD (Conery *et al.*, 2004) and FOXO (Seone *et al.*, 2004) proteins might lead to alterations in kinase activity of these PI-3K pathway and therefore explain reduced phosphorylation of elements of this pathway when SMAD2/3 signalling is elicited by myostatin binding to activin type II receptors. Furthermore, there is evidence that SMAD2 activation induces SHIP-1 to inhibit PPK signalling (Valderrama-Carvajal *et al.*, 2002)

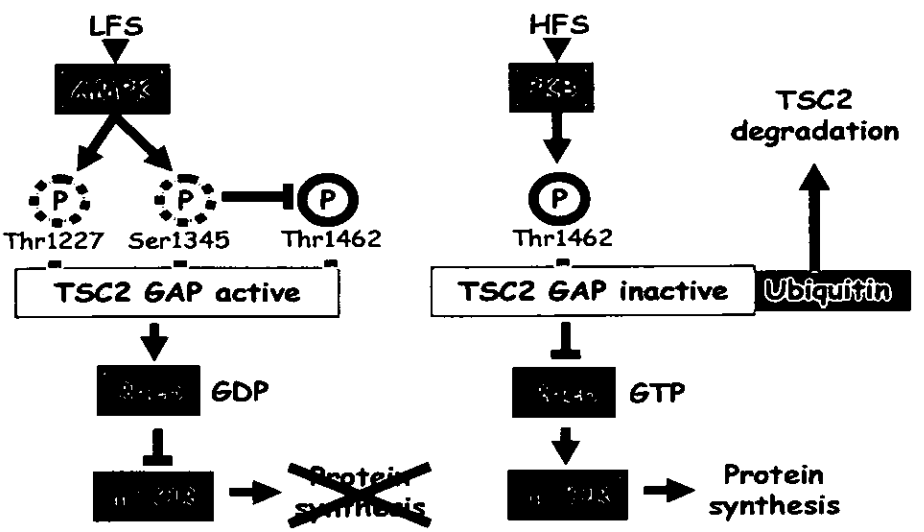
5) *Myostatin and contrasting exercise:* It would be interesting to see the effect of HFS and LFS on myostatin expression in order to assess its role in adaptation to resistance and endurance exercise training. Furthermore, larger studies on myostatin expression in humans in response to different exercise types would aid in identifying whether myostatin has a role in adaptive processes to disparate exercise types.

6) *Signalling protein concentrations in human muscle:* It would be interesting to see whether humans possess different concentrations of signalling proteins controlling transcription and translation of exercise-relevant responses. An endurance athlete for example might contain a higher concentration of AMPK which could potentially maximise the signal and thus exhibit heightened mitochondrial biogenesis, whereas those with higher concentrations of elements of the PI-3K-PKB pathway may exhibit larger increases in MPS in response to resistance exercise. If this were true then the concentrations of signalling proteins could prove good indicators of trainability.

7) *Optimising exercise-nutrition strategies:* Interactions between timing of nutritional intake and exercise need further attention. In order for athletes to gain maximum hypertrophy or optimum glycogen resynthesis for strength and endurance athletes respectively, nutritional timing may be able to give an extra edge. Studies should examine these strategies on a cellular level and understand the kinetics of the cellular signalling system controlled in synergy by exercise and nutrition.

8) *Adaptation of connective tissues to physical activity:* Studies on adaptation to exercise should be extended to the muscle extracellular matrix, bone and tendons (Kjaer, 2004). It has been shown that these areas regulated by exercise and loading, and as skeletal muscle adapt. However, many of the physiological changes are not characterised. Moreover the cell signalling and genetics are completely unknown, and thus this is an area that will prove a 'hot –bed' of research in the near future.

9) *PKB and AMPK signalling to TSC2:* It was hypothesised in this thesis that specific AMPK or PKB phosphorylation sites on TSC2 could opposingly affect proteins synthesis through Rheb (below). To substantiate this model one could activate AMPK by AICAR during HFS which should abolish mTOR Ser2448 phosphorylation.



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ORAL PRESENTATIONS:

1) The 2004 Physiological Society Meeting at the University of Cambridge, two abstracts presented.

2) The 2004 Physiological Society Meeting at the University of Glasgow, two abstracts presented.

3) The 2004 Focussed Physiological Society Meeting at Derby Medical School, University of Nottingham, one abstract presented.