Acute regulation of skeletal muscle protein metabolism by nutrients, exercise and hypoxia

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For Elenie, Mum and Dad.

ABSTRACT

Muscle mass adapts in response to changing functional and metabolic demands of the organism and its maintenance is important for movement, health and survival. However, many questions remain regarding the acute response of muscle to feeding, exercise and altered environmental conditions. Thus, the aim of the present thesis was to investigate how muscle responds acutely to changes in some of these stimuli in humans. The first experiment aimed to understand the temporal response of muscle protein metabolism and associated molecular anabolic signalling to a single oral protein feed. Rates of myofibrillar protein synthesis (MPS) exhibited a biphasic response to an acute protein meal. Myofibrillar and sarcoplasmic protein synthesis approximately tripled and doubled, respectively by 90 min post-feed before returning to postabsorptive values by 180 min, despite continued availability of plasma essential amino acids (+80%). Activity-related phosphorylation of proteins involved in anabolic signalling mirrored the increase in MPS (e.g. p70S6K and 4EBP1 increased 2.1±0.2 and 2.6±0.5-fold, respectively; mean ± SEM), and remained elevated after MPS had declined to baseline. The second study reports the recovery of muscle function and plasma markers of muscle damage following delayed onset muscle soreness (DOMS)-inducing exercise when combined with an oral protein meal. Protein ingestion accelerated muscle force and power recovery by 48 h post-exercise vs. placebo (muscle force = -0.25% vs. -10.7%; power = -0.15% vs. -8.7% for protein and placebo trials, respectively). The augmented functional recovery was not, however, matched by improvements in perceived muscle soreness or plasma markers of damage. Finally, the third study examined the response of

MPS to reduced O_2 supply (hypoxia, 12.5% O_2). After 3.5 h hypoxia at rest, MPS was unaffected from normoxic values. However, although MPS increased after normoxic resistance exercise (224±49%), this was blunted during hypoxia (184±268%, $p>0.05$) despite increased anabolic signalling (p70S6K and ACC-β increased 3.4±1.1-fold and decreased 0.7±0.1-fold, respectively), and was correlated with the measured blood O_2 saturation ($r^2 = 0.48$, $p<0.05$).

It is concluded that (i) after a single oral protein feed, muscle protein synthesis demonstrates a 'muscle-full' effect where, following rapid activation, MPS is switched-off despite continued elevated amino acid concentrations and anabolic signalling through the mTOR pathway, (ii) a single post-exercise protein meal increases muscle function recovery, but not perceived muscle soreness or blood markers of damage, and (iii) reduced muscle O_2 supply suppresses protein synthesis after exercise, even in the presence of increased anabolic signalling. These findings have significant implications for both athletic and clinical populations in terms of nutritional and exercise strategies employed, and for the efficacy of exercise regimens for increasing / maintaining muscle mass in conditions of reduced O_2 supply.

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DECLARATION

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree. The following published material has resulted from work contained within this thesis:

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Signed

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CHAPTER I. INTRODUCTION

1.1. Skeletal muscles

In healthy adults skeletal muscle is the major contributor to lean body mass (Bohé *et al.* 2003), with the musculoskeletal system accounting for approximately half of total body weight (Wackerhage and Rennie, 2006). Muscles serve to fulfil a variety of tasks in response to changing functional and metabolic demands of the organism (Bassel-Duby and Olsen, 2006). One of muscles' primary functions is to enable locomotion of the body by transmitting the force generated during muscular contractions, via connective tissue, to pull on the body's bony lever system (McArdle *et al.* 1996). This system allows fine control of the rate and extent of force development to move the body or for the propulsion of objects in order not only to perform basic mechanical activities associated with the demands of daily living but also to complete complex dynamic movements, such as during exercise and sport for competition (MacIntosh *et al.* 2006).

In addition to this role in mobility, skeletal muscle plays a central role in the control of whole-body protein, fat and carbohydrate (CHO) metabolism, contributing to ~25-30% of basal energy expenditure (Radegran *et al.* 1999; Welle and Nair, 1990). Muscle constitutes the bodies' largest mobilisable source of protein, containing approximately half of the total body protein content of a healthy human (Rooyackers and Nair, 1997); a reserve that can be catabolised in times of organismal need to provide the free amino acids for the synthesis of molecules required by other parts of the body (e.g. Zhang *et al.* 2008b). Furthermore, muscle contributes to temperature

regulation of the body (MacIntosh *et al.* 2006), and is the predominant site of whole-body glucose uptake (Horowitz *et al.* 2001; Kraegen *et al.* 1990; Zierler, 1999), the disregulation of which may lead to pathologic complications such as type II diabetes mellitus (Handberg *et al.* 1996; Hilder *et al.* 2005; Hirose *et al.* 2000; Turinsky, 1987). Muscle, therefore, not only serves mechanical functions but also contributes to metabolic and energy homeostasis (Smith *et al.* 2008). As such, the adequate maintenance of skeletal muscle is pivotal to preserve contractile function, health and survival (Tipton and Ferrando, 2008). Indeed, significant loss of muscle mass (atrophy) correlates strongly with loss of locomotive capacity, metabolic dysfunction and increased frailty-associated mortality (Farre *et al.* 2008; Jatta *et al.* 2009; McDermott *et al,* 2008; Miller and Wolfe, 2008; Regensteiner *et al,* 1993; Wagner, 2008).

Despite the physiological and pathophysiological importance of skeletal muscle, the mechanisms regulating its mass are incompletely understood. Thus, the aim of this thesis is to highlight and investigate gaps in the knowledge relating to regulation of muscle by factors known to be important in affecting muscle protein metabolism and muscle mass.

1.1.1. Skeletal muscle structure

The structure of a tissue reflects its function. In the case of skeletal muscles that function is primarily to generate contractile force for movement (MacIntosh *et al.* 2006). Skeletal muscles possess specialised functional units, the sarcomeres, which enable contraction by converting chemical energy (in the form of adenosine

triphosphate; ATP) into mechanical energy, movement or resistance to stretch (McArdle *et al.* 1996).

Muscle fibres are long, cylindrical, multinucleated cells (Berchtold *et al.* 2000), the number and size of which dictate total muscle volume (Wackerhage and Rennie, 2006). These fibres lie parallel to each other; each fibre being composed of smaller units, termed myofibrils, which run parallel to the long axis of the fibre through which force is transmitted. The myofibrils are composed of smaller subunits, the myofilaments, and comprise the contractile apparatus responsible for force production. The predominant proteins of the myofilaments are the thin filament, actin, and the thick filament, myosin, which together account for ~85% of the total myofibrillar complex (McArdle *et al.* 1996). Sarcomeric myosins comprise two heavy chains that protrude from the myosin filament by a neck region which contains two pairs of myosin lights chains; the essential and regulatory (or phosphorylatable) light chains (Pette and Staron, 1990). These filaments determine the contractile heterogeneity of fibre types observed in human skeletal muscle, which may be categorised into three broad classes; slow-oxidative (SO, or type I), fast-oxidative-glycolytic (FOG, or type IIa), and fast-glycolytic (FG, or type IIx) fibres.

Actin and myosin, along with associated titin and nebulin filaments, interdigitate between successive Z disks to form the sarcomere; repeating units that are arranged in series within the myofibril (Schiaffino and Reggiani, 1996). The characteristic striated appearance of muscle results from alternating myosin-containing (Anisotropic, A-band) and actin-containing (Isotropic, I-band) regions within the sarcomere. The centre of each I-band contains the actin-anchoring Z disks, which

adhere to the sarcolemma of the cytoskeleton to provide stability to the entire structure and anchors the filament group ultimately to skeletal structures to provide muscle cell contractions, which effects movement of the body.

1.1.2. E-C coupling and the sliding filament theory

Since Luigi Galvani's studies of frog muscle first raised the hypothesis of intrinsic 'animal electricity' as inducing muscular contractions (Galvani, 1791), great progress has been made in understanding how electrical phenomena (i.e. action potentials / depolarisation) result in muscle contraction. Combined with the advent of electron microscopy and x-ray diffraction technologies, a detailed explanation has been developed of how an electrical discharge at the muscle stimulates chemical and mechanical events leading to muscle action; a process first termed excitation-contraction coupling (E-C coupling) by Sandow (1952).

E-C coupling originates with neuronal input from the central nervous system, generating at the motor nerve an action potential; brief (< 1 millisecond) electrical impulses transmitted along motor units. The action potential is received at the motor end plate, which releases acetylcholine (ACh). ACh then diffuses across the synaptic cleft and binds to ACh receptors on the sarcolemma which, in turn, conducts the action potential into the interior of the muscle cell, via dihydropyridine receptors (DHPRs), to the transverse tubule (T tubule) system; an extensive network of interconnecting channels running perpendicular to the myofibril, which ends in the terminal cisternae of the sarcoplasmic reticulum (SR) where Ca^{2+} is stored (Ebashi, 1976; Protasi, 2002; Rios *et al.* 1991). This depolarisation subsequently induces Ca^{2+}

release from the SR, mediated by interaction of DHPRs with SR ryanodine receptors (Berchtold *et al.* 2000; Masamitsu, 1999; Meissner and Lu, 1995). Thereafter Ca^{2+} binds to troponin, removing the inhibitory action of troponin-tropomyosin on actin-myosin interaction. Thus, the active sites of actin and myosin are joined, forming the actin-myosin crossbridge (actomyosin). This then activates myosin ATPase and catalyses the hydrolysis of ATP to adenosine diphosphate + inorganic phosphate (P_i), thus releasing energy. The energy released in this process causes movement of the actin-myosin crossbridge, and the muscle generates tension. Actin dissociates from myosin when ATP binds to actomyosin, leading to a relative movement or sliding of the thick and thin filaments, and the muscle shortens and contracts. Coupling and uncoupling continue as long as the Ca^{2+} concentration remains at a sufficient level to inhibit the troponin-tropomyosin complex. Thus, when neural input is removed, the Ca^{2+} pump moves Ca^{2+} back into the SR and, in the presence of ATP, actin and myosin remain separated in a relaxed state (Wilmore *et al.* 2008).

The cyclical oscillating nature of actomyosin attachment, rotation, and detachment, causes muscle fibres to shorten without actin or myosin actually changing length. Actin filaments slide past the myosin filaments and into the A band during shortening, thus decreasing the length of the I band region as the Z disks are pulled towards the M line, producing force at the Z disks, which is ultimately transduced to the skeleton to generate limb movement. This process is termed the sliding filament theory and provides the molecular mechanism by which muscles contract to produce force (McArdle *et al.* 1996).

1.1.3. Types of muscle contraction

The ultimate effect of E-C coupling and the mechanistic actions of the sliding filament theory is to shorten the muscle fibres in unison along the muscles' longitudinal axis, thereby contracting the whole muscle and producing force. There are two principle categories of muscle contraction: isometric and isotonic (or dynamic). Isometric contractions are produced when there is no change in muscle (sarcomere) length during muscular activation, and are associated with the highest level of force production. Isotonic muscle actions occur when the length of the muscle is altered, and are divided into two forms: concentric and eccentric. Concentric contractions involve the muscle shortening, and joint movement occurring as tension develops, for example in the quadriceps muscle during knee extension exercise. Eccentric actions occur when the muscle lengthens while developing tension, such as in the quadriceps during knee flexion (McArdle *et al.* 2006).

1.1.4. Skeletal muscle plasticity

The functional and structural properties of skeletal muscle are crucial to meet the demands of daily living. Skeletal muscle constitutes a highly adaptable tissue, capable of remodelling its structure and function in response to demands imposed by physiological (e.g. physical activity) and environmental (e.g. altitude exposure) challenges (Durieux *et al.* 2007; Hilber, 2008; Hoppeler and Flück, 2002). To detect these changes, mechanical, metabolic, neuronal and hormonal signals are transduced over signalling pathways (Hoppeler *et al.* 2007), which alter cellular gene

expression, biochemical properties and morphology (Flück, 2006; Murton *et al.* 2008). Muscle plasticity is reflected in profound alterations in muscle mass and function (i.e. force, power and endurance capabilities) in response to modifications in contractile activity (endurance exercise, electrical stimulation, denervation, inactivity), increased or decreased load (resistance training and microgravity, respectively), nutrition (amino acid and insulin supply), environmental factors (hypoxia, thermal stress), and a range of pathologies (Flück and Hoppeler, 2003; Murton *et al.* 2008). However, the understanding of how these factors regulate muscle remains poorly understood (Greenhaff *et al.* 2008).

1.2. Regulation of muscle protein homeostasis by nutrients

1.2.1. Circadian rhythm and protein balance

Skeletal muscle mass is regulated by the net balance between accrual and loss of muscle proteins (e.g. mitochondrial, sarcoplasmic and myofibrillar proteins), termed net protein balance (NPB). NPB is defined as the difference between rates of muscle protein synthesis (MPS) and muscle protein breakdown (MPB), which must be equal over a diurnal cycle for muscle mass to remain constant. However, when MPS exceeds MPB, NPB is positive which, over time, results in a gain in muscle mass (hypertrophy). Conversely, NPB is negative when MPB is greater than MPS and results in loss of muscle (atrophy) (Biolo *et al.* 1997; Eley and Tisdale, 2007; Phillips *et al.* 1997). Of MPS and MPB, it appears that the rate of MPS, which is consistent between muscles in humans (Mittendorfer *et al.* 2005), serves as the primary determinant of changes in NPB throughout a circadian rhythm (Phillips *et*

al. 2005). Feeding-induced increases in MPS / decreases in MPB are responsible for maintaining the relatively stable levels of body protein, and thus skeletal muscle mass, in healthy young adults eating regular meals and not performing exercise (Phillips *et al.* 2005; Tomé and Bos, 2000). This is due to the undulating diurnal pattern of NPB created by repeated postprandial periods of positive NPB and subsequent postabsorptive declines into negative NPB, illustrated in Figure 1.1 (Paddon-Jones *et al.* 2005; Phillips *et al.* 2005).

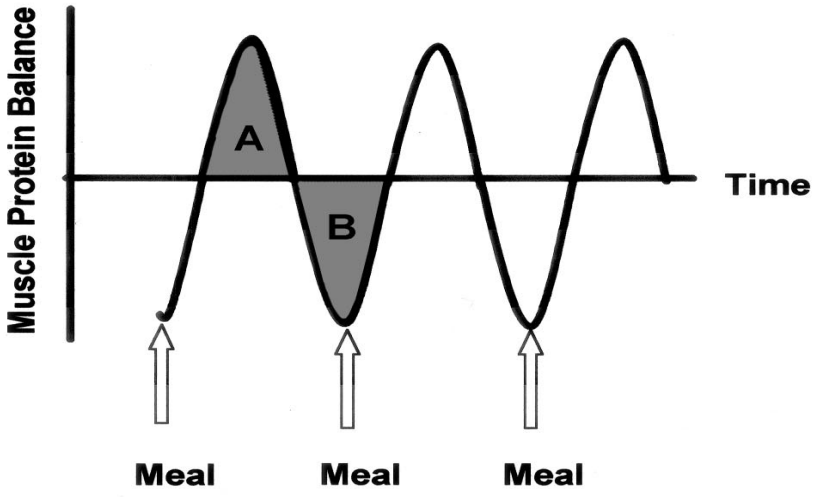

Fig. 1.1. Schematic representation of normal fed-state gains and fasted-state losses in skeletal muscle protein balance (synthesis minus breakdown). Note that the area under the curve in the fed state (A) would be equivalent to the fasted loss area under the curve (B); hence, skeletal muscle mass is maintained by normal feeding. Adapted from Phillips (2004).

All organisms appear to possess the ability to sense nutrient availability on a moment-to-moment basis, and to adjust flux via metabolic and signalling pathways accordingly (Gulati and Thomas, 2007). Thus, it has long been recognised that provision of a mixed meal switches NPB from negative to positive (Bennet *et al.* 1989; Rennie *et al.* 1982). This effect results primarily from an approximate 2 – 3 fold increase of basal rates of MPS (Bohé *et al.* 2001) and, to a lesser extent, ~50% fall in MPB (Gelfand *et al.* 1990; Rasmussen *et al.* 2000; Rennie *et al.* 2002). In

particular, the actions of increased postprandial concentrations of amino acids (AA) and insulin have been implicated as important in regulating rates of muscle protein turnover in humans (Boirie *et al.* 2001). However, despite the importance of feeding-induced changes in protein metabolism for the maintenance of muscle mass, and a wealth of research conducted in the area, the precise responses of MPS to food and the molecular signalling mechanisms regulating this response remain poorly understood.

1.2.2. Regulation of protein metabolism by amino acids

Since amino acids are required for protein biosynthesis, it is logical to propose that amino acids should control components involved in the regulation of protein synthesis (mRNA translation) (Proud, 2007). Indeed, the ability of AA alone to increase AA transport (Lundholm *et al.* 1987) and subsequently MPS is well understood (Fujita *et al.* 2007; Bohé *et al.* 2001, 2003; Rennie *et al.* 1982; Paddon-Jones *et al.* 2004). The effect of mixed meals on MPS appears almost entirely due to increased AA availability, since either orally or intravenously administered AA alone are able to stimulate MPS to ~80% of the level observed with mixed meal ingestion (Bennet *et al.* 1989, 1990; Cheng *et al.* 1985; Fryburg *et al.* 1995; Smith *et al.* 1992), with little or no effect on MPB (Adegoke *et al.* 2009; Rennie, 2007). Whilst all twenty AA are required to synthesise proteins, only the essential amino acids (EAA; i.e. those not capable of being synthesised *in vivo*) are required to stimulate MPS (Rasmussen *et al.* 2000; Smith *et al.* 1998), since administration of non-essential amino acids alone (NEAA; i.e. those synthesised de novo) does not increase MPS (Rennie, 2007; Tipton *et al.* 1999b). In fact, a large flooding dose of

individual EAA, in particular leucine (one of the branched-chain amino acids; BCAA), is capable of stimulating MPS (Smith *et al.* 1992; Escobar *et al.* 2006), though this effect is short-lived and presumably dependent on the availability of other EAA released into the plasma by entry from the gut after feeding or by proteolysis in other tissues (Rennie, 2007).

1.2.2.1 Latency, duration, and dose response relationship between amino acid availability and MPS

To date only one study has reported the temporal rise and subsequent decline of MPS after provision of AA (Bohé *et al.* 2001). It was shown that following the onset of continuous intravenous AA infusion (to plasma concentrations ~70% above postabsorptive values) there exists a latency in stimulation of MPS, whereby a lag period of ~30 min is followed by a steep rise in MPS, peaking at ~2 h and declining rapidly thereafter to basal values, despite continued AA availability (Bohé *et al.* 2001); the fate of excess AA not incorporated to protein being ureagenesis (Rennie *et al.* 2002) and recycling of carbon into intermediates of the citric acid cycle (e.g. α-ketoglutarate, succinyl-CoA, oxaloacetate) and glycolysis / gluconeogensis (pyruvate, acetyl-CoA). This phenomenon has been termed the 'muscle full' phenomenon, by which MPS returns to basal values despite continued AA availability and may explain the inability to build muscle mass simply by eating more protein (Millward, 1995; Rennie, 2007). This pattern of MPS is also similarly observed post-feeding in different sub-cellular protein fractions, including sarcoplasmic, myofibrillar and mitochondrial proteins (Bates and Millward, 1983; Moore *et al.* 2009; Rennie *et al.* 2002).

The protein synthetic response to increased amino acid availability also exhibits a dose response relationship. Firstly, an almost linear rise in MPS occurs with increased plasma EAA concentration within the normal physiologic range (Bohé *et al.* 2001). Secondly, in accordance with the 'muscle full' phenomenon, maximal stimulation of MPS is achieved with relatively small doses of EAA. Cuthberston *et al.* (2005) showed that both myofibrillar and sarcoplasmic protein synthesis were increased in a dose-dependent manner from 2.5-10 g EAA, with no further increase elicited with 20 g EAA. This is supported by the findings of Moore *et al.* (2009) utilising whole protein, who reported a similar dose-dependent increase in MPS with 5, 10 and 20 g egg protein (approximately 2.5, 5 and 10 g EAA, respectively), which was not further augmented by 40 g protein (~20 g EAA). Together these studies indicate that MPS is maximally stimulated by the consumption of just 10 g EAA, where leucine acts as the primary stimulator of the system (Greiwe *et al.* 2001), with excess protein intake being catabolised to urea (Rennie *et al.* 2002). Such a narrow range of concentrations over which amino acids have their effect on protein synthesis and breakdown is congruent with known physiologic responses to feeding, where the diurnal variation in amino acid concentration in the blood is only ~ ±50 % of the daily mean (Bergstrom *et al.* 1990), and may suggest a desensitisation of the signalling mechanisms that detect and transmit AA availability with protein ingestions above the concentration required to maximally stimulate MPS (Rennie *et al.* 2006).

1.2.2.2. Amino acids and control of translational signalling

The acute synthesis of muscle proteins is primarily regulated at the level of messenger RNA translation. Since translation consumes AA during peptide elongation, it makes physiological sense for AA availability to regulate signalling pathways controlling this process (Proud, 2007). As such, accompanying the AA-stimulated increases in MPS are alterations in the phosphorylation, and presumably activity, of signalling molecules involved in assembly of the translational apparatus, which results in translation of pre-existing mRNA (Rennie, 2005). AA regulate mRNA translation through multiple mechanisms, though of particular importance is the mammalian target of rapamycin (mTOR) pathway. Due to its crucial role in nutrient sensing, and in regulating cell growth, the cell cycle and gene expression, the mTOR signalling cascade has been the focus of intense research over recent years. Consequently, much progress has been made in delineating the complex network of events surrounding mTOR signalling in response to feeding.

It has been known for over 10 years that AA serve as positive regulators of mTOR signalling. Various cell lines of mammalian origin demonstrate that when transferred from medium containing physiological concentrations of AA to a medium devoid of AA, signalling through the mTOR pathway is rapidly (within minutes) suppressed (Kimball and Jefferson, 2002; Proud, 2002). This, at least in part, occurs through accumulation of uncharged tRNA activating the general control nonrepressed 2 (GCN2) pathway, which subsequently phosphorylates and inhibits the eukaryotic initiation factor 2 α (eIF2α) to prevent translation initiation (Wek *et al.* 1995). Conversely, addition of a mixture of amino acids (Wang *et al.* 2005), or leucine

alone (Peyrollier *et al.* 2000) stimulates components of the mTOR pathway; an increase which is prevented by treatment with the mTOR inhibitor rapamycin (Peyrollier *et al.* 2000). The mechanism through which leucine stimulates mTOR signalling is incompletely defined, though likely involves promotion of mTOR binding with one of its regulatory proteins, regulatory associated protein with TOR (raptor), in order to form a nutrient-sensitive complex (Kimball and Jefferson, 2006). Activation of mTOR by nutrients have also been translated to humans; a 2 h infusion of leucine (Greiwe *et al.* 2001), and a 6 h infusion of branched-chain AA (Liu *et al.* 2001) or a mixture of EAA and NEAA (Liu *et al.* 2002) increases the phosphorylation of mTOR. More recently, measures of MPS in conjunction with assessment of activity of the mTOR signalling cascade have been made, demonstrating that oral ingestion of 10 g EAA (Cuthbertson *et al.* 2005) and approximately 19.5 g of leucine-enriched EAA mixture plus 27 g carbohydrate (Fujita *et al.* 2007) augmented both MPS and signalling through mTOR.

The mechanisms by which AA stimulate mTOR activation remain elusive. One potential candidate includes the conversion of ras-homolog enriched in brain (Rheb) to its active GTP-bound form via AA modulated activation of the Rheb guanosine-nucleotide exchange factor, translationally controlled tumour protein (TCTP) (Corradetti and Guan, 2006; Kimball and Jefferson, 2006; Proud, 2007). AA availability is also sensed by a novel class 3 phosphoinositide-3 kinase (PI3K), which enhances phosphorylation of mTOR by an unknown mechanism (Byfield *et al.* 2005; Nobokuni *et al.* 2005; Peyrollier *et al.* 2000). Other possible mechanisms include the vacuolar protein sorting 34 (Vps34) and mitogen activated protein kinase kinase kinase kinase 3 (MAP4K3), though direct evidence for a role of these

molecular events in underlying the AA induced mTOR activation is yet to be reported (Gulati and Thomas, 2007; Proud, 2007).

When phosphorylated, mTOR controls translation initiation (Gingras *et al.* 1999, 2001), translation elongation (Kimball, 2002), and ribosomal biogenesis (Avruch *et al.* 2001). The first mTOR target protein to be identified was the 70-kDa ribosomal protein S6 kinase (p70S6K) which, when phosphorylated, promotes upregulation of translation initiation via phosphorylation of multiple substrates (e.g. eukaryotic initiation factor 4B; eIF4B, and eukaryotic initiation factor 4G; eIF4G) and also by disassociating with the eukaryotic initiation factor 3 (eIF3), which allows assembly of the preinitiation complex and its subsequent tethering to the 5' cap structure of mRNA (Peterson and Sabatini, 2005). Phosphorylation of p70S6K also increases the translation elongation phase of protein synthesis by regulating the activity of elongation factors, such as the eukaryotic elongation factor 2 (eEF2) (Balage *et al.* 2001). Thus, p70S6K activation likely leads to increased protein synthesis (Avruch *et al.* 2001; Mayer and Grummt, 2006; Proud, 2004a). Indeed, increased activity of the mTOR/p70S6K pathway has been shown to correlate with increased muscle growth (Atherton *et al.* 2005; Baar and Esser, 1999; Bodine *et al.* 2001; Bolster *et al.* 2003). Another well characterised target of mTOR is the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1); a translational repressor protein whose phosphorylation and inhibition is a key step in stimulating translation initiation (Gingras *et al.* 1999; Lawrence and Abraham, 1997; Wang and Proud, 2006). 4E-BP1 binds to and sequesters eIF4E, preventing eIF4E from engaging with the scaffold protein eIF4G, since 4E-BP1 and eIF4G bind to eIF4E at overlapping sites (Gingras *et al.* 1999). Dephosphorylated 4E-BP1 thus prevents formation of the

eIF4F complex (comprised of eIF4E, eIF4G and eIF4A), which mediates mRNA binding to the 40S ribosomal subunit and, therefore, cap-dependent mRNA translation initiation (Beugnet *et al.* 2003; Kimball, 2002; Wang *et al.* 2005). However, phosphorylated 4E-BP1 allows eIF4F formation and sequential increases in translation initiation (Proud, 2007). A further point of translational control via mTOR is of translation elongation through the eukaryotic elongation factor 2 (eEF2). Phosphorylation of eEF2 by its upstream kinase, eEF2 kinase, blocks its ability to bind ribosomes, thus inhibiting peptide chain elongation (Browne and Proud, 2002; Carlberg *et al.* 1990). However, activated mTOR phosphorylates and inhibits eEF2 kinase, which relieves inhibition of eEF2 to allow continuation of its physiological function (Browne and Proud, 2002; Redpath *et al.* 1996). P70S6K also phosphorylates and inhibits eEF2 kinase, underscoring the importance of the p70S6K protein kinase in regulation of mRNA translation (Proud, 2007). A summary of the signalling pathways thought to control protein synthesis, and the response of these to feeding, is provided in Figure 1.2.

Congruent with feeding-induced increases in mTOR activity, both *in vitro* and *in vivo*, p70S6K, 4E-BP1 and eEF2 are positively regulated by amino acids in cell culture (Wang *et al.* 2005), in rats (Anthony *et al.* 2000a; Kimball *et al.* 2004; Yoshizawa *et al.* 1998) and in humans following EAA administration (Cuthbertson *et al.* 2005; Fujita *et al.* 2007; Greiwe *et al.* 2001).

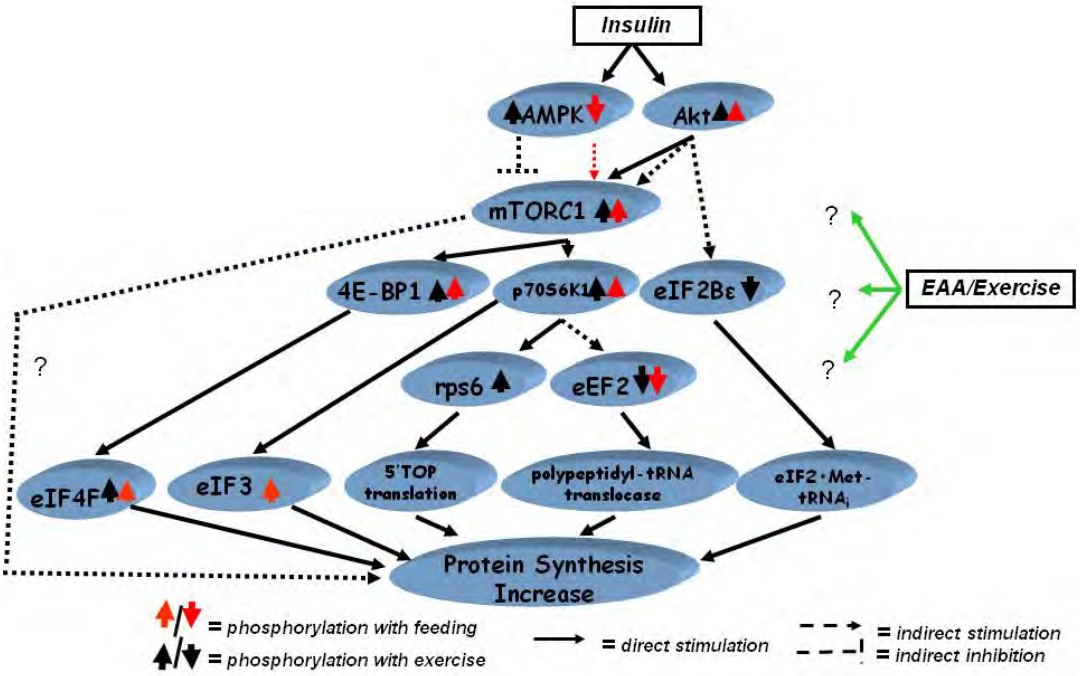

Figure 1.2. Translational signalling pathways involved in the regulation of muscle protein synthesis. The response of these pathways to both essential amino acid provision (EAA; red arrows) and acute resistance exercise (Exercise; black arrows) is also included. Adapted from Kumar *et al.* (2009a).

In addition to translational regulation through mTOR, AA appear to upregulate translation initiation via mTOR independent mechanisms. In particular, the α-subunit of the eukaryotic initiation factor 2 (eIF2α) has been implicated as important in the AA mediated increase in protein synthesis. Phosphorylated eIF2α phosphorylates and inhibits the guanosine-nucleotide-exchange factor, eIF2B, thereby preventing binding of the initiator methionyl-tRNA to the 40S ribosomal subunit and, subsequently inhibits recognition of the start codon (Herbert, 2007; Kimball and Jefferson, 2005). Conversely, reduced eIF2α phosphorylation activates eIF2B by dephosphorylation, leading to increased translation initiation (Cuthbertson *et al.* 2005; Kimball and Jefferson, 2004). In contrast to mTOR-mediated upregulation of translational signalling, however, evidence of the role AA play in increasing translation through eIF2α/eIF2B remain equivocal. In rat L6 myotubes, increased

AA availability was shown to reduce eIF2α phosphorylation (Kimball *et al.* 1998). However, studies in rat skeletal muscle (Anthony *et al.* 2000a; Crozier *et al.* 2005) and C2C12 myotubes (Atherton *et al.* 2009) contradict this, showing no alteration in eIF2α or eIF2B activity following leucine administration, despite significant increases in protein synthesis (Anthony *et al.* 2000a; Crozier *et al.* 2005). The role of this pathway following AA intake is further confounded by human investigations demonstrating that ingestion of 10g EAA significantly reduced eIF2B phosphorylation, suggesting an increase in translation (Cuthbertson *et al.* 2005), though Drummond *et al.* (2010) found no change in eIF2α phosphorylation with the same supplementation. Further investigation into the role this mechanisms plays in feeding-induced increases in protein synthesis are thus required.

1.2.3. Role of insulin in the regulation of muscle protein turnover

The role of insulin in regulating MPS has been a topic of some controversy over recent years. Traditionally insulin has been thought to serve as an anabolic stimulus independently of amino acid availability. For example, intravenous infusion of insulin to supra-physiological concentrations stimulated MPS in young, food-deprived rats (Garlick *et al.* 1983) and neonatal pigs (Suryawan *et al.* 2004). Furthermore, rats infused with mixed AA failed to enhance MPS unless insulin levels were concomitantly raised via glucose infusion (Preedy and Garlick, 1986). However, interpretation of these early studies may be limited due to the use of immature or growing animals in which the influence of development may alter insulin-mediated responses (Rennie, 2007). Nevertheless, recent studies in cell culture and *in vivo* have reported that insulin activates elements of the PKB / mTOR

pathway which regulate MPS; provision of insulin activates 4E-BP1, p70S6K and eEF2 via activation of the phosphatidylinositol-3 kinase (PI-3K) / Akt signal transduction pathway (Avruch *et al.* 2001; Browne and Proud, 2002; Gingras *et al.* 2001; Martin and Blenis, 2002; Redpath *et al.* 1996; Schmelzle and Hall, 2000; Suryawan *et al.* 2007, 2008). Similarly in humans, a hyperinsulinaemic infusion for six hours ($20\mu U.kg^{-1}.h^{-1}$) causes increased phosphorylation of p70S6K (Liu *et al.* 2001), though measures of protein synthesis were not obtained.

In humans, the majority of data support the notion that insulin is not stimulatory for MPS. Firstly, increasing AA to postprandial concentrations increases MPS to similar values as those observed following a mixed meal, despite only modest rises in plasma insulin availability, from 7-10 μU/ml (Bennet *et al.* 1989). This was followed demonstrations that insulin is incapable of stimulating MPS unless sufficient availability of AA are present (Bell *et al.* 2005; Bennet *et al.* 1990; Biolo *et al.* 1995a; Fujita *et al.* 2006; Gelfand *et al.* 1987) and only at the highest insulin concentrations (100 μU/ml) (Rennie *et al.* 2006; Suryawan *et al.* 2004). Similarly, physiologic increases in plasma insulin via carbohydrate feeding do not independently stimulate MPS (Anthony *et al.* 2000a). Furthermore, clamping insulin at postabsorptive concentrations (<5 μU/ml), thereby preventing the AA-regulated transient increase in plasma insulin, does not prevent increases in MPS (Bohé *et al.* 2003; Cuthbertson *et al.* 2005), though this may attenuate the response (Anthony *et al.* 2002; Kimball and Jefferson, 2006). Finally, although increasing postprandial insulin availability to levels >10 μU/ml enhances the activity of key components of the translational signalling cascade beyond that of AA alone, for example increased mTOR and 4EBP1, and decreased eEF2 phosphorylation (Greenhaff *et al.* 2008),

there is no concomitant increase in MPS (Fujita *et al.* 2006; Greenhaff *et al.* 2008; Rasmussen *et al.* 2006; Rennie *et al.* 2006). It, therefore, appears that only postabsorptive concentrations of insulin are necessary for stimulation of MPS and that the role of insulin is permissive rather than modulatory (Kimball and Jefferson, 2006; Rennie *et al.* 2002).

Rather than increasing MPS, the principle action of insulin may be to inhibit MPB. Basal rates of MPB are reduced ~50% with modest increases in plasma insulin concentrations (Louard *et al.* 1992). Indeed, raising systemic insulin levels to 30 mU/L (Greenhaff *et al.* 2008), or just 15 mU/L (Wilkes *et al.* 2009), approximately halves the rate of basal protein breakdown, with no further effect at higher insulin concentrations (Greenhaff *et al.* 2008). This effect is likely to be due to insulin-mediated activation of the phosphatidylinositol 3 kinase (PI3K) – PKB pathway, since upregulation of PI3K-PKB via insulin supresses expression of the E3 ubiquitin ligases muscle atrophy F-box (MAFbx/atrogin-1) and muscle ring-finger 1 (MuRF1) by inhibiting Foxo (Forkhead box subfamily O) transcription factors, both *in vitro* (Latres *et al.* 2005; Lee *et al.* 2004; Sacheck *et al.* 2004) and *in vivo* (Greenhaff *et al.* 2008), therein lowering proteasomal protein degradation (Sandri *et al.* 2004). The effect of reduced proteolysis by insulin is to increase NPB and thus increase muscle growth (Sacheck *et al.* 2004).

As such, it may be that the common link between MPS and MPB is the concentration of circulating AA (Rennie, 2005), which stimulate MPS directly, and inhibit MPB through an AA-mediated transient rise in plasma insulin levels (Anthony *et al.* 2002; Crozier *et al.* 2005; Escobar *et al.* 2005). AA intake, therefore,

augments MPS and decreases MPB in order to maximise the anabolic effect of feeding.

1.3. Regulation of muscle by physical activity

1.3.1. Effects of resistance exercise on skeletal muscle

Resistance exercise (RE) is defined as the performance of dynamic or isometric muscular contractions against external resistance of varying intensities (Phillips, 2007). Guidelines published by the American College of Sports Medicine (ACSM) recommend that in order to induce gains in muscle mass and strength, resistance training should consist of one-to-three sets of 8-12 repetitions over 8-10 different exercises that cover all the major muscle groups, performed 2-3 $d \cdot wk^{-1}$ (ACSM, 1998). However, the necessity of performing multiple sets for inducing gains in muscle mass and function has come under some criticism. Carpinelli (2002) showed by meta-analysis of prior published resistance training studies that little evidence exists to support the use of multiple sets, concluding that a single set may be as beneficial. Later studies confirmed this, reporting a 34% increase in strength following 12 wks of resistance training consisting of one set of six repetitions, over six muscle groups (Winett *et al.* 2003). Nevertheless, the majority of studies investigating the effects of chronic RE training have utilised multiple (i.e. three) sets, performed 3 – 5 $d \cdot wk^{-1}$ which, after 12 wk stimulates muscle hypertrophy as shown by increased quadriceps cross-sectional area (CSA) by ~8%, with concomitant improvements in one repetition maximum (1-RM) of ~36% (Green *et al.* 1999; Holm *et al.* 2008; Moore *et al.* 2007; Petrella *et al.* 2008). In fact, as little as 3 wk

resistance training can increase quadriceps CSA by 5.2% and 1-RM by 39%, demonstrating the rapid plasticity of skeletal muscle to RE (Seynnes *et al.* 2007). There does exist, however, large variability in muscle adaptations following resistance training programs; Hubal *et al.* (2005) reported a wide range of changes in muscle size (from -2 to +59% cross-sectional area of the biceps brachii muscle) and strength (0 to +250% for 1 repetition maximum) following 12 weeks resistance training. As such, it may be expected that inter-subject variations in the response of muscle to acute resistance exercise occur at the molecular level (i.e. changes in protein turnover and signalling). Indeed, Kumar et al. (2009a) reported wide variations in the responses of anabolic signalling proteins (e.g. mTOR, p70S6K, 4EBP1, eIF4E) to acute resistance exercise.

The required intensity for adaptations in muscle to occur as a result of resistance training is generally accepted to be in the region of 60 – 70% of one repetition maximum 1RM (Campos *et al.* 2002; Phillips, 2007, 2009). 12 wk resistance training at 60% 1RM increased lean leg mass 6%, quadriceps CSA 9%, and 1RM 13%, whereas training at 15.5% 1RM only increased CSA 3%, and 1RM was unchanged (Dreyer et al. 2006; Holm *et al.* 2008; Verdijk *et al.* 2009). Moreover, rises in MPS are only seen when the load lifted reaches 60% 1RM but does not further increase at 90% 1RM (Kumar *et al.* 2009b).

The primary mechanism through which hypertrophy is achieved is via repeated increases in MPS following each acute bout of RE in the fed state, as illustrated in Figure 1.3 (Mayhew *et al.* 2009).

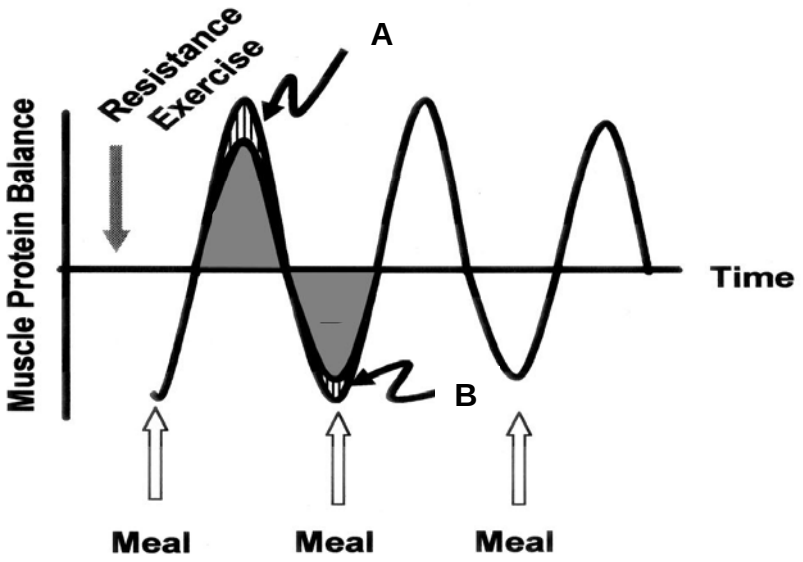

Fig. 1.3. Fed-state gains and fasted-state losses in skeletal muscle protein balance with performance of resistance exercise. Fed state gains are enhanced by an amount equivalent to the stimulation of protein synthesis brought about by exercise (A). Additionally, fasted-state losses appear to be less (B), due to persistent stimulation of protein synthesis in the fasted state.

An acute bout of resistance-type exercise, even in the postabsorptive state, potently stimulates MPS between 200 – 300% above basal values (Biolo *et al.* 1995b; Chesley *et al.* 1992; Kumar *et al.* 2009; MacDougall *et al.* 1995; Phillips *et al.* 1997, 1999; Yarasheski *et al.* 1993). This stimulation is detectable within 1-2 h post-exercise and may last for up to 72 h (Dreyer *et al.* 2006; Miller *et al.* 2005), though recent work shows MPS returns to baseline values within 4 h post-RE even in the fed state (Kumar *et al.* 20091). Whilst the precise mechanism(s) regulating the RE-mediated elevations in MPS remain unclear, a wealth of studies suggest that, as with feeding, activation of the Akt-mTOR-p70S6K signal transduction pathway is integral to the response (Dreyer *et al.* 2006; Drummond *et al.* 2009; Glover *et al.* 2008; Kumar *et al.* 2009a; Tannerstedt *et al.* 2009). Increased phosphorylation of these molecules has been associated with the rise in MPS post-exercise, and at 24 h and 72 h post-exercise (Deldicque *et al.* 2008). This has led to postulation that the extent of activation of this pathway may be predictive of contraction-mediated

muscle growth in rats (Baar and Esser, 1999; Dreyer *et al.* 2006; Drummond *et al.* 2008) and in humans (Kumar *et al.* 2009b; Phillips, 2009; Terzis *et al.* 2008). The responses of components of translational signalling to resistance exercise are presented in Figure 1.2.

In addition to increases in MPS with RE, there occurs a concurrent rise in MPB to allow remodelling and to provide some of the free amino acids (in addition to those derived from the diet) required to fuel increased MPS (Burd *et al.* 2009; Kumar *et al.* 2009a). It is well established that post-exercise MPB increases, as indicated by arteriovenous tracer dilution measure of fractional breakdown rates (Biolo *et al.* 199b, 1997; Tipton *et al.* 1999a). However, the increase in mixed-muscle protein breakdown is of a smaller magnitude than the increase in MPS; between 30 – 50% by 3 h (Biolo *et al.* 1995b; Phillips *et al.* 1997, 1999) and is more short-lived than the elevated MPS (Phillips *et al.* 1997). The resultant effect is an increase in NPB in the postabsorptive state, though NPB remains negative.

The proteolytic signals controlling increases in post-exercise MPB in human muscle remain poorly characterised, but likely involve calpains, caspases, metalloproteinases, and ubiquitin-proteasomal degradation. In humans, expression of MAFbx and MuRF1 messenger RNA (mRNA) is upregulated post-exercise (Louis *et al.* 2007; Mascher *et al.* 2008; Raue *et al.* 2007). However, other markers of protein degradation, such as mRNA levels of the transcription factor forkhead box-O 3A (Foxo3A) are unaffected 4 h following an acute resistance exercise bout (Raue *et al.* 2007). Myostatin, a member of the transforming growth factor-β superfamily and inhibitor of skeletal muscle growth, is downregulated ~45% following acute

resistance exercise (Louis *et al.* 2007; Mascher *et al.* 2008), consistent with a shift in muscle towards anabolism and remodelling. Furthermore, mRNA expression of the C2 proteasome subunit has been shown to be upregulated in response to acute resistance exercise in rat hindlimb (Dupont-Versteegden *et al.* 2006). Thus, the C2 proteasome subunit may be important in regulating muscle remodelling in the post-exercise period, though this remains untested in humans.

Finally, such acute investigations of resistance exercise-mediated changes in protein synthesis and translational signalling provide useful information for estimating the efficacy of anabolic agents (Tipton and Ferrando, 2008). Indeed, some evidence exists that in carefully controlled studies, acute post-exercise changes in MPS and MPB are, at least qualitatively, predictive of chronic adaptations to training (Hartman *et al.* 2007; Wilkinson *et al.* 2007). As such, single studies are often used to predict long-term responses to not only exercise training, but also a variety of other stimuli which affect muscle, such as feeding and altering environmental interventions (Burd *et al.* 2009).

1.3.2. Effects of dynamic eccentric exercise on skeletal muscle

Unfamiliar, particularly eccentric, muscular contractions commonly result in muscle discomfort for up to 24–96 h after exercise (Cheung *et al.* 2003; Morgan and Allen 1999). This phenomenon, termed delayed-onset muscle soreness (DOMS), peaks 48 - 72 h after exercise and is characterized by tenderness to palpation and movement in the damaged muscle (Armstrong 1984; Jones *et al.* 1986). There are also declines in

flexibility (Gulick *et al.* 1996), force production (-38%; Prasartwuth *et al.* 2005), and power-generation (-16%; Twist and Eston 2005).

Despite the high incidence of DOMS, the mechanisms underlying this physiologic state remain uncertain (Cheung *et al.* 2003). Since the early 20th century (Hough, 1902) the debilitating symptoms associated with DOMS has been the focus of much research (Abraham, 1977; Cobb *et al.* 1975; Friden and Sjostrom, 1981; Komi and Buskirk, 1972; Waltrous *et al.* 1981). As a result, a variety of theories on the causes of DOMS have been proposed. For example, lactic acid accumulation as a product of anaerobic glycolysis was originally thought to induce muscle soreness following intense exercise (Assmussen, 1956), though this has since been discredited as causative (Francis, 1983; Schwane *et al.* 1981; Waltrous *et al.* 1981). Muscle spasms resulting from ischemia-induced stimulation of pain nerve endings have also been purported to underlie muscle soreness (deVries, 1961; 1966), though this theory has also lost favour due to a lack of reproducibility (Abraham, 1977; 1979). Inflammation-induced muscle soreness has gained credence over recent years as contributing to sensations of muscle soreness post-eccentric exercise. Magnetic resonance imaging analysis shows significant muscle swelling 1 d after eccentric exercise (Nosaka and Clarkson, 1996), and plasma inflammatory markers such as interleukin-6 (IL-6), and C-reactive protein are significantly elevated and correlate with soreness during the DOMS period, and thus may at least partially account for the sensations of pain associated with DOMS (Clarkson *et al.* 1992; Crameri *et al.* 2004a; Malm *et al.* 2004; Miles and Clarkson, 1994; Nosaka *et al.* 2006). However, muscle inflammation does not appear to explain the DOMS-related loss of muscle function and damage, since increases in inflammatory markers (IL-6) do not

correlate with the rise in damage markers (MHC fragments in plasma) or reduced muscle torque production (MacIntyre *et al.* 2001; Miles *et al.* 2008). Other proposed mechanisms include cytoskeletal and contractile protein damage, and damage to connective tissue (discussed below).

Given the breadth of hypothesised mechanisms regulating the onset of DOMS, there is now a general consensus that no single hypothesis can explain the phenomenon's onset, and an integration of two or more theories likely combine to explain DOMS (Cheung *et al.* 2003). It has long been proposed that a unique sequence of events may explain the occurrence of DOMS which, with advancing knowledge, has gained general acceptance over recent years (Armstrong, 1984; Cheung *et al.* 2003; Peake *et al.* 2005). The current understanding is that following a single bout of dynamic, eccentric exercise, mechanical disruption of sarcomeres and surrounding connective tissue, and subsequent increases in inflammation of these tissues, results in loss of contractile function and concurrent sensations of muscle soreness. Additionally, this sarcomeric damage results in Ca^{2+} influx into the cell, causing increased activity of Ca^{2+}-activated proteases (the calpains) and phospholipases, and production of reactive oxygen species, the generally accepted active agents involved in degradation of the myofibre contractile apparatus and sarcolemma (Allen *et al.* 2005; Ebbeling and Clarkson, 1989). Neutrophil recruitment to muscles further contributes to muscle degradation (Belcastro *et al.* 1998; Raj *et al.* 1998), and exacerbates the contractile deficits of DOMS. However, the precise sequence of events remains to be determined.

Clearly, a key aspect of events involved in DOMS is structural damage to the contractile proteins. The possibility of 'torn tissue' following lengthening contractions has long been hypothesised to explain the loss-of-function phenotype associated with DOMS (Hough, 1902). This remained largely speculative until the late 1970's, when histological evidence of degenerating muscle fibres following exhaustive exercise was reported (Vihko *et al.* 1978). Subsequently, Friden *et al.* (1981) demonstrated an eccentric exercise-induced broadening and even total disruption of the Z-line along with widespread disruption of sarcomere architecture. Such irreversible myofibrillar damage of the Z-line has since been confirmed by numerous authors, along with loss of proteins of the cytoskeleton (Friden and Lieber, 1992; Morgan, 1990; Proske and Allen, 2005; Sorichter *et al.* 1999) after, for example, 210 maximal isokinetic contractions of the quadriceps (Crameri *et al.* 2007). Thus, there is little doubt about the occurrence of muscle damage following eccentric exercise (Mackey *et al.* 2008), although this is not always observed (Crameri *et al.* 2004a; Yu *et al.* 2002).

While this structural damage to the cytoskeletal and contractile proteins of muscle cells would likely result in reductions in functional capacity of the whole muscle, it may not be expected to induce significant sensations of muscle soreness to the individual (Kjaer, 2004) since there are no pain receptors intramuscularly. This conclusion is further supported by observations that the level of muscle soreness experienced does not accurately reflect the magnitude of indirect indices of eccentrically-induced muscular damage (Nosaka *et al.* 2002). Thus, other mechanisms may account for the perceptions of soreness following eccentric-exercise. A potential candidate is damage to intramuscular connective tissue (the

extracellular matrix, ECM). During eccentric-exercise, shearing forces are placed on the ECM in order to relieve some of the stress placed on cytoskeletal structures, with the ECM distributing up to 70% of intramuscular strain during contraction (Crameri e*t al.* 2007; Kjaer, 2004). This force results in damage to collagen structures of the ECM, and has been appreciated for many years following observations of increased connective tissue breakdown (e.g. hydroxyproline in urine) (Abraham, 1977; Brown *et al.* 1997). Recent work has provided support for these observations, demonstrating ECM disruption following eccentric-exercise, as indicated by increased expression of the ECM adhesion-modulating molecule tenascin C (Crameri e*t al.* 2007; Kjaer, 2004) and increases in collagen-localised inflammation (Crameri *et al.* 2004b). Though measures of perceived soreness were not taken in these studies, it is this inflammation of the ECM, more so than of cytoskeletal / contractile protein damage, that is thought to account for sensations of muscle soreness associated with DOMS (Kjaer, 2004). Thus, exercise-induced damage to both the ECM and myofibrils may account for differential induction of muscle soreness and functional declines, respectively; acting synergistically to cause the principle symptoms of DOMS.

1.4. Regulation of muscle by low atmospheric oxygen concentrations (hypoxia)

A constant adequate supply of oxygen (O_2) is essential to the survival of all vertebrates (Essop, 2007). O_2 acts as an environmental / developmental signal that regulates growth, differentiation processes, and cellular energy homeostasis (Haddad, 2004). Oxidative phosphorylation also generates about 90% of total cellular ATP (De Palma *et al.* 2007). Thus, sufficient O_2 supply is essential to provide the ATP required for all anabolic processes within the body. O_2, therefore,

plays a central role in nearly all higher life processes and, as such, its availability regulates interdependent cell metabolism, growth, and survival and is important in maintaining cell and tissue integrity (Brahimi-Horn *et al,* 2007; Cai *et al,* 2006; Douglas and Haddad, 2008; Liu *et al,* 2008; Lum *et al,* 2007). However, despite the physiologic importance of O_2 availability, the protein synthetic response of human skeletal muscle to decreased O_2 supply, and the intracellular mechanisms involved in these responses, is incompletely understood.

1.4.1. Hypoxia, hypoxaemia and tissue hypoxia

The term hypoxia describes conditions of decreased O_2 availability and results from an imbalance between O_2 supply and demand (Essop, 2007). Ultimately, it is the intracellular hypoxia, and associated O_2 sensing mechanisms, within specific tissues that stimulate metabolic and/or morphological adaptations. Reduced tissue O_2 supply precedes this and is causative of tissue hypoxia, and can result from one, or a combination of, three scenarios: 1) reduced ability of blood to carry O_2, as in anaemia; 2) reductions in tissue blood flow, such as that which occurs with ischaemia / vascular dysfunction or; 3) low partial pressure of O_2 in arterial blood which occurs in, for example, patients suffering pulmonary disease or healthy individuals during high altitude exposure (Tansey, 2008; West, 2008). Of these, the latter two scenarios present non-pathological, physiologically relevant conditions for which to study the *in vivo* effects of tissue hypoxia.

Acute (12 min) blood flow occlusion of the radial and ulnar arteries reduces haemoglobin O_2 saturation, which plateau after 6 min (Hamaoka *et al.* 2000) and

leads to significant forearm muscle tissue deoxygenation at a rate of -37.4 %.min^{-1} by stopping tissue O_2 supply (Pareznik *et al.* 2006). This is consistent with studies in pathological ischaemia that also report tissue (cardiac and skeletal muscle) deoxygenation (Pareznik *et al.* 2006).

While blood flow occlusion causes significant tissue hypoxia, it is not suitable for studying the effect of prolonged hypoxic exposure upon muscle metabolism. A complete loss of blood supply causes rupturing of the sarcolemma, loss of contractile filaments, plasma exudation, oedema and infiltration of leukocytes in skeletal muscle (Scholz *et al.* 2003). This morphological damage begins 3 h after occlusion and is nearly complete following 6 h (Blaisdell, 2002). Furthermore, while anaemia can be simulated acutely via blood volume reduction in healthy humans (Gonzalez-Alonso *et al.* 2006), this method is not suitable for prolonged investigation of the effects of hypoxia. As such, exposure to sub-atmospheric O_2 concentrations is an alternative to ischaemia / anaemia for studying the effects of low O_2 supply to allow insight into the pathophysiology of hypoxia (Tansey, 2008). Additionally, simulated hypoxia (e.g. normobaric / hypobaric hypoxia) is a valuable model for understanding human adaptations to high-altitude environments.

Using near infrared spectroscopy it has been demonstrated that a fall in the partial pressure of peripheral blood O_2 saturation (SpO_2) to ~84%, from normoxic values of ~ 98%, is sufficient to mimic the effects of moderate altitude exposure (Breen *et al.* 2008; DeLorey *et al.* 2004; Heubert *et al.* 2005). Acute reductions in SpO_2 become prominent from 2000 m (~16% O_2; partial pressure of O_2 (PO_2) = 121 mmHg) altitude (Bartsch and Saltin, 2008). This effect worsens with increasing altitude, with

SpO_2 falling to approximately 79 ± 3% at 5000 m (~11.5 % O_2; PO_2 = 81 mmHg), 66 ± 6% at 6500 m (~9 % O_2; PO_2 = 63 mmHg), and 52 ± 2% at 8000 m (~7.5 % O_2; PO_2 = 49 mmHg) (Anholm *et al.* 1992). However, reports citing the severity of hypoxia required to stimulate the hypoxaemia necessary for tissue deoxygenation are varied. Heubert *et al.* (2005) found 16% inspired O_2 decreased SpO_2 to 84±1% at rest. In contrast, despite inspiring a lowered concentration of O_2 than Heubert and colleagues, Kinsman *et al.* (2002) reported a decline in SpO_2 to just 91±1% when breathing 15% O_2 (~2650 m; PO_2 = 113 mmHg). Sustained falls in SpO_2 have, however, been reported with greater levels of hypoxia, where acute inspiration of ~12% O_2 (~4300 m; PO_2 = 90 mmHg) lowered SpO_2 to 88±1% (DeLorey *et al.* 2004), 75±1% (Jones *et al.* 2008), and 85%±1% (Kawahara *et al.* 2008) of normoxic values. This response occurs within 30 min normobaric hypoxic exposure (Loeppky *et al.* 1996; Savourey *et al.* 2007), and is adequate to cause muscle deoxygenation (i.e. reduced oxyhaemoglobin levels, as measured by near-infrared spectroscopy) (DeLorey *et al.* 2004; Kawahara *et al.* 2008; Salman *et al.* 2005).

1.4.2. Effects of hypoxia on skeletal muscle mass and protein metabolism

Altering the concentration of O_2 in inspired air may affect muscle protein metabolism and muscle mass. There are several examples of situations in which this may be studied. Firstly, humans living at (Kayser *et al.* 1991) or returning from expeditions to (Fulco *et al,* 1992; Rose *et al,* 1988) high altitude display alterations in muscle mass that indicate a role of hypoxia in regulation of muscle. Secondly, comparisons can be made with symptoms described in certain pathologies that are characterised by chronic intermittent hypoxia (termed ‘pathological hypoxia’) (e.g.

Debigare and Maltais, 2008; Jatta *et al*, 2009; Raguso *et al*, 2004). Thirdly, *in vitro* studies into the influence of hypoxia, i.e. <0.2% O_2 from normal values of 2 – 5% at the tissue level (Roy *et al.* 2003), provide insight into the possible role reduced O_2 availability may play in regulating processes involved in protein metabolism (Bi *et al*, 2005; Koumenis *et al*, 2002). Fourthly, limited studies conducted *in vivo* indicate the potential importance of hypoxia in muscle protein synthesis (Rennie *et al*, 1983).

1.4.2.1. Muscular adaptations during high altitude exposure

Comparative studies show that humans living at altitude for all or most of their lives are lighter (body mass = 67.8±10.8 versus 60.3±8.4 kg for sea-level residents and high altitude sherpas, respectively), have lower muscle mass (upper arm muscle cross-sectional area = 41.2±5.1 vs. 34.5±5.6 cm^2) (Fiori *et al*,. 2000; Weitz *et al.* 2000) and possess smaller muscle fibre cross-sectional area (3,186±521 μm^2) compared with than lowland counterparts (4,170±710 μm^2) (Kayser *et al.* 1991).

It is well documented that transition from low- to high-altitude results in losses of body weight (Abdelmalki *et al*, 1996; Boyer and Blume, 1984; Cerretelli, 1992; Ferretti *et al*, 1990; Green, 1992; Green *et al*, 1989; Hamad and Travis, 2006; Kayser, 1992; MacDougall *et al*, 1991; Wagenmakers, 1992; Westerterp and Kayser, 2006). Despite reports of reduced body mass at altitude, a lack of continuity in the protocols utilised render it difficult to determine the precise effects of varying altitudes / duration of exposure upon body composition. It is clear from early reports that ascent to altitudes >5,000 m (~11 – 11.5% inspired O_2; PO_2 = ~81 mmHg) for approximately six to eight weeks induces significant reductions in body size of

7.4±2.2 kg (Boyer and Blume, 1984; Green *et al*, 1989; Hoppeler *et al*, 1990; Rose *et al*, 1988) at a rate of 1-2 $kg \cdot wk^{-1}$ (Westerterp, 2001). Later studies indicate, however, that exposure to altitudes as low as 4,300 m (~12% O_2; PO_2 = ~90 mmHg) for three weeks (Tanner and Stager, 1998) or as few as sixteen days (Fulco *et al*, 1992) can cause significant losses in body weight (-4.2 kg and -5.9 kg, respectively) compared to sea-level controls. Thus, short-duration (16-21 d) stays at ≥4,300 m consistently causes reductions in body weight (Fulco *et al*, 1992; Fusch *et al*, 1996; Tanner and Stager, 1998), though losses in body mass appears to worsen with increasing altitudes.

The reduction in body mass during chronic hypoxia is principally due to loss of lean (skeletal muscle) mass (Fulco *et al*, 1992; Rose *et al*, 1988; Tanner and Stager, 1998). For example, simulated ascent to >8,000 m for 40 d showed a decrease in body mass of 7.4±2.2 kg, of which 4.9 kg (~66%) was fat-free mass and was associated with a 17% reduction in thigh muscle cross-sectional area (Rose *et al*, 1988). Furthermore, 32 d at >5,400 m induced a mean 4 kg reduction in body weight, of which ~73% was fat-free mass (Boyer and Blume, 1984). As with effects on body mass, lower altitudes also appear to induce losses in fat-free mass, where 21 d at 4,300 m induced a 4.2±2.8 kg reduction in body mass of which 77% was fat-free mass (Tanner and Stager, 1998), and 18 d at 4,300 m reduced fat-free mass significantly by 2.06 kg, whereas fat mass was unaltered (Fulco *et al*, 1985). However, as with body mass losses, it seems that the loss of fat-free mass is greater at higher altitudes (e.g. >8,000 m) compared to moderate (e.g. ~4,500 m) altitudes.

Losses of muscle mass at altitude are reflected in alterations in muscle morphology at the ultrastructural level. Muscle fibre cross-sectional area (CSA) was reduced 10% after six wk (Hoppeler *et al,* 1990) and -15% following ten weeks (Mizuno *et al,* 2008) exposure to 5,350 m. Reductions in fibre CSA may also be fibre specific, with Type I muscle fibres showing a significant decrease in cross-sectional area (-25%), while Type II fibres also decreased, though non-significantly during a simulated ascent >8,000 m (MacDougall *et al,* 1991). Presumably, given the degree of change observed in fat-free mass at 4,300 m, lower altitudes may cause comparable alterations in muscle morphology, though this remains untested. Furthermore, it appears that the loss of muscle function and mass during chronic stays at altitude may persist for long after the initial loss of muscle mass. Sedentary controls and long-distance runners assessed for morphological adaptations pre-, and 2-12 months post-high altitude (up to 8,000 m) sojourns exhibit reduced muscle fibre cross-sectional area of 15% and 51% for sedentary controls and endurance runners, respectively (Oelz *et al,* 1986). The net effect of these changes with exposure to altitude is impairments in aerobic exercise performance (Cerretelli, 1992; Heubert *et al,* 2005; Hoppeler and Vogt, 2001), reductions in maximal isometric voluntary force / power generating capabilities (Caquelard *et al,* 2000; Dousset *et al,* 2001; Ferretti *et al,* 1990; Fulco *et al,* 2001), and reduced muscle mass.

The mechanisms regulating the reduction in muscle mass following altitude expeditions have been scarcely investigated. Various hypotheses have, however, been proposed to explain this phenomenon, including: 1) increased energy expenditure that is not matched by increased energy intake (Butterfield, 1990; Gill and Pugh, 1964; Grover, 1963; Rose *et al,* 1988); 2) a loss of appetite and thus

reduced dietary intake at altitude (Boyer and Blume, 1984; Bradwell *et al,* 1986; Consolazio *et al,* 1968; Hannon *et al,* 1976; Sridharan *et al,* 1982; Surks *et al,* 1966); 3) loss of body water due to increased insensible loss via increased ventilation, decreased fluid intake, or abnormal water metabolism (Fulco *et al,* 1985; Krzywicki *et al,* 1971; Pugh, 1962); and 4) a decreased absorption of nutrients from the gastrointestinal tract (Milledge, 1972; Rai *et al,* 1975). Most of these hypotheses have, however, since been disproved. For example, increased dietary intake can alleviate (Pulfrey and Jones, 1996) but not fully prevent continued body mass losses during high altitude expeditions (Hoyt *et al,* 1994; Westerterp *et al,* 1994), even when dietary energy intake is matched for energy expenditure (Hamad and Travis, 2006; Worme *et al,* 1991). Also, no loss of body water has been shown after prolonged high altitude exposure (Fusch *et al,* 1996; Westerterp *et al,* 2000). The role of fat, carbohydrate and/or protein malabsorption from the gut in controlling altitude-related alterations in body composition remains controversial, with reports both supporting (Boyer and Blume, 1984; San Miguel *et al,* 2002) and rejecting (Guilland and Klepping, 1985; Kayser *et al,* 1992; Rai *et al,* 1975; Sridharan *et al,* 1982) the hypothesis. Thus, the mechanisms underlying reductions in muscle mass during altitude / hypoxic exposure remain undetermined, although it is apparent that there must exist an imbalance between MPS and MPB in these situations.

1.4.2.2. Muscle mass in conditions of clinical hypoxia

Comparisons can be drawn between decreased O_2 supply due to environmental hypoxic exposure and in patients hypoxic as a result of critical illness. Indeed, early attempts to understand human adaptation to low atmospheric O_2 were conducted in

individuals suffering pathological hypoxia, though conclusions were clouded by virtue of the illness itself (Davies and Gazetopoulos, 1981; Robin, 1980).

Clinically, numerous conditions are associated with hypoxaemia / tissue hypoxia. For example, patients suffering chronic obstructive pulmonary disease (COPD) endure recurring hypoxic episodes due to interrupted breathing patterns where resting SpO_2 declines to 87.8 ± 2.7% (Nasilowski *et al,* 2008), which is analogous to the decreases in SpO_2 seen in healthy individuals at ~4,500 m (~12% O_2) altitude (DeLorey *et al,* 2004). Similarly, obstructive sleep apnoea (OSA) is associated with regular (5 nocturnal respiratory disturbances per hour) falls in SpO_2 to 88.3±5.0%, both at night and during the daytime (Chiang, 2006; Clause *et al,* 2008; Noda *et al,* 1998), and is a common symptom of obesity (Alam *et al,* 2007) and type II diabetes mellitus (T2DM) (Chasens, 2007 Tasali *et al,* 2008). Furthermore, vascular resistance also may exacerbate the consequence of hypoxia in obesity and T2DM (Capla *et al,* 2007; Mohler *et al,* 2006; Prompers *et al,* 2007; Wong *et al,* 2008) via ischaemia-induced localised tissue hypoxia. Pathological blood flow restriction also underlies the skeletal muscle hypoxia observed in peripheral arterial occlusive disease (PAD) patients (Dehne *et al,* 2007; Hauser *et al.* 1984; Pareznik *et al,* 2006).

Interestingly, muscle wasting is a common symptom in all of the above conditions where pathological hypoxia is present. For example, atrophy is prevalent in COPD (Debigare and Maltais, 2008; Jatta *et al,* 2009; Palange *et al,* 1998; Pereira *et al,* 2004; Raguso *et al,* 2004), with 25% of male patients having fat-free body mass (FFM) index <17 $kg \cdot m^{-2}$ (Wagner, 2008), versus >18 $kg \cdot m^{-2}$ in healthy controls (Schols *et al,* 2005). Those with COPD also show decreased muscle fibre cross-

sectional area (Gosker *et al,* 2002; Pereira *et al,* 2004; Whittom *et al,* 1998) and reduced thigh muscle cross-sectional area of 83.4 ± 16.4 versus 109.6 ± 15.6 cm^2 for healthy individuals (Bernard *et al,* 1998). A complication of OSA (Pillar and Shehadeh, 2008), T2DM and obesity (Brash *et al,* 1999; Mastrocola *et al,* 2008; Severinsen *et al,* 2007) is also accelerated muscle loss particularly of the lower extremities. Furthermore, PAD has been shown to promote muscle weakness and fibre atrophy (Killewich *et al,* 2007; Regensteiner *et al,* 1993; Steinacker *et al,* 2000). This myopathy is independent of other co morbidities associated with PAD (Pipinos *et al,* 2008) and ultimately causes reduced muscle size (Hourde *et al,* 2006; McDermott *et al,* 2007; Regensteiner *et al,* 1993). Consequently, these conditions are associated with loss of locomotive capacity, decreased muscle function and, therefore, reduced quality of life (Dourado *et al.* 2009; Wüst and Degens, 2007).

Increasing our understanding of the molecular pathways underpinning this muscle wasting will lead to the development of new therapeutic agents and strategies to combat it. Various mechanisms have been proposed to explain the muscle wasting observed in each of these respective disease states. However, the underlying cause(s) of atrophy in many cases remain incompletely understood. In those conditions where hypoxaemia occurs as a result of interrupted breathing patterns, muscle wasting has been hypothesised to be caused by energy imbalance via increased resting energy expenditure due to increased work of breathing in COPD (Sergi *et al,* 2006) and OSA (Ryan *et al,* 1995). Aggressive nutritional supplementation, however, does not prevent or reverse muscle wasting in these conditions (Guinot-Bourquin *et al,* 2004; Schols, 2003). Furthermore, the finding that only one in four COPD patients suffers muscle wasting led to investigation of genetic susceptibility to atrophy, with the

focus primarily directed towards genes encoding inflammatory molecules (Broekhuizen *et al,* 2005; Hopkinson *et al,* 2006). However, few associating polymorphisms have been found, and cause and effect relationships between specific genotypes in these diseases remain to be established (Wagner, 2008). Finally, inactivity may be partially responsible for reductions in muscle mass in hypoxaemic conditions, since COPD patients, diabetics and obese individuals all display lowered activity levels versus healthy controls (Zuwallack, 2009). However, resistance training does not appear to fully restore muscle strength or lead to improvements in exercise capacity (Bernard *et al.* 1998; Man *et al.* 2009), and leads to blunted hypertophic responses in hypoxaemic pathologies versus healthy individuals (e.g. 2 – 4% increases in quadriceps cross-sectional area in COPD patients versus 8 – 10% increases healthy individuals) (Casaburi *et al.* 2004; Kongsgaard *et al.* 2004), suggesting activity levels alone may not account for muscular abnormalities in these conditions.

With regards to atrophy in conditions of vascular disease / ischaemia, impaired blood flow and associated decreases in substrate supply has been implicated as a principle cause of lower limb locomotor muscle dysfunction (Killewich *et al.* 2007). A reduced nutritional supply may not, however, provide an exhaustive explanation for muscle loss in ischaemic diseases, since body weight loss and muscle fatigability are often difficult to reverse despite an optimal nutritional intake, suggesting atrophy cannot fully be explained by malnutrition alone (Guinot-Bourquin *et al,* 2004). Lastly, denervation has also been implemented as a mechanism of atrophy in ischaemia, with histological evidence of denervation found in PAD legs (Regensteiner *et al,* 1993) suggesting ischaemic damage to these nerves may

contribute to muscle dysfunction and atrophy in the long-term. However, electromyographic analysis of the tibialis anterior muscle in PAD shows muscle action potential activities are within normal limits (Papapetropoulou *et al,* 1998).

Thus, the mechanisms investigated to date do not appear to fully explain the loss of muscle observed in diseases where pathological hypoxia is a feature. Recently a direct role for hypoxia in reducing rates of protein synthesis has been proposed to explain muscle loss in conditions of environmental / pathological hypoxia (Hochachka *et al,* 1996; Hoppeler and Vogt, 2001; Wüst and Degens, 2007), though to date this has received little investigative attention *in vivo*. Furthermore, whilst the physiological responses to hypoxia have been appreciated for a long time (as detailed above), the molecular processes that occur within cells have only recently come under investigation (Kenneth and Rocha, 2008). *In vitro* studies provide an opportunity for greater insight into the regulation of skeletal muscle protein metabolism by low O_2 supply for subsequent application in humans.

1.4.2.3. Hypoxia and control of protein synthesis and translational signalling in vitro

Giving credence to the hypothesis that intramuscular hypoxia may contribute to the occurrence of muscle wasting are studies in various cell lines investigating the role of hypoxia (< 0.2% O_2) on protein turnover. Reduced O_2 availability can be closely monitored at both the cellular and organismal levels (Liu *et al,* 2006), leading to activation of O_2-responsive intracellular signalling pathways, with the aim of reinstating energy status for continued cell survival (Kenneth and Rocha, 2008). Delineating this cellular hypoxic response and the underlying molecular signalling

pathways has been the focus of intense research over recent years, primarily as a result of the role hypoxia plays in embryonic development, and in conditions such as tumor growth, ischaemia, stroke and wound-healing (Semenza, 2001b; Simon and Keith, 2008; Wouters *et al*, 2005). Findings of such studies help towards better understanding the response of human skeletal muscle to hypoxia.

During periods of O_2 limitation, most excitable cells cannot meet the energy demands of the cell due to a reduction in oxidative metabolism, resulting in rapid depletion of ATP (Boutilier, 2001; Hochachka and Lutz, 2001; Pierce and Czubryt, 1995; Stanley *et al*, 1997). As an adaptive response cells undergo two key metabolic alterations: 1) increasing anaerobic glycolysis in order to maximise the yield of ATP per mol O_2 and, 2) down regulating non-essential energy consuming processes, including protein synthesis (Arsham *et al*, 2003; Boutilier, 2001; Jibb and Richards, 2008; Michiels, 2004). Although the former of these mechanisms is undoubtedly useful in surviving O_2 lack , evaluation of such defence strategies indicates that suppression of energy turnover provides the greatest protection against hypoxia (Hochachka, 1986; Hochachka *et al*, 1996; Storey and Storey, 1990; Wouters *et al*, 2005). This is because protein synthesis is the dominant ATP-consuming process in mammalian skeletal muscle (Michiels, 2004; Rolfe and Brand, 1996), requiring approximately 4 ATP molecules per peptide bond and accounting for 25-30% ATP usage at basal metabolic rate (Welle and Nair, 1990). Furthermore, anaerobic ATP production cannot sustain the pre-existing energy demands of mammalian cells and tissues for more than a couple of hours in muscle (Boutilier, 2001), requiring the conservation of ATP via alternative mechanisms, e.g. decreased protein synthesis. As such, inhibition of protein synthesis promotes energy homeostasis and may

sustain survival during periods of hypoxia where ATP production may be limited (Breen *et al,* 2008; Liu *et al,* 2006; van den Beucken *et al,* 2006).

It is widely accepted that, *in vitro*, mRNA translation is severely but reversibly inhibited during hypoxia (Arsham *et al,* 2003; Bi *et al,* 2005; Boutilier, 2001; Jibb and Richards, 2008; Koumenis *et al,* 2002; Michiels, 2004), which has been demonstrated in all cell lines tested to date (Barnhart *et al.* 2008). This is further supported by a recent study in L6 myotubes showing that atrophy of these cells occurs over 24 – 48 h in supra-physiological levels of hypoxia (1% O_2), due to a combination of decreased protein synthesis and increased protein breakdown (Caron *et al,* 2009). The hypoxic down-regulation of this ATP-consuming process is arranged hierarchically, with protein synthesis and RNA/DNA synthesis being the first to suffer rapid, widespread inhibition (Buttgereit and Brand, 1995), occurring within minutes to hours of the initiation of a hypoxic stimulus and preceding ATP depletion (Lefebvre *et al,* 1993; Michiels, 2004). However, in mammalian cells this response may be slower; in L6 myotubes protein synthesis is unaltered after 24 h hypoxic exposure but declined significantly (49% decrease) after 48 h hypoxia (Caron *et al,* 2009).

In mammalian systems, hypoxia affects cell metabolism and gene expression in two ways: 1) through slower transcriptional responses that are largely dependent on the hypoxia-inducible factor (HIF) family of O_2-sensitive transcription factors (Proud, 2004b; Semenza, 2001a), and; 2) via rapid and reversible effects on cell signalling events that serve to acutely balance energy supply and demand (Arsham *et al,* 2003). Chronic adaptations to hypoxia invariably involve changes in the transcription of

genes regulated by a major hypoxia-responsive transcription factor, HIF-1 (Semenza and Wang, 1992). HIF-1 is a heterodimer composed of a HIF-1β subunit, which is constitutively expressed, and a HIF-1α subunit, the expression and transcriptional activity of which are under tight regulation by cellular O_2 concentration (Wang *et al,* 1995) and requires the chromatin remodelling complex SWI/SNF for its activation (Kenneth *et al,* 2009). In normoxia (normal O_2 concentrations, ~20.9%) HIF-1α is controlled by a class of 2-oxoglutarate dioxygenases called prolylhydroxylases (PHDs) that target this subunit for ubiquitination by the Von Hippel Lindau system and subsequently proteasomal degradation (Kenneth *et al,* 2009). Under hypoxic conditions, however, PHD activity is inhibited and HIF1-α is stabilised, allowing levels to increase and subsequently translocate into the nucleus to promote transactivation of target genes (Brahimi-Horn *et al,* 2007; Giaccia *et al,* 2003; Hu *et al,* 2003). These include more than 100 genes (Chavez *et al,* 2008) encoding proteins involved in glycolysis and the tricarboxylic acid cycle (Kim *et al,* 2006; Papandreou *et al,* 2006), erythropoieses and angiogenesis (Kaelin, 2002; Semenza, 2001b), as well as cell cycle arrest (Gardner *et al,* 2001; Koshiji *et al,* 2004), autophagy (Zhang *et al,* 2008a) and apoptosis (Greijer and van der Wall, 2004). However, because HIF-1α mediated transcription primarily represents a mechanism for adaptation to prolonged hypoxia (Ryan *et al,* 1998; Tang *et al,* 2004; Leek *et al,* 2005), alternative responses must take place in the short-term to account for the acute alterations in mRNA translation during hypoxia.

Early adaptations to the onset of hypoxia must, therefore, entail modulated activity of preexisting proteins (Bi *et al,* 2005; Koritzinsky *et al,* 2006; Semenza, 2001a), which can undergo rapid alterations (van den Beucken *et al.* 2006). Although HIF-1

is generally associated with transcriptional changes that regulate chronic adaptations to hypoxia, relieved inhibition of HIF-1α at the early onset of hypoxia may rapidly increase the expression of specific proteins that are normally translationally repressed (van den Beucken *et al.* 2006). One such mechanism that has received much attention recently is the protein, REgulated in Development and DNA Damage responses (REDD1), first discovered by Ellisen *et al.* (2002). REDD1 has a half-life of <5 min (Kimball *et al.* 2008) and, as such, can undergo rapid alterations in total content of the protein. During hypoxia, REDD1 expression is elevated in a HIF-1α dependent manner (DeYoung *et al.* 2008; Schwarzer *et al.* 2005) and, by activating the TSC 1/2 complex, functions to suppress mTOR activity and thus rates of protein synthesis (Brugarolas *et al.* 2004; DeYoung *et al.* 2008) and subsequent mTOR, p70S6K and 4E-BP1 inhibition (Arsham *et al.* 2003; Ellisen, 2005; Sofer *et al.* 2005; van den Beucken *et al.* 2006). HIF-1α independent mechanisms of translational inhibition have also been revealed. During hypoxic exposure, increases in AMP concentrations leads to increased activity-related phosphorylation of the AMP-activated protein kinase (AMPK) at Thr172 (Liu *et al.* 2006) through phosphorylation of the upstream kinase LKB1 (Emerling *et al,* 2009). The result is translation inhibition via TSC 1/2-mediated inhibition of the mTOR / 4E-BP1 / p70S6K pathways (Liu *et al,* 2006). Additionally, AMPK phosphorylation leads to eEF2 and eIF2α inhibition, which also contributes to reduced rates of protein synthesis. Contradicting this, however, are reports that AMPK activation is not required for hypoxia-induced suppression of protein synthesis, suggesting that REDD1 activation is the primary mechanism involved (Brugarolas *et al.* 2004).

Thus, it is clear that acute hypoxia causes skeletal muscle cell dysfunction *in vitro*, but little is known about the effects of hypoxia on muscle function and metabolism *in vivo* (Degens *et al*, 2006). In light of findings from *in vitro* experiments, and the high O_2 and ATP cost of protein synthesis, hypoxia-induced suppression of protein synthesis remains a plausible, but not directly tested, hypothesis. Therefore, understanding of the molecular signalling changes that accompany hypoxia may reveal novel therapeutic strategies for the treatment of altitude-related and pathological muscle wasting.

1.4.2.4. In vivo studies of the effects of hypoxia on rates of protein synthesis and translational signalling

Very few investigations have reported whether the *in vitro* conclusions drawn of the effect hypoxia has upon translational signalling and protein synthesis also occur within *in vivo* models. Supporting a role for hypoxia in inhibiting protein synthetic rates, early reports in the rat alluded to diminished rates of protein synthesis in brain and skeletal muscle tissue during inspiration of 5% O_2 (Klain and Hannon, 1970; Sanders *et al*, 1965). However, these studies have been criticised for imperfect application of radio-active labelled isotope tracer methodologies, for example: tracer was not administered directly into the bloodstream; only trace amounts of labelled amino acids were used; no measurement was made of tissue unbound labelled amino acid radioactivity, and; inappropriate labelled amino acids were utilised, such as alanine and glutamate (Preedy *et al*, 1985). Subsequently, studies using more physiologically relevant inspired O_2 concentrations (10% O_2, ~6,300 m) in rats, and correcting for past methodological mistakes to determine fractional protein synthetic

rates, demonstrated 15-35% reductions in protein synthesis in several tissues, including skeletal muscle after just 6 h hypoxic exposure in the fed state (Preedy *et al,* 1985). A single study conducted in humans is congruent with these observations, showing reduced rates of protein synthesis (as indicated by forearm leucine uptake) in the rested, fasted state following 6.5 h exposure to approximately 4,500 m (~12% O_2), using a hypobaric chamber (Rennie *et al,* 1983). This affect, if continued over chronic hypoxia, would be sufficient to cause muscle wasting, providing MPB was concomitantly unchanged / increased. This was also independent of changes in plasma hormones, glucose and amino acid profile and thus appeared the result of hypoxia *per se*. However, despite its potential importance, this study used a sample size of just four; therefore further investigation is required to confirm these findings. Furthermore, the underlying mechanisms regulating this response were not elucidated in these early studies.

Only one other study to date has attempted to investigate the effect of hypoxia *in vivo*. Imoberdorf *et al.* (2006) measured protein synthesis in human subjects following either a passive (i.e. flown by helicopter) or active (i.e. ascent by cable-car to 3220 m followed by a 4 – 5 h walk the next day) ascent to altitude (4,559 m). In contrast to Rennie *et al.* (1983) their findings showed that hypoxia did not affect basal / postabsorptive rates of protein synthesis when compared to normoxia in the passive group, and that protein synthesis increased when at altitude in the active group, possible was a result of increased activity. This would suggest that hypoxia has no affect on protein synthesis *in vivo*. There are, however, a number of errors inherent in the design of this study that place the findings into question. Firstly, the active ascent group did not perform a normoxic exercise control trial, thus it is

unknown whether the increase in protein synthesis observed at altitude was comparable to the increase observed at sea level, disguising the potential for an altered protein synthetic response to exercise in hypoxia. Secondly, the subject groups studied were regular mountaineers and thus may have been partly acclimatised, trained, and/or non-responders to the hypoxic stimulus. This notion is reinforced by the fact that no symptoms of acute mountain sickness were reported. Thirdly, fasted rates of protein synthesis were obtained at 19-23h post-exercise / arrival at altitude, thus any acute effects would have been missed. Fourthly, no assessment of activity of translational signalling was made, thus any underlying molecular adaptations are not known. Lastly, the method employed to measure protein synthesis was the [$^{2}H_{5}$ring] phenylalanine flooding dose technique, i.e. giving a large (3 g) bolus of phenylalanine, which has been shown to be sufficient to stimulate incorporation of constantly infused tracer (i.e. increase protein synthesis) independent of any other stimuli; an effect that is not observed when using a primed constant infusion approach (Smith *et al,* 1998). It may thus be that the methodology applied in this study masked any inhibitory affect of hypoxia on protein synthesis, through stimulation of MPS. Other confounding variables inherent with any research conducted at altitude may also have been influential, such as gastroenteritis, malnutrition, and low temperatures; all of which are known to induce muscle atrophy during altitude exposure (Kayser, 1992). However, mTOR activity has been shown to be significantly reduced after 7-9 days hypoxic exposure at 4,559 m, suggesting a possible reduction in protein synthesis (Vigano *et al,* 2008). In contrast, however, De Palma *et al.* (2007) have reported no change in the activity of mTOR during chronic hypoxic exposure in rat skeletal muscle exposed to 10% O_2 concentrations, thus

limiting any extrapolation of the effects of hypoxia upon protein synthesis and translational signalling.

1.5. Summary of introduction

The balance between protein synthesis and degradation determines muscle size, contractile function and health. This balance is maintained by positive postprandial, and negative postabsorptive, periods of net protein balance throughout a diurnal cycle. When circadian net protein balance is positive (i.e. synthesis exceeds degradation), over time muscle mass increases. Conversely, a negative protein balance eventually leads to loss of muscle. Input from a number of external stimuli, particularly feeding and exercise have been established as key modulators of protein synthetic and breakdown processes. However, whilst much progress has been made over recent years in characterising the protein synthetic responses to feeding and exercise, numerous questions remain unanswered pertaining to the precise changes in protein metabolism, associated translational signalling and ultimately muscle function. Thus the initial aims of this thesis were two-fold. Firstly, despite it being well documented that acute protein feeding potently stimulates protein synthesis, the precise temporal response of MPS and that of putative molecular regulators of MPS, have not been elucidated. Therefore, the first aim of this thesis was to detail the temporal response of MPS and translational signalling to an acute protein bolus. Secondly, since the protein synthetic and breakdown response to acute resistance exercise plus protein feeding has been well characterised, but little is known of the functional consequences of these changes, this thesis investigated the functional

recovery of muscle following acute muscle-damaging exercise when combined with post-exercise protein ingestion.

Thirdly, despite the importance of oxygen availability to all cellular processes, and in particular that of peptide elongation, the effects of reduced oxygen supply on protein synthesis has received very little attention amongst researchers to date. As such, the final aim here was to determine the protein synthetic and accompanying molecular signalling changes to reduced O_2 supply at rest and following acute exercise. Collectively, attainment of these aims by utilising an integrated approach would enhance our understanding of the regulation of skeletal muscle protein homeostasis and function. Such knowledge would have important practical implications for advancing the design / implementation of intervention and therapeutic strategies for both clinical and athletic populations.

CHAPTER 2. GENERAL EXPERIMENTAL METHODS

The following sections describe the materials and methods employed in multiple experimental chapters of the present thesis. Where additional materials, modified methods, or specific protocols were utilised, details are provided within the specific methods section of the relevant experimental chapter. All experiments took place inside the Welkin Laboratories of the Chelsea School, University of Brighton.

2.1. Health and Safety

All studies were approved by the University of Brighton Research Ethics and Governance Committee, and complied with all standards set by the *Declaration of Helsinki* on the use of human research subjects. The availability of medical personnel was ensured throughout all testing sessions in case of emergency. Participants were free to withdraw from testing at any time, without giving reason. Intravenous infusions were carried out by trained phlebotomists to minimise the possibility of adverse reactions (e.g. local irritation of the infusion site; sweating; irregular breathing; and increased heart rate).

For all experimentation where muscle biopsy samples were collected, two experimenters were present at all times. All muscle biopsies were performed by trained and experienced personnel in a designated clinical investigation room and were conducted using sterile techniques. To minimise any likelihood of discomfort, all subjects underwent an ultrasound to determine the precise location from which muscle biopsies were collected. In the event of substantial subject discomfort for any

reason, testing was to cease immediately and participants allowed to recover fully under close observation by a qualified physician.

2.2. Human participants

Participants were recruited from the University of Brighton undergraduate and postgraduate student cohort. Subjects were all recreationally active (≤ 3 days activity week^{-1}) and were required to refrain from taking any form of nutritional supplement or medication at the time of testing and for > 12 weeks prior to the study. Participants were required to complete a health questionnaire and based on responses were deemed healthy. Subjects abstained from alcohol consumption, caffeine ingestion, and exhaustive exercise 72 h before each testing session and on the day of the study. For each study, subjects were also required to arrive at the laboratory in a fasted state, which was defined as a complete avoidance of food consumption for 12 h prior to testing, with water intake allowed *ad libitum*. Each volunteer was advised of the purpose of the study and associated risks both verbally and in writing. Participants subsequently provided written informed consent (Appendix 1) prior to the initiation of testing with the understanding that participants were free to withdraw from experimentation at any time without providing reason. Personal details and test results remained confidential and were stored in a lockable filing cabinet and/or on a password protected computer. Anonymity was maintained at all times.

2.3. Muscle biopsy collection

The muscle biopsy procedure was performed as described previously (Dietrichson *et al.* 1987). The area of the thigh (vastus lateralis) to be sampled was anaesthetised using 1% Lidocaine, after which a small (approximately 1 cm wide and 2 cm deep) incision was made through the skin and fascia. The conchotome technique was then used to remove a muscle sample (approximately 100 mg) from the vastus lateralis muscle, which was then washed free of blood in ice-cold saline, blotted, and snap frozen in liquid nitrogen. Samples were subsequently stored at -80°C until analysis. The incision was then stitched and covered with an adhesive dressing. For collection of repeated biopsies, subsequent incisions were made in a distal to proximal direction on the thigh approximately 5 cm apart. Participants were informed not to remove the dressing, to refrain from strenuous exercise, and to avoid excessive alcohol consumption for at least 2 days after the study. Participants returned approximately one week after the studies for the removal of biopsy stitches. In the event of the appearance of signs of infection (identified as redness or swelling to the area of incision), participants were referred to the resident physician. Contact details were made available for non-biased, independent advice regarding the biopsy procedure prior to provision of informed consent.

2.4. Evaluation of myofibrillar protein synthesis

2.4.1. Primed, constant infusion of stable isotope labelled tracer

On the morning of the study participants arrived at the laboratory at ~0800 h in the postabsorptive state for insertion of 18-g catheters into the antecubital veins of both

arms for tracer infusion and blood sampling. A baseline blood sample was collected to determine the background enrichment of leucine and α-ketoisocaproic acid (KIC) in plasma. A primed, continuous infusion (priming dose 7.8 μmol·kg body wt^{-1}, infusion rate 0.13 μmol · kg body wt^{-1} · min^{-1}) of [1,2-$^{13}C_2$] leucine tracer (99 Atoms %, Cambridge Isotopes, Cambridge, MA, USA) was then started at a time specific to the individual studies' protocol (see relevant experimental chapters).

2.4.2. Analysis and calculation of myofibrillar protein synthesis

To determine plasma KIC and leucine enrichment and concentration [atoms percent excess; APE], a known amount of norleucine and KIC internal standards were added to plasma. Proteins were then precipitated and the supernatant, containing free amino and imino acids, was collected to prepare the *tertiary*-butyldimethylsilyl [*t*-BDMS (leucine)] and *ortho*-phenylenediamine-*tertiary*-butyldimethylsilyl [OPDA-*t*-BDMS (KIC)] derivatives for analysis by gas chromatography-mass spectrometry (GC-MS; MD800, Thermo Finnison, Hemel Hempstead, UK) by using electron-ionization and selective ion monitoring (Babraj *et al.* 2002; Schwenk *et al.* 1984; Watt *et al.* 1991).

To prepare the myofibrillar fraction, frozen muscle (20-30 mg) was homogenised using scissors in buffer containing 50 mM Tris-HCL pH 7.5, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, and then centrifuged at 3000×g, 4ºC for 20 min to pellet the myofibrils and collagen. The pellet was then washed with 0.3 M NaOH to solubilise myofibrillar proteins, centrifuged as above, and the collagen pellet was washed with acetic acid and acetic acid-pepsin leaving an insoluble mature collagen pellet. Myofibrillar proteins were precipitated from the 0.3 M NaOH supernatant with 1 ml perchloric acid (0.5 M) and then 1 ml ethanol (Bohe *et al.* 2001). The myofibrillar

protein extracts were hydrolyzed in 0.1 M HCl : Dowex 50W-X 8-200 slurry (Sigma Ltd, Poole, UK) at 110 º C overnight and the liberated amino acids were purified on cation-exchange columns (Dowex 50W-X8-200) (Balagopal *et al.* 1997; Bohe *et al.* 2001).

The amino acids were then converted to their *N*-acetyl-*n*-propyl ester (NAP)-derivative and the leucine enrichment (APE) determined by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS; Delta-plus XL, Thermofinnigan, Hemel Hempstead, UK) (Rennie *et al.* 1996; Meier-Augenstein, 1999).

The fractional synthetic rate (FSR) of myofibrillar proteins was calculated based on the incorporation rate of [1,2-$^{13}C_2$] leucine into muscle proteins using a standard precursor-product model as follows:

$$FSR = \Delta E_m / E_p (1 / t) \times 100$$

where ΔE_m is the change in enrichment (APE) of protein-bound leucine in two subsequent biopsies, E_p is the mean enrichment over time of the precursor for protein synthesis (plasma KIC was chosen to represent the immediate precursor for muscle protein synthesis, i.e. leucyl-*t*-RNA (Matthews *et al.* 1982; Watt *et al.* 1991; Chinkes *et al.* 1996), and t is the time between biopsies. Values for FSR are expressed as percent per hour ($\%\cdot h^{-1}$).

2.5. Evaluation of signalling events regulating myofibrillar protein synthesis

Western blot analysis was used to measure the phosphorylation and/or total protein concentration of components of the translational signalling cascade. Frozen muscle tissue (~20 mg) was rapidly homogenised with scissors in ice-cold buffer (50 mM Tris-HCL pH 7.5, 1 mM EDTA, 1 mM EGTA, 10 mM glycerophosphate, 50 mM NaF, 0.1% Triton-X, 0.1% 2-mercaptoethanol, 1 complete protease inhibitor tablet [Roche Diagnostics Ltd, Burgess Hill, UK]) at 6 $\mu l \cdot mg^{-1}$ tissue. Proteins were extracted by shaking for 15 min at 4ºC and samples were then centrifuged at 13000×g for 10 minutes at 4ºC and the supernatant containing the proteins was collected. The protein concentration in the supernatant was determined by the Bradford method with a commercial reagent (B6916, Sigma-Aldrich, St. Louis, MO) and adjusted to 1 $\mu g \cdot \mu l^{-1}$ in 3×Laemmli buffer. Fifteen micrograms of protein (15µl) from each sample were loaded onto 12% NuPAGE Novex Bis-Tris gels (Invitrogen, Paisley, UK), separated by SDS PAGE, and transferred on ice at 100 V for 45 min to methanol pre-wetted 0.2 µm PVDF membranes. Blots were then incubated sequentially in 5% (w/v) BSA in TBST for 1 h, primary antibodies (diluted in 5% BSA in TBST) overnight shaking at 4ºC, and then secondary antibody (1:2000 anti-rabbit; New England Biolabs, Ipswich, MA) for 1 h. Membranes were developed using Immunstar (Bio-Rad Laboratories, Richmond, CA) and the protein bands were visualized and quantified by densitometry on a Chemidoc XRS (Bio-Rad Laboratories, Inc. Hercules, CA) ensuring no pixel saturation. Data were expressed in relation to β-actin or pan-actin loading controls (see specific experimental chapters).

2.6. Determination of maximal isometric voluntary contraction (MVC)

MVC of the quadriceps were tested using a custom-made isometric rig that could be adjusted to fix subjects' knee joint angle at 90° (1.57 rad), measured using a goniometer. A strap was attached to the ankle of subjects' dominant leg, connected via chain to a strain gauge (Tedea Ltd., Huntleigh, UK) that measured the amount of isometric force developed in the contracting quadriceps performing the knee extension. The strain gauge signal (sample rate = 0.1 kHz) was amplified, filtered, and converted from analog to digital (model Micro 1401, CED). Results were recorded and analysed on a Microsoft Windows 98 compatible personal computer using Spike II software. Subjects performed three maximal 5 s efforts with the peak value taken as the highest value achieved.

CHAPTER 3. THE MUSCLE FULL EFFECT AFTER ORAL PROTEIN: TIME DEPENDENT CONCORDANCE AND DISCORDANCE BETWEEN MUSCLE PROTEIN SYNTHESIS AND mTOR SIGNALLING

3.1. Introduction

For many years it was speculated that increased protein availability was likely to stimulate muscle protein synthesis. However, the first demonstration that a mixed meal was able to increase human muscle protein synthesis was not made until 1982 (Rennie *et al.* 1982). It was later shown that amino acids alone made the major (~80%) contribution to this stimulation (Bennet *et al.* 1990; Watt *et al.* 1992). When these early measurements were made, instrument limitations made it difficult to measure incorporation of stable- isotope labelled tracer amino acids into muscle protein over short periods. As such, most of the measurements reported were for periods of 6-8 h or more (Nair *et al*, 1988). With improvements in the sensitivity of analytical methods, it became possible to make measurements of tracer incorporation into muscle over much shorter periods (~1 h) using either gas chromatography – mass spectrometry (GCMS) or gas chromatography – combustion – isotope ratio mass spectrometry (GC-C-IRMS). Taking advantage of this, Bohé *et al.* (2001) demonstrated the latency and duration of MPS responses to increased amino acid availability. In response to a constant intravenous infusion of mixed amino acids it was shown that there was a lag in the response of ~30 min before MPS was stimulated by 2- fold for ~120 min, thereafter returning to postabsorptive rates despite continued elevated availability of amino acids for up to 6 h (Bohé *et al.* 2001). This phenomenon has been linked to the "muscle full" hypothesis of

Millward (1995), though has received little investigative attention since. Recently, however, this effect has been confirmed in rat muscle in response to a single oral feed, whereby MPS was shown to rise and fall within 180 min of meal feeding despite plasma leucine concentrations remaining elevated (Norton *et al.* 2009). This increase in MPS was mirrored by phosphorylation of components of the mTOR signalling pathway, where 4E-BP1, p70S6K and eIF4G phosphorylation increased in a similar fashion to MPS. However, mTOR signalling became disconnected with MPS during the return to basal values in MPS; 4E-BP1, p70S6K and eIF4G activity remained elevated at 180 min, i.e. after the switch off of MPS. This suggests that, whilst plasma leucine levels and subsequent increases in translational signalling may mediate increases in MPS, the down turn in MPS following a single meal response may be influenced by other factors in addition to leucine concentrations and increased mTOR signalling alone. This phenomenon whereby increases in translational signalling outlasts that of MPS post-feed is yet to be demonstrated in humans and is of importance in understanding more fully the complex regulation of the skeletal muscle mass.

Clearly, therefore, stimulation of MPS is related to blood concentration of amino acids, especially of EAA (Bohé *et al*. 2003) and in particular leucine in a dose-dependent saturable fashion and that a similar relationship exists between MPS and the amount of orally administered EAA (Cuthbertson *et al.* 2005). Recently, similar saturable relationships have been uncovered for whey protein ingested at rest and after resistance exercise (Moore *et al.* 2009), whereby it was demonstrated that 20 g whey protein is sufficient to maximally stimulate MPS. The resultant increased blood and muscle amino acid availability not only provides substrate for protein

synthesis but also directly modulates intracellular signalling events regulating initiation and elongation phases of mRNA translation; particularly that of signalling through the mammalian target of rapamycin complex 1 (mTORC1) (Cuthbertson *et al.* 2005; Smith *et al.* 2009). To date these signalling components comprises mTOR, regulatory associated protein of TOR (Raptor), mammalian LST8/G-protein β-subunit like protein (mLST8/GβL) and the recently identified partner proline- rich PKB substrate 40 (PRAS40).

Nevertheless, neither the temporal responses of human MPS to a single oral meal of protein of a nutritionally meaningful size, nor the signalling responses that may be involved in the control of translation of mRNA have been delineated (Anthony *et al.* 2000b). Thus, the aim of this study was to fill this gap by studying the effects of a meal of 48 g whey protein sufficient to maximally stimulate human MPS and maintain elevated plasma and intramuscular AA concentrations over an extended period (Moore *et al*. 2009). It was hypothesized that (a) a protein meal would, like amino acid infusion, produce a short-lived response, despite causing longer more sustained elevations in plasma and muscle intracellular AA, beyond the peak MPS response; and (b) molecular signalling responses regulating mRNA translation would predict those of MPS during the upswing, but not during the subsequent decline to postabsorptive rates.

3.2. Methods

3.2.1. Study Design

Eight healthy young men (age 21±2 y; body mass index 22.9±0.9 kg.m^2) were studied after an overnight fast and were asked to refrain from heavy exercise for 72 h prior to the study day. On the morning of the study subjects arrived at ~0800 h for insertion of catheters into both arms for tracer infusion and blood sampling (see section 2.4.1.). Blood samples and muscle biopsies were taken according to the protocol below (Figure 3.1).

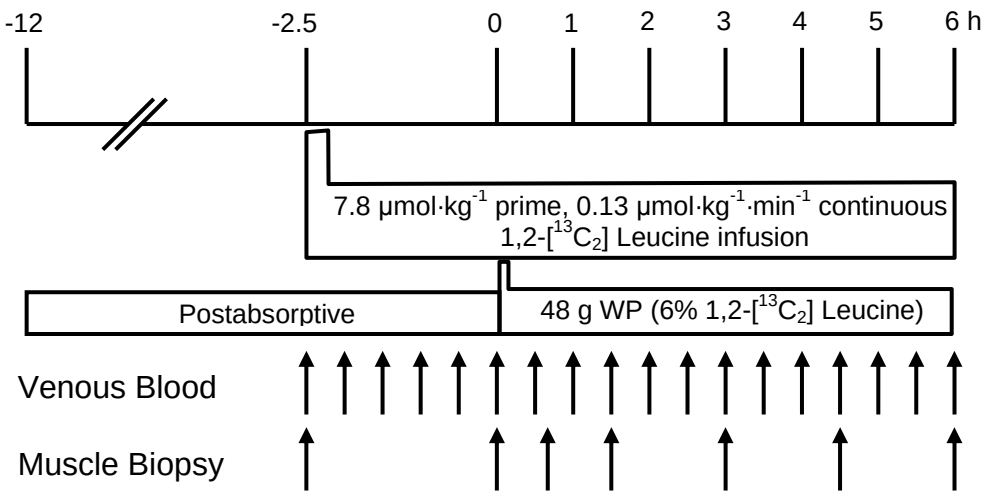

Figure 3.1. Protocol for the measurement of muscle protein synthesis and anabolic signalling phosphorylation in response to ingestion of 48 g whey protein (WP).

Muscle biopsies were taken intermittently (Figure 3.1.) from the quadriceps under sterile conditions using the conchotome technique (Dietrichson *et al.* 1987), as described in section 2.3. A primed, continuous infusion of [1, 2-$^{13}C_2$] leucine tracer (see section 2.4.1.) was started (at -2.5 h) immediately after the first biopsy and maintained until the end of the study. A second biopsy was taken after 2.5 h (0 h) in the postabsorptive state, (so that the basal rate of MPS could be determined),

whereupon the subjects drank a preparation of 48 g of whey protein isolate powder in 500 ml water (98% whey, 1% CHO, 1% fat); chosen for its richness in EAA (equivalent to ~20 g EAA). After the study, both catheters were removed and subjects were fed and monitored for 30 min before being allowed to go home. Subjects were reimbursed with an inconvenience allowance for participating in the study.

3.2.2. Measurement of plasma amino acids, glucose and insulin

Plasma glucose was measured using iLab plus 300 Clinical Chemistry System. Plasma insulin was measured using human high sensitivity ELISA (IDS). For the measurement of plasma concentrations of amino acids, equal volumes of plasma and 10% sulfosalicyclic acid were mixed and cooled at 4°C for 30 min. The samples were spun at 10,000 × *g* to remove the precipitated protein and passed through a 0.22 µm filter prior to analysis by a dedicated amino acid analyser (Biochrom 30, Biochrom, Cambridge, UK) using a lithium buffer separation. All 20 AA concentrations were determined by comparison to a standard sample, using norleucine as an internal standard.

3.2.3. Myofibrillar and sarcoplasmic protein synthetic rates

Muscle tissue (~25 mg) was prepared as outlined in section 2.4 for evaluation of myofibrillar protein synthesis. The supernatant produced following extraction of the myofibrillar fraction was used for determination of sarcoplasmic FSR after precipitating out protein using 1 M PCA and washing with 70% ethanol, prior to acid hydrolysis as for the myofibrillar fraction. Rates of myofibrillar and sarcoplasmic protein synthesis were calculated as stated in section 2.4.

3.2.4. Intramuscular amino acid analysis

Muscle tissue (~25 mg) was prepared as outlined in section 2.4 for evaluation of myofibrillar protein synthesis. Muscle intracellular leucine was determined after precipitating the sarcoplasmic protein fraction with ethanol and centrifugation, before drying and centrifuging the resulting supernatant and taking up the intramuscular AA in lithium buffer. Concentrations of leucine were calculated according to the method of Bergstrom *et al.* (1974).

3.2.5. Immunoblotting, eIF4E·4EBP1 association and PKB kinase activity assay

Western blots were prepared and analysed as outlined in section 2.5. Phosphorylated protein of PKB Ser473, mTOR Ser2448, p70S6K Thr389, 4EBP1 Thr37/46, eIF4E Ser209, eEF2 Thr56 and eIF4G Ser1108, AMPK Thr172 and pan-actin (loading control) were measured (New England Biolabs; all 1:2000).

eIF4E·4EBP1 interaction was determined by incubating 100 – 200 µg protein lysates (prepared as for western blots) with 30 µl of m^7GTP-Sepharose containing a cap structure that recognises eIF4E (mixed in homogenisation buffer) for 2 h at 4 °C whilst rotating. Sepharose beads were then collected by centrifugation (4,000 rpm for 1 min) before washing in homogenisation buffer (4,000 rpm for 30 sec) three times. Bound proteins were then eluted in 50 µl 1 x Laemmli buffer by heating at 100 °C for 7 min. After centrifugation (4,000 rpm for 30 sec) eluted eIF4E and bound proteins were loaded onto a gel (15 µl) and blotted for eIF4E and 4EBP1, as outlined in section 2.5.

PKB was immunoprecipitated from 200 µg protein before incubation with 1 mM ATP and GSK3β Ser9 fusion protein substrate (New England Biolabs, Herts) in order to measure its kinase activity.

3.2.6. Statistical Analysis

Phosphorylation data were normalized to pan-actin to correct for loading anomalies and GSK3β phosphorylation from the PKB kinase assays according to total PKB recovery from immunoprecipitates. Data were tested for normality and, if normally distributed, differences were detected by repeated measures One-Way ANOVA using Tukey's Post Hoc test with GraphPad software (La Jolla, San Diego CA). $P<0.05$ was considered as significant. For temporal comparisons between FSR and other measures e.g. signalling protein phosphorylation, data sets were normalized over a range of 0-100% according to data span (i.e. for each data set 0% represents the lowest value and 100% the highest). Data are presented as means ± SEM.

3.3. Results

3.3.1. Feeding-induced changes in plasma concentrations of insulin and glucose

After protein feeding, the plasma insulin concentrations increased sharply, being elevated at 30 and 60 min (296 and 303%, respectively; $P<0.01$). However by 90 min after feeding, plasma insulin concentrations returned to postabsorptive values, where they remained for the rest of the study. Plasma glucose was steady, at typical postabsorptive values (~5.5 mM), throughout (figure 3.2).

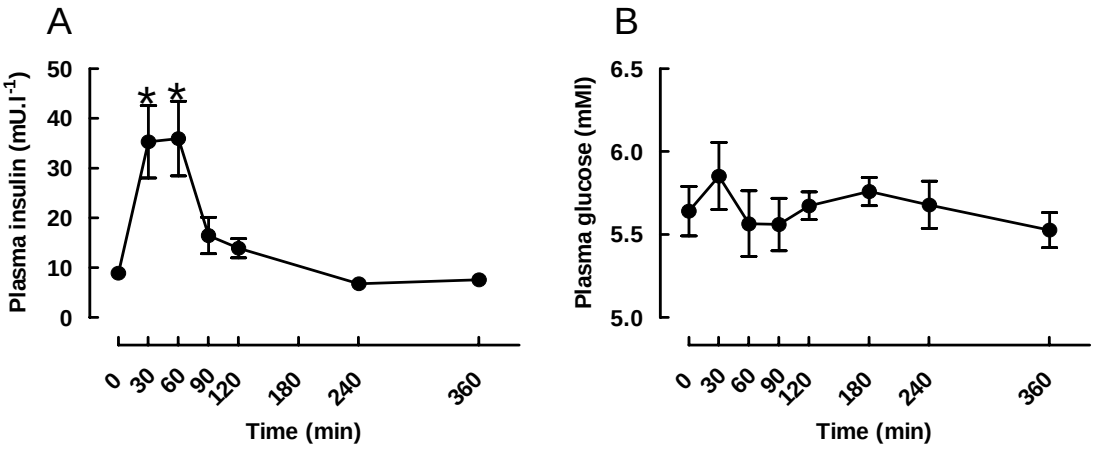

Figure 3.2. Plasma insulin (A) and glucose (B) in response to a 48 g whey protein bolus. * represents significant increase from postabsorptive values (P<0.05). Data are presented as means ± SEM; n = 8.

3.3.2. Feeding-induced changes in plasma concentrations of amino acids and intramuscular concentrations of leucine

Plasma alpha-ketoisocaproate (KIC) was increased by 66 % at 60 min and remained above postabsorptive concentrations throughout the measurement period i.e. up to at least 360 min (Figure 3.3). Plasma EAA were significantly increased after 30 min, peaking at 60 min (131%; P<0.01) and remaining elevated for 180 min (Figure 3.4A) whereas non essential amino acids (NEAA) despite increasing at 30 min (31%; P<0.05), returned to basal values by 120 min and by 360 min were significantly depressed (-13%, P<0.05) compared to postabsorptive values (Figure 3.4B). Plasma leucine was increased by 30 min, peaking at 60 min (229%; P<0.01) and remained significantly elevated until 240 min (Figure 3.4C) whereas intracellular leucine (Figure 3.4D) was increased between 90 and 180 min, peaking at 90 min (88%; P<0.01); both plasma and intracellular leucine concentrations decreased to postabsorptive values thereafter.

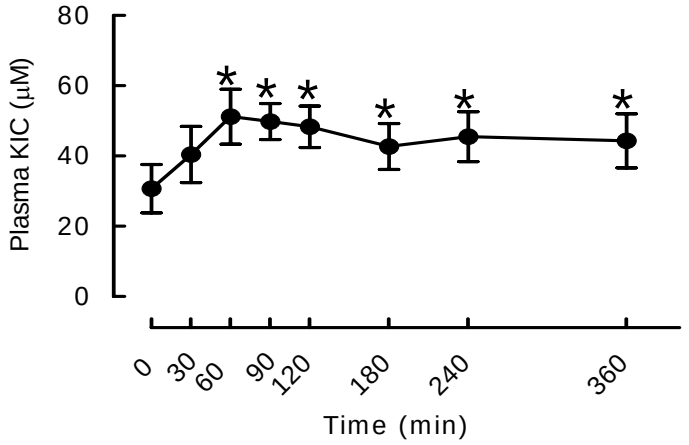

Figure 3.3. Plasma concentrations of alpha-ketoisocaproate (KIC) in response to a 48 g whey protein bolus. * represents significant increase from postabsorptive values (P<0.05). Data are presented as means ± SEM; n = 8.

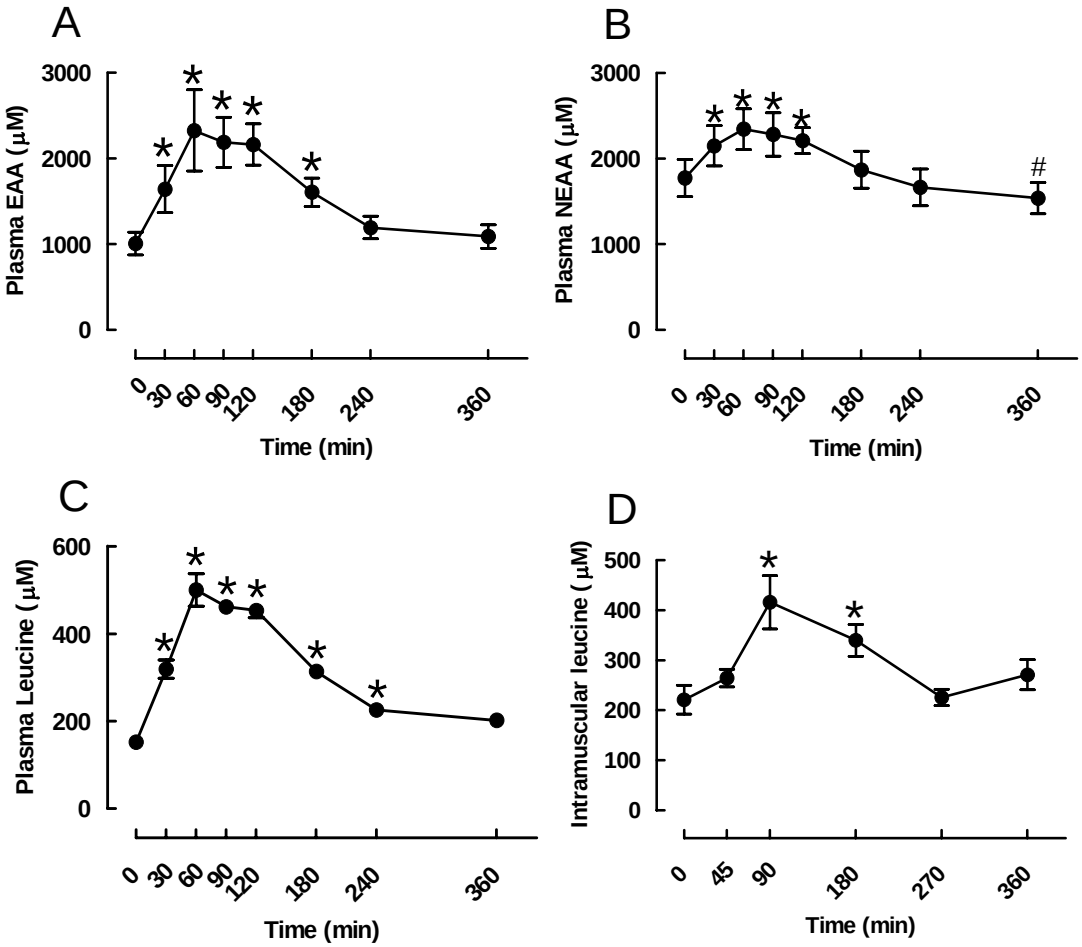

Figure 3.4. Plasma EAA (A), NEAA (B), leucine (C) and intramuscular concentrations of leucine (D) in response to a 48 g whey protein bolus. * represents significant increase from postabsorptive values; # represents a significant decrease from postabsorptive levels (P<0.05). Data are presented as means ± SEM; n = 8.

3.3.3. Temporal response of myofibrillar and sarcoplasmic FSR to feeding

Myofibrillar FSR increased significantly by 90 min post-feed (from 0.034±0.003 to 0.104±0.015 %·h^{-1}; P<0.05), returning to within baseline values by 180 min, where it remained for the duration of the study (Figure 3.5). Sarcoplasmic FSR followed an identical pattern, peaking by 46-90 min post-feed (from 0.052±0.006 to 0.106±0.012 %·h^{-1}; P<0.05) before returning to baseline values.

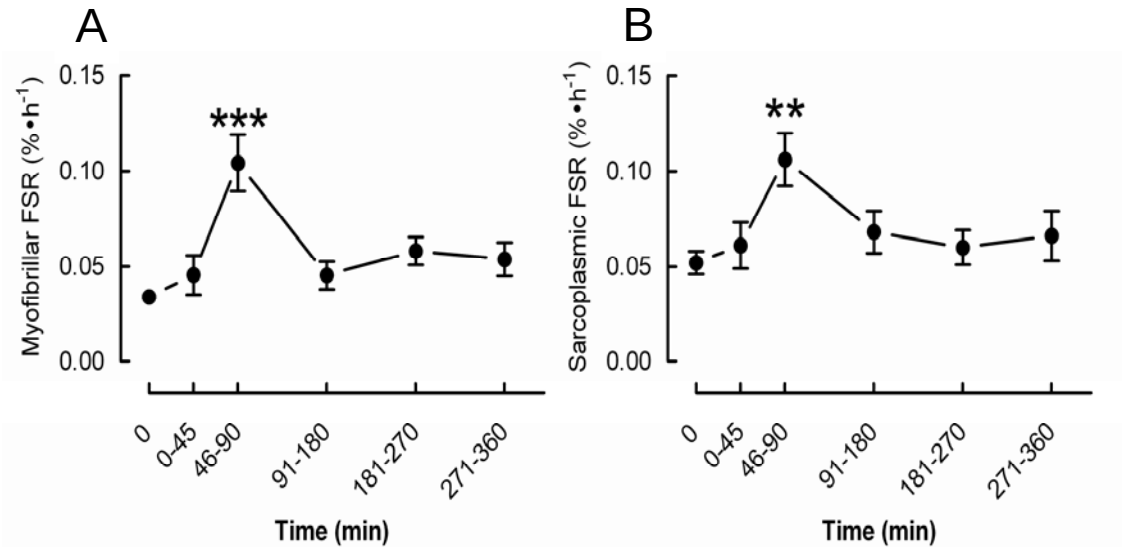

Figure 3.5. Myofibrillar (A) and sarcoplasmic (B) FSR in response to a 48 g whey protein bolus. * represent significant increase from postabsorptive levels (P<0.001). Data are presented as means ± SEM; n = 8.

3.3.4. Comparison of normalized feeding-induced changes in plasma insulin, leucine and intracellular leucine with MPS

When normalized for data span, the rise in insulin preceded MPS responses and fell with a similar pattern and mirrored PKB kinase activity (Figure 3.6A, B). In contrast, even though increases in plasma leucine concentration preceded FSR responses, it remained significantly elevated even though FSR had returned to postabsorptive values (Figure 3.6C). As with myofibrillar FSR, intracellular leucine was increased by 90 min, although the decline to postabsorptive values in myofibrillar FSR preceded the drop in intracellular leucine (Figure 3.6D).

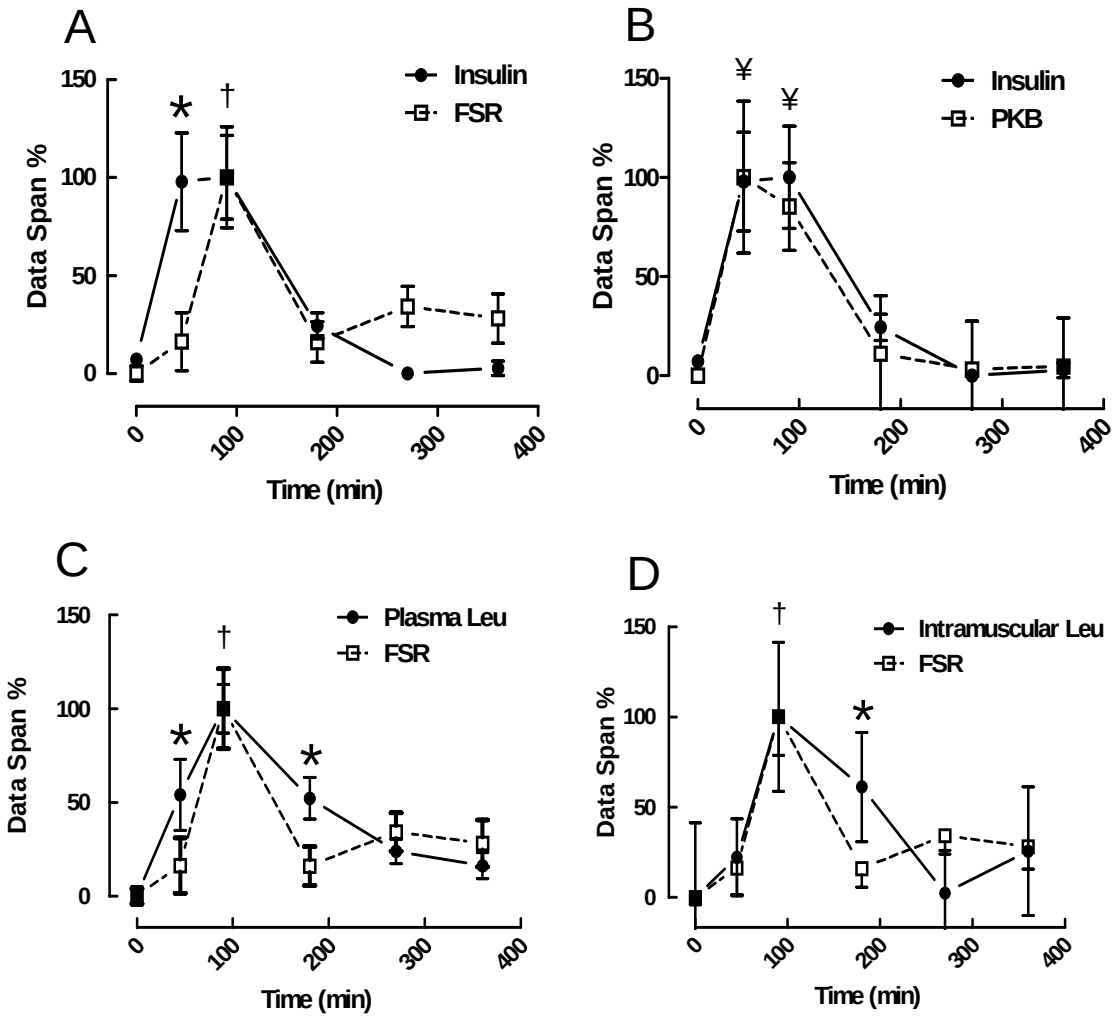

Figure 3.6. Normalized (data span = 100%) responses of myofibrillar FSR plotted with plasma insulin (A), plasma insulin plotted with PKB (B) and myofibrillar FSR plotted with plasma (C) and intracellular (D) leucine in response to a single 48 g whey protein bolus. * represents significant increase from postabsorptive values in plasma or intramuscular leucine, or plasma insulin; † represents a significant increase in both FSR and plasma insulin, leucine or intramuscular leucine; ¥ represents significant increase in both PKB kinase activity and plasma insulin (all where $P<0.05$). Data are presented as means ± SEM; $n = 8$.

3.3.5. Comparison of normalized feeding-induced changes in PKB, p70S6K1, 4EBP1 and eIF4G phosphorylation with MPS

After the protein feed, phosphorylation of PKB increased rapidly (1.6±0.2-fold and 1.5±0.1-fold at 45 and 90 min post-feed, respectively) and returned to postabsorptive levels at later time points. The time-course of the fold changes of PKB preceded the upswing in myofibrillar protein synthesis, though mirrored the decrease in MPS

(Figure 3.7A). p70S6K1 phosphorylation mirrored that of MPS initially increasing at 90 min (2.1±0.2-fold) but unlike FSR, remained elevated at 180 min (1.5±0.2-fold) post-feed, before falling towards postabsorptive levels at 270 and 360 min (Figure 3.7B). However the fall was slower than that of MPS and no longer matched the changes in MPS. 4EBP1 and eIF4G phosphorylation followed a time course similar to that of p70S6K1, increasing 2.6±0.5 and 2.2±0.3-fold at 90 and 180 min, respectively (Figure 3.7C, D).

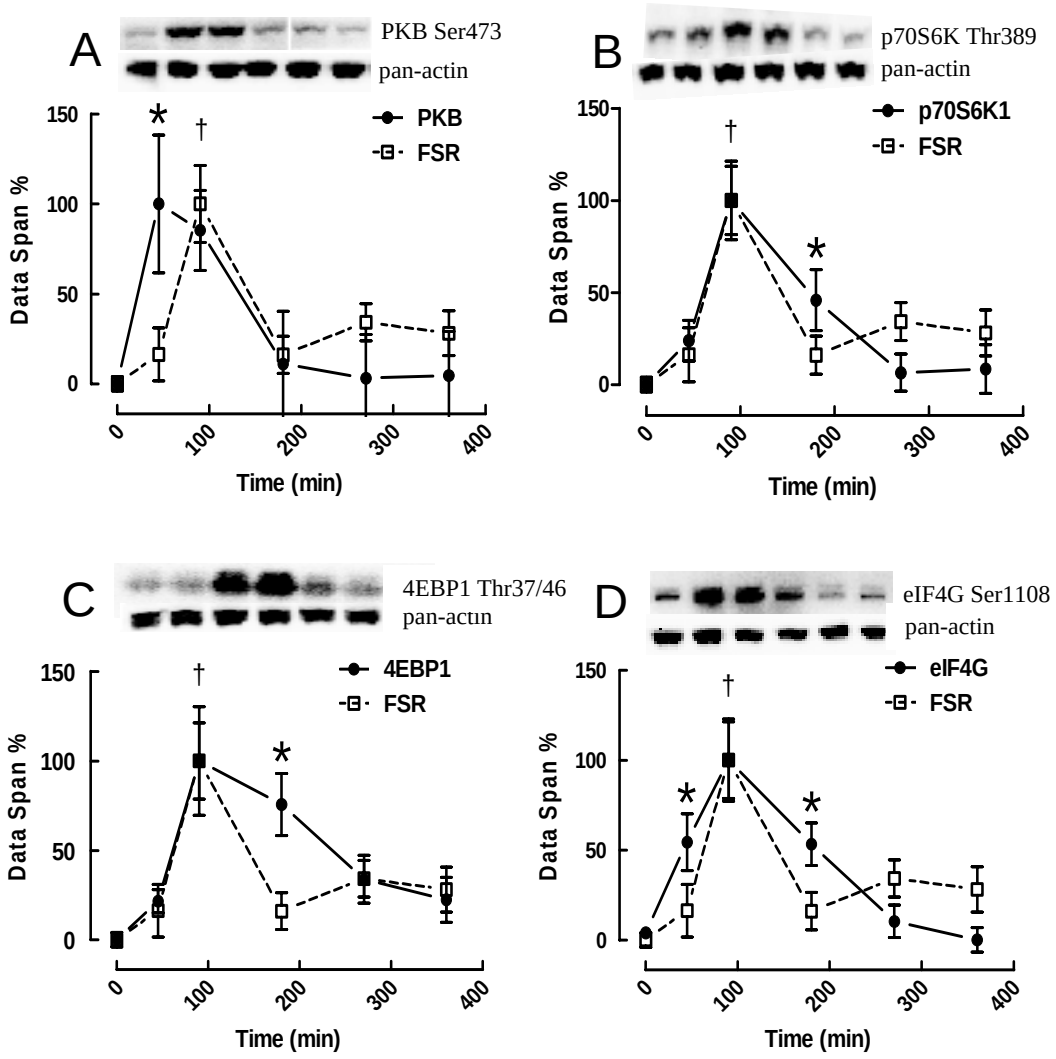

Figure 3.7. Normalized responses of myofibrillar FSR against PKB (A), p70S6K1 (B), 4EBP1 (C) and eIF4G (D) in response to a single 48 g whey protein bolus. Representative blots show all time points from one subject with corresponding loading controls, both obtained from one western blot. * represents significant increase from postabsorptive levels in signalling protein phosphorylation; † represents a significant increase in both FSR and signalling protein phosphorylation (P<0.05). Data are presented as means ± SEM; n = 8.

3.3.6. Feeding-induced changes in eIF4E·4EBP1 association

After the protein feed, eIF4E·4EBP1, normalized to eIF4E recovery, decreased by 90 min (0.33±0.05-fold) and remained depressed up to 180 min (0.21±0.08-fold, $P<0.05$) (Figure 3.8).

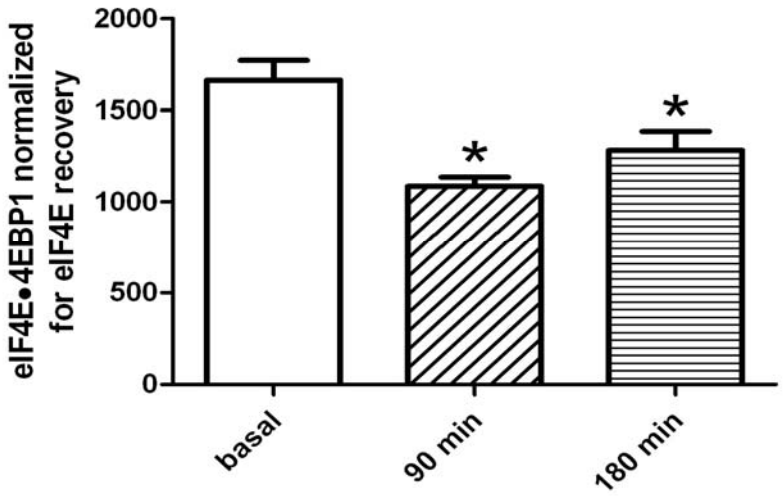

Figure 3.8. Changes in eIF4E association with 4EBP1 in response to a single 48 g whey protein bolus. * denotes a significant reduction in from postabsorptive values in eIF4E association with 4EBP1 ($P<0.05$). Data are presented as means ± SEM; $n = 8$.

3.3.7. Specific feeding-induced responses of AMPK, eEF2, ERK1/2 and eIF4E phosphorylation

After ingestion of the protein meal, phosphorylation of AMPK (Figure 3.9A) and eEF2 (Figure 3.9B) remained within postabsorptive values throughout; phosphorylation of ERK1/2 (Figure 3.9C) and eIF4E (Figure 3.9D) were also unchanged.

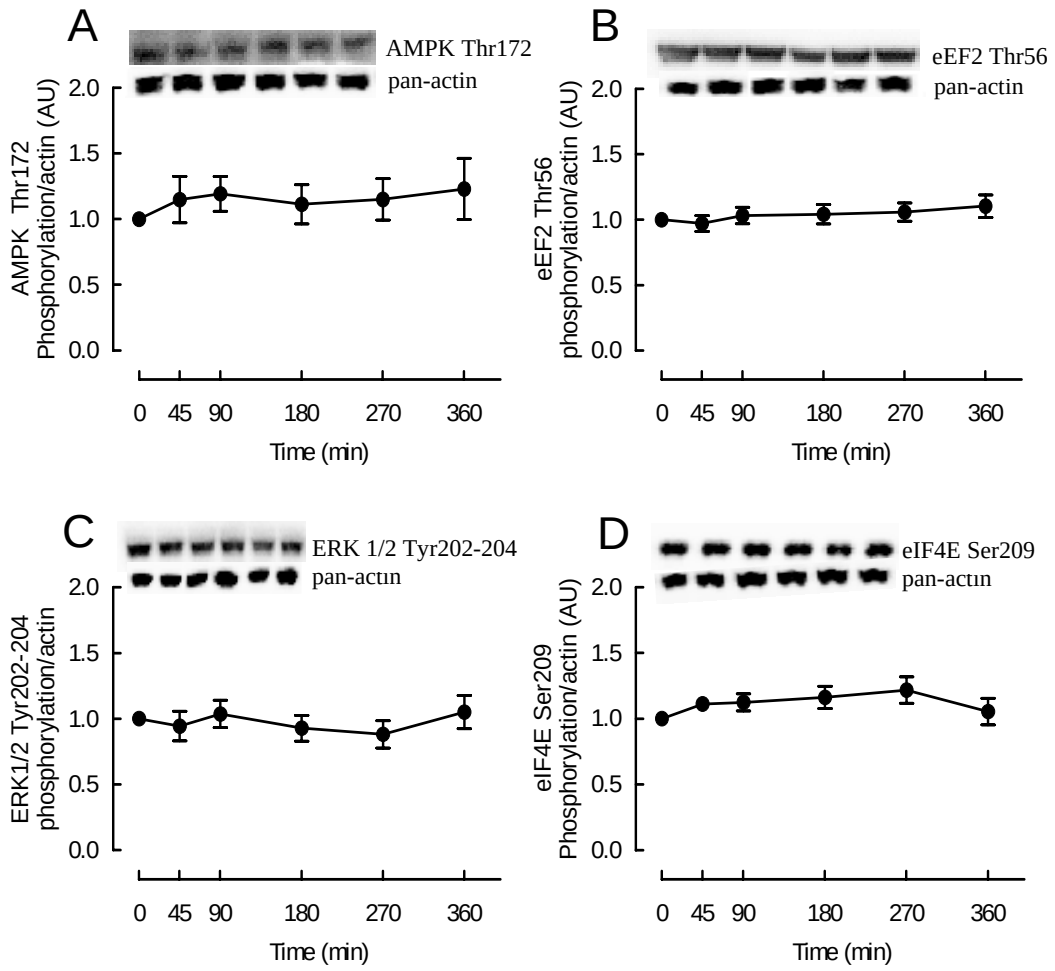

Figure 3.9. Phosphorylation of AMPK (A), eEF2 (B), ERK1/2 (C) and eIF4E (D) in response to a 48 g whey protein bolus. All values remained unchanged from postabsorptive values throughout the study (P>0.05). Representative blots show all time points from one subject with corresponding loading controls, both obtained from one Western blot. Data are presented as means ± SEM; n = 8.

3.4. Discussion

This study reports novel findings representing the time-course of response in MPS and associated signalling to a physiologically relevant protein feed. We have shown that in response to a single oral bolus of whey protein (i) after a latent period of ~45 min, MPS is approximately tripled, and sarcoplasmic protein synthesis doubled, between 45 and 90 min before rapidly returning to postabsorptive rates, despite continued availability of intracellular leucine and plasma EAA, (ii) the stimulation of

MPS is preceded by the stimulation of PKB kinase activity and phosphorylation of eIF4G and coincides with S6K1 and 4EBP1 phosphorylation, and (iii) congruent with the original hypothesis, the rapid return to basal values in MPS precedes both p70S6K1, 4EBP1 and eIF4G dephosphorylation.

The latency and duration of MPS in human muscle using constant infusions of amino acids has been reported previously, whereby myofibrillar and sarcoplasmic MPS are stimulated approximately three-fold for a period of ~2 h before rates returned to basal values despite continued amino acid availability (Bohé *et al.* 2001). This study provides further support to these findings in response to an oral protein feed where the response of MPS is similar in both amplitude and duration to that seen after AA infusion. Thus, it appears that by shortening the period of measurement post-feeding (i.e. 45-90 min), myofibrillar protein synthesis is approximately tripled, and sarcoplasmic doubled after consuming maximal doses of AA and that the lower values for MPS measured by others are likely due to more prolonged measurement periods (Moore *et al.* 2009). Furthermore, it is now apparent taking these studies together that the return of MPS to basal values is not a consequence of amino acid depletion from plasma or intracellular pools. These findings make it likely that muscle can gauge its capacity to synthesize new proteins (i.e. reduce rates of MPS back to basal levels) even when the activating stimulus (i.e. increased amino acid availability) in both plasma and muscle cells continues; thus supporting previous reports of the existence of a 'muscle full' phenomenon in response to feeding (Millward, 1995).

What signals could be regulating the switch on and off in MPS after feeding? A number of studies in both rodent (Anthony *et al.* 2000b; Vary *et al.* 2007) and human

models (Fujita *et al.* 2007; Cuthbertson *et al.* 2005; Smith *et al.* 2009) have reported that the mTORC1 pathway is central to the regulation of feeding-induced changes in MPS. These findings provide further evidence for this involvement, at least as indexed by phosphorylation of rapamycin (mTORC1) sensitive substrates, S6K1 and 4EBP1 after feeding. Nevertheless, whether or not EAA directly regulate mTORC1 activity is unresolved with it becoming increasingly well recognized that other proximal regulators exist including mitogen-activated protein kinase kinase kinase kinase 3 (MAP4K3) (Findlay *et al.* 2007), vacuolar protein sorting 34 (vps34) (Nobukuni *et al.* 2005) and the recombination activating G proteins (Rags) (Sancak *et al.* 2008). Importantly, no increase in mTOR phosphorylation at Ser2448 was observed in the present study, suggesting that such putative proximal regulators (Rags, MAP4K3 etc) may control mTORC1 via means independent of this site; perhaps instead via regulating raptor•mTOR interaction (Beevers *et al.* 2009) or via phosphorylation of another site, such as Thr2446 (Cheng *et al.* 2004).

Perhaps the most comparable study to that of the present investigation was that by Fujita and colleagues in which some similarities with our data exist e.g. increases in S6K1 and 4EBP1 phosphorylation ~ 1 h after feeding a meal containing ~20 g EAA (Fujita *et al.* 2007). However, these studies also described increases in mTOR Ser2448 phosphorylation and depressions (i.e. stimulatory) in that of eEF2 Thr56 and AMPK Thr172 which was not seen here. There were subtle differences between this study and that of Fujita and colleagues including biopsy timing (45 vs. 60 min) , and feed composition and resultant plasma glucose (provision of whey protein isolate vs. EAA + carbohydrates). Therefore, as insulin responses and EAA load were similar in both studies, it should perhaps have been expected for similar

changes to be observed in mTOR, AMPK, and eEF2 phosphorylation; that is unless glucose metabolism could play a role. For example, internalized glucose could signal independently of insulin, or if plasma glucose availability were translated into increased muscle glycogen storage, the activity of AMPK, which has a glycogen binding domain which when bound inhibits its activity (McBride *et al.* 2009), could be suppressed and promote dephosphorylation of eEF2K at Thr398 (Horman *et al.* 2004), and subsequently of eEF2 at Thr56. That said it is perhaps unlikely that muscle glycogen was significantly increased over a 1 h postprandial period (Carey *et al.* 2003), especially as the present study and that of Fujita and colleagues were performed in the overnight fasted (and thus partially glycogen depleted) state.

If one is to discriminate regulatory from non-regulatory phosphorylation events it is essential to provide a time course in order to match changes in MPS (spanning both the upward and downward curves) to those of the putative regulatory signals. The present study provides this data for the first time in response to feeding in humans and identify, at least for the feeding-induced *increase* in MPS, a potential role for PKB/mTORC1 signalling. For example, the activity of PKB and eIF4G preceded that of MPS, increasing by 45 min. For PKB at least, it is likely that the increase in its activity was insulin mediated since it mirrored the rise and fall in plasma insulin and EAA do not activate PI3K or PKB (Hara *et al.* 1998). The fact that eIF4G preceded other indices of mTORC1 signalling may indicate that its activity is PKB dependent and/or independent of mTORC1 signalling. Support for this preposition may be found in work showing that dose-responses in MPS responses to leucine are closely reflected by the amplitude of eIF4G but not S6K1 and 4EBP1 phosphorylation (Bolster *et al.* 2004). It may be speculated that an increase in those

signals that preceded a measured incorporation of labelled leucine in to myofibrillar protein (i.e. PKB and eIF4G) could be important in the response of MPS to feeding since one would expect the regulatory signal (i.e. phosphorylation) to head the biological response (i.e. MPS). In addition to sustained PKB activity and eIF4G phosphorylation for up to 90 min, two of the best characterized mTORC1 substrates, S6K1 and 4EBP1, were increased by 90 min which suggests that their activity may be required for sustaining increased MPS, rather than regulating the initial increase.

What of the decline in MPS? Much less of an explanation for this was found presently because aside from PKB, mTORC1 substrates (i.e. S6K1 and 4EBP1) remained phosphorylated, and the eIF4F complex remained assembled (as indicated by eIF4E·4EBP1 association), long after MPS had returned to baseline, which likely explains past reports of phosphorylation increases >2 h of feeding (Cuthbertson *et al.* 2005) despite the decline in MPS. It is tempting to speculate that the raised concentrations of either or both plasma and intracellular amino acid availability are responsible for the continued stimulation and that another signal may exist to essentially 'override' amino acid-induced signalling. But what could this be, and how would it signal? One possibility is through negative feedback via metabolites generated by the deamination of excess amino acids; indeed, prolonged plasma KIC concentrations were present throughout this study suggesting ongoing oxidation. Nonetheless, if this were the case, how these metabolites would signal muscle full remains to be investigated. In conclusion, mTORC1 signalling may control the increase in MPS after protein ingestion with the caveat being, if these signals are regulating MPS responses to amino acids it remains puzzling as to the functional significance of anabolic signalling outlasting the MPS responses.

CHAPTER 4. THE EFFECTS OF A SINGLE ORAL PROTEIN MEAL ON RECOVERY OF MUSCLE FUNCTION FOLLOWING AN ACUTE ECCENTRIC EXERCISE BOUT

4.1. Introduction

The decrements in muscular flexibility, force and power production, as well as increases in sensations of muscle pain, following a single bout of unaccustomed exercise are well documented (Armstrong 1984; Gulick *et al.* 1996; Jones *et al.* 1986; Prasartwuth *et al.* 2005; Twist and Eston 2005). The result is a reduction in individuals' capacity to meet the demands of activity associated with daily living and work. Additionally, athletic populations may only be able to train with reduced intensity, leading to sub-maximal total training-induced improvements in performance. Thus, any practice that restricts the decrement in function or augments the recovery process would have valuable practical implications for therapists, trainers, and athletes alike. Despite, however, the practical importance of therapeutic discovery for post-exercise muscle damage and/or soreness (hereafter collectively termed 'DOMS') effective treatment strategies have remained elusive.

Establishing a causal mechanism(s) of DOMS has historically proved difficult. This scenario has impaired the discovery of effective treatment strategies for alleviating the negative symptoms associated with DOMS, and has led to a large number of treatment strategies aimed at addressing the various proposed mechanisms of DOMS to be investigated. Many of these have yielded insignificant or, at best, equivocal results. For example, stretching (Johansson *et al.* 1999), cryotherapy (Gulick and

Kimura 1996), massage (Lightfoot *et al.* 1997), hyperbaric oxygen therapy (Harrison *et al.* 2001), ultrasound (Ciccone *et al.* 1991), and carbohydrate supplementation (Close *et al.* 2005) all translated to little or no improvement in perceptions of muscle soreness, performance, or haematological markers, such as creatine kinase (CK). Thus investigation of other possible methods of alleviating the symptoms associated with DOMS is required.

It is known that early intake of protein after exercise stimulates positive NPB following both resistance (Rasmussen *et al.* 2000) and endurance exercise (Rodriguez *et al.* 2007). The potent stimulatory effect of 40 g (Tipton *et al.* 1999b) and 6 g (Rasmussen *et al.* 2000; Borsheim *et al.* 2002) of essential amino acids (EAAs) on increasing protein synthesis and, to a lesser extent, decreasing protein breakdown may augment muscle repair following acute damage by increased turnover and net deposition of contractile proteins (Biolo *et al.* 1997; Phillips *et al.* 1997). Measures of muscle function were not taken in these studies and so application of this strategy to performance (for example, strength and power production) is currently unknown.

Chronic protein supplementation to enhance recovery of blood markers of muscle damage and perceptions of pain during the DOMS period has received some attention over recent years. Prolonged amino acid intake, administered over a number of days to weeks, has been shown by various authors to attenuate the rise of plasma CK and sensations of muscle soreness induced by a heavy bout of resistance or endurance exercise (Coombes and McNaughton 2000; Knitter *et al.* 2000; Kraemer *et al.* 2009; Nosaka *et al.* 2006; Ohtani *et al.* 2001; Skillen *et al.* 2008). The

haematological and perceived soreness response to acute protein supplementation, however, is less clear. For example, 3 g of the leucine metabolite β-hydroxy-β-methylbutyrate (Wilson *et al.* 2009) or 25 g whey protein isolate (Buckley *et al.* 2008) ingested either pre- or post-resistance exercise, and the amino acid mixture of Nosaka *et al.* (2006) administered pre- and post-exercise failed to attenuate the exercise-induced rises in CK and perceptions of muscle soreness. Conversely, 42 g whey protein ingested before and after whole-body resistance exercise at of 80% 1 RM (Hoffman *et al.* 2009); acute BCAA intake pre- and 60 min during three 90 min cycling exercise bouts at 55% VO_2peak (Greer *et al.* 2007), BCAA ingestion (77 $mg \cdot kg^{-1}$ body mass) prior to 140 squat exercise (Shimomura *et al.* 2006) and; protein-enriched mixed meal consumption after repeated sprint cycling exercise (Rowlands *et al.* 2007) all attenuated levels of perceived muscle soreness and plasma CK concentrations at 24 h and 48 h (Greer *et al.* 2007; Hoffman *et al.* 2009; Rowlands *et al.* 2007), up to 4 d post-exercise (Shimomura *et al.* 2006). As such, the precise effects of acutely ingested protein meals on indices of DOMS remain equivocal.

These studies provide valuable insight into the potential of protein-based supplements to affect haematological indicators and perceptions of pain associated with DOMS. However, the effect of protein supplementation for recovering indices of muscle function following acute DOMS-inducing exercise is less clear. Recently, this topic has gained increased investigative attention, though the precise effects of protein intake on functional recovery remain controversial. Longitudinal studies suggest that mixed amino acid supplementation reduces the initial loss of strength and power associated with the initiation of a 4 wk resistance training regimen

(Ratamess *et al.* 2003). Furthermore, 10 $g \cdot d^{-1}$ protein added to regular dietary intake over a 54 d training period improves health and reduces the incidence of muscle soreness (Flakoll *et al.* 2004), and 14 g whey protein plus 6 g mixed amino acids ingested pre- and post-exercise over ten weeks resistance training resulted in increased fat-free mass, thigh cross-sectional area, serum concentrations of growth hormones and levels of muscle myofibrillar proteins versus a placebo control (Willoughby *et al.* 2007). Supporting these findings are acute studies demonstrating that BCAA ingestion pre- and 60 min-during three 90 min moderate (55% VO_2max) cycling exercise bouts improves functional recovery of leg-flexion torque at 48 h (Greer *et al.* 2007), though the exercise mode utilised may not induce a large muscle-damaging response owing to the lack of an eccentric-component, and the inclusion of CHO makes determination of any effect of protein alone difficult due to the potential for insulin mediated alterations in protein metabolism. Furthermore, whey protein ingestion (25 g) recovered peak isometric torque at 6 h post-resistance exercise (Buckley *et al.* 2008), though lack of a cross-over design and small subject numbers make inference of this observation difficult. Contradicting these studies, however, Green *et al.* (2008) found that a single CHO-protein drink (protein content = 0.3 $g \cdot kg^{-1}$ body mass) consumed immediately after 30 min downhill treadmill running had no affect on maximal voluntary isometric quadriceps force at 24, 48 and 72 h post-exercise. Similarly, Rowlands *et al.* (2007) showed no improvement in recovery of MVC force and sprint power output at 24 h post-exercise following ingestion of a mixed meal containing 218 g protein, though 24 h may have been an insufficient timeframe in which to observe any beneficial effects of protein ingestion upon functional recovery. It is further observed that a common limitation to the above studies is that only measures of muscle isometric force were recorded as an

indicator of muscle function. Whilst informative, this may not be indicative of the type of muscular contractions performed in many sporting/daily living activities. One exception to this is the study of Rowlands *et al.* (2007), which measured muscular power, though was only assessed at 24 h post-exercise. Additionally all the above studies, with the exception of Buckley *et al.* (2008), administered a protein-based supplement that also contained CHO, which may have influenced results due to the potential for insulin-mediated effects on protein metabolism (Greenhaff *et al.* 2008; Wilkes *et al.* 2009). The effects of protein intake alone, therefore, remain obscure.

Thus, the effects of a single post-exercise protein bolus on recovery, particularly of muscle force and power following DOMS-inducing exercise remains to be determined. The aim of this study was, therefore, to investigate whether ingestion of a single high-protein dose immediately following an eccentric exercise bout could enhance recovery of perceived muscle soreness, haematological markers of damage, or muscle force and power in the 72 h period during which DOMS occurs. It was hypothesized that post-exercise protein ingestion would augment recovery of indices of muscle damage during the DOMS period.

4.2. Methods

4.2.1. Study design

Nine recreationally active male subjects (age 21 ± 1 y; BMI 23.5 ± 0.8 $kg.m^2$) took part in the study. Each subject visited the laboratory on two occasions, separated by a minimum of 28 days. This recovery period was chosen based on pilot data showing a

return to baseline values in all measured parameters. Prior to exercise, a 10 mL venous blood sample was obtained. MVC was determined as detailed in section 2.6. Peak power output (PPO) was determined as the highest value recorded from three maximal 5 s sprints on a cycle ergometer fitted with SRM power cranks (Powermeter V cranks, SRM training systems, SRM Germany). Subjects cycled from a stationary position against a resistance equal to 7.5% body mass and were instructed to remain seated throughout each sprint to reduce contribution from muscles other than the quadriceps group. Subjects returned to the laboratory 24, 48, and 72 h after the downhill running protocol, at which times a blood sample, assessment of muscle soreness, and performance measurements were recorded.

4.2.2. Downhill running protocol

Prior to the beginning of each exercise bout, subjects completed a 5 min warm up at a gradient of 0% using self-selected speeds. On completion of the warm up the treadmill gradient was set to –10% and subjects ran for a 30 min period maintaining a target heart rate of 75% predicted heart rate maximum (= 220 – age; beats·min^{-1}). This intensity is consistent with previous literature that used downhill running to induce DOMS (Braun and Dutto, 2003). Treadmill speed was altered accordingly during the 30 min to maintain the target heart rate. These speeds, and the time at which they occurred, were recorded and reproduced when subjects returned for their second trial.

4.2.3. Supplement intervention

The study's supplementation protocol followed a double-blind, randomized, cross-over design. Immediately following the 30 min downhill run, subjects ingested either

100 g milk protein concentrate solution containing 40 g EAAs (http:// www.myprotein.co.uk for amino acid sequence) with no carbohydrate component (PRO), or a flavoured water placebo solution (CON). This amount of EAAs was chosen based on previous studies showing an increase in postexercise protein synthesis using this concentration (Rasmussen et al. 2000; Tipton et al. 1999b). Post-exercise supplementation was chosen in light of recent research by Tipton et al. (2007) reporting that, in contrast to the response of net protein balance to timing of EAA ingestion (Tipton et al. 2001), post-exercise intake of a whole whey protein solution stimulated increases in protein synthesis comparable to those stimulated by pre-exercise ingestion. No carbohydrate was included in the placebo drink to avoid any confounding insulin-mediated effects on protein metabolism (Borsheim *et al.* 2004). Both solutions were made to a final volume of 1 L. Subjects were required to replicate their dietary intake 24 h prior to, and in the 72 h post-exercise for the two experimental trials, and record their diets during the first 24 h post-exercise. Consumption of additional meals was allowed 3 h after supplement ingestion.

4.2.4. Assessment of DOMS

Delayed-onset muscle soreness (DOMS) values were obtained using a visual analogue scale (Lee *et al.* 2002). The scale ranged from no muscle soreness (value of 1) to very very sore (value of 10). Subjects were asked to assess the pain in both legs, and the mean value of the two scores was taken. Sensitivity to muscle pain was assessed using a strain gauge algometer fitted with a 1 cm foam pad (Force Dial1 FDK/FDN series, Wagner instruments, Greenwich, Conn.). The algometer was used to assess deep muscle pain and trigger point tenderness of the quadriceps muscle group. The reliability and validity of the algometer has been established previously

(Fischer, 1987). The algometer was pressed parallel to the skin with increasing pressure at the approximate mid-point of the vastus lateralis, vastus medialis, and rectus femoris. These points were marked prior to exercise on day 1 of each testing period to allow consistency between measurements. The test was stopped when the sensation of pressure became uncomfortable, though not painful, for the subject. All measurements were taken in triplicate from the right thigh with the subject lying supine, and then repeated in a seated position with the knee at a 908 angle. The values of the three sites were averaged, allowing expression of muscle tenderness as a single value.

4.2.5. Blood markers and analysis

A 10 mL blood sample was taken from an antecubital vein before the eccentric exercise, and at 24, 48, and 72 h after exercise. The collected sample was placed in a clear tube and allowed to clot, centrifuged at 5000 $r \cdot min^{-1}$ for 10 min, and the separated serum drawn off and frozen at –20°C until analysis. Creatine kinase (CK) concentrations were determined in duplicate using a VITROS1 DT60 II dry slide clinical chemistry system (Ortho-Clinical Diagnostics, Amersham, UK). Serum protein carbonyl (PC) content was determined in triplicate using a commercially available enzyme-linked immunosorbent assay (ELISA; Zenith Technology Corporation Ltd., Dunedin, New Zealand) following previously described methods (Buss *et al.* 1997). The coefficient of variation was 4.8% for CK and was 3.1% for PC from ten separate samples. The intra-assay variation for the PC ELISA was 2% determined from five separate samples analysed in triplicate. CK was chosen as a marker of muscle damage for its sensitivity to discrete lesions in the cellular membrane, whereas PC was chosen as a marker of oxidative stress, as its release has

previously been shown to demonstrate a strong correlation with the time-course effects of DOMS (Lee *et al.* 2002).

4.2.6. Statistical analyses

Results are reported as mean ± SEM. Differences in blood markers, performance variables, and assessment of DOMS were analysed between conditions by a repeated-measures analysis of variance (ANOVA) following confirmation of normal distribution of data (SPSS version 12). When a significant time or condition interaction was obtained a Tukey's post hoc test was performed to identify the point of difference. Pearson's product–moment correlation test was performed to assess relationships between plasma CK and PC concentration and subjects' perceptions of muscle soreness.

4.3. Results

4.3.1. Post-eccentric exercise changes in perceived muscle soreness with and without protein ingestion

The effect of the downhill run on rating of muscle soreness and perceived pain is presented in Table 4.1. A significant increase in pain sensation and perceived soreness was experienced by all participants under each condition ($P< 0.05$), suggesting that the downhill running protocol was sufficient in eliciting muscle soreness and DOMS from the exercise. There were no differences observed in rating of muscle soreness at any time point between the two experimental conditions.

Table 4.1. Algometry and perceptual assessment of muscle soreness during the 72 h post-exercise period. Note: All values were significantly elevated from pre-eccentric exercise values ($P< 0.05$). Data are presented as means ± SEM; $n = 9$. Units are pounds (lb) of pressure applied to the muscle to obtain sensations of soreness.

Time (h)	Trial	Perceived soreness at 90° (lb)	Perceived soreness at 180° (lb)	Visual analogue score
24	CON	26.2±0.9	23.1±0.9	4.9±0.5
	PRO	26.4±1.4	23.8±1.3	5±0.3
48	CON	25.1±1.1	22.7±1.5	4.8±0.3
	PRO	26.8±1.2	26.7±1.2	5.4±0.4
72	CON	28.6±1.5	26.7±1.5	5.6±0.3
	PRO	30.6±1.3	27.9±1.4	5.9±0.4

4.3.2. Influence of post-eccentric exercise protein intake on serum concentrations of muscle damage markers

Serum CK was significantly elevated after the downhill run, peaking at 24 h in both conditions ($P< 0.01$), and declining to within pre-exercise values at 48 and 72 h ($P> 0.05$), though remaining elevated (Figure 4.1A). No significant differences in CK concentrations were observable between PRO and CON. Serum PC concentrations were significantly raised post-eccentric exercise ($P< 0.05$), peaking after 24 h in the protein trial and after 72 h in the control trial. There were no differences in the concentration of PC between the two experimental conditions (Figure 4.1B). A strong correlation existed between PC concentrations and the time course of the muscle soreness response in the CON condition ($R_2 = 0.98$), which was replicated in the PRO trial ($R_2 = 0.94$). The CK response was very different, peaking after 24 h and returning to near-baseline levels after 48 and 72 h in both conditions. Accordingly, the CK correlation with the perceived pain response was weak for both the CON ($R_2 = 0.36$) and PRO supplements ($R_2 = 0.34$).

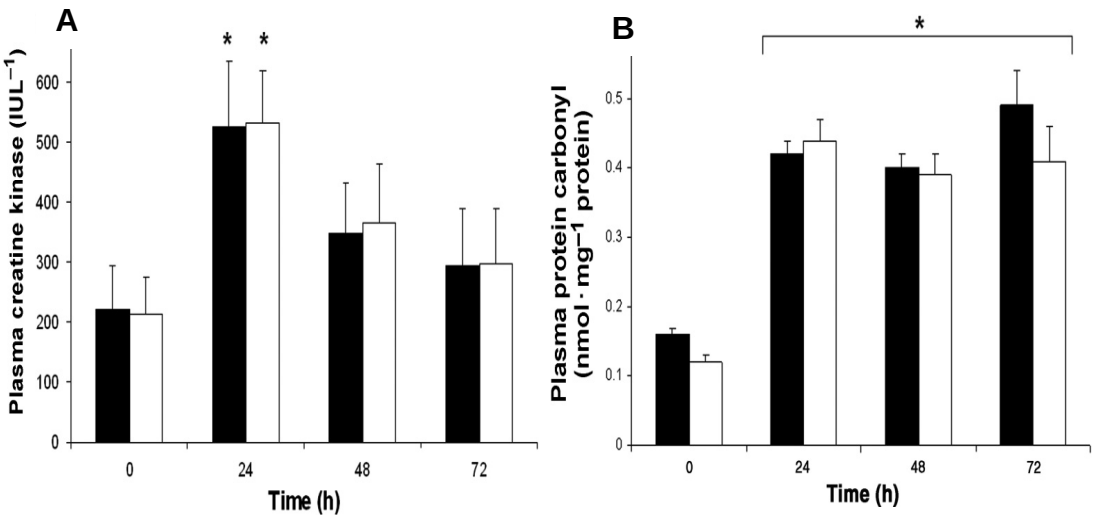

Figure 4.1. Mean change in plasma creatine kinase (A) and protein carbonyl (B) concentrations during the 72 h post exercise period. □, placebo group; ■, protein group. * denotes significant difference from baseline values ($P < 0.05$). Data are presented as means ± SEM; $n = 9$.

4.3.3. Effects of protein ingestion on functional recovery of muscle post-eccentric exercise

The absolute changes in peak MVC and PPO are reported in Figures 4.2A and 4.2B, respectively. Absolute values for MVC and PPO showed a significant difference between the PRO and CON conditions for both measures of muscle function ($P< 0.05$). This difference was found at the 48 h time point for both MVC and PPO. MVC declined following the downhill run in both experimental conditions, and a significant decline in force production occurred at 24 h (–7.9%) and 48 h (–10.7%) in the control trial ($P< 0.05$). Peak MVC remained within pre-exercise values at all time points in the PRO trial, reaching a non-significant nadir of –6.6% after 24 h and recovering to –0.25% at 48 h. PPO decline followed a similar pattern to MVC following the CON condition with the greatest decline observed at 48 h (–8.7%, $P< 0.05$,) whereas PPO was lowest at 24 h during the PRO trial (–7.9%, $P< 0.05$), but had returned to baseline at 48 and 72 h.

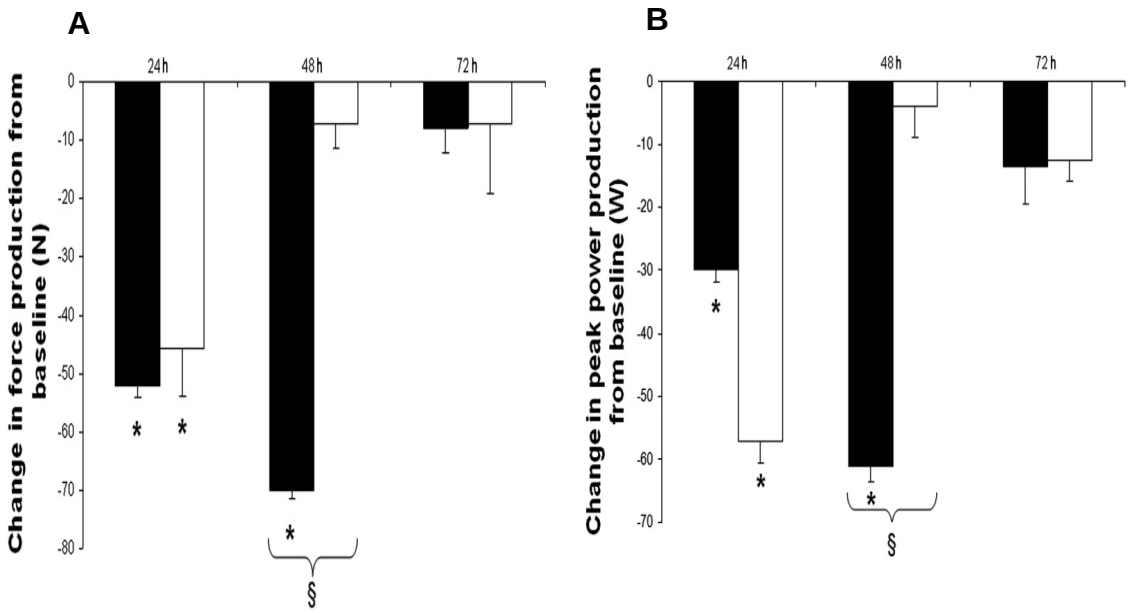

Figure 4.2. Absolute change in MVC (A) and PPO (B) during the 72 h post-exercise period. □, placebo group; ■, protein group. Asterisk (*) indicates significant difference from baseline values (P< 0.05); section symbol (§) indicates significant difference between conditions (P< 0.05). Data are presented as means ± SEM; n = 9.

4.4. Discussion

The principal finding of this study is that ingesting a single dose of 100 g of protein containing 40 g of EAAs immediately after an eccentric exercise bout increases the recovery rate of isometric force and dynamic power production during the DOMS period. Both MVC and PPO reached a significant nadir at 48 h in the placebo trial (–10% and –8.7% for MVC and PPO, respectively; p < 0.05), compared with a return to within pre-exercise values for both measures of muscle function following protein intake. This increased rate of recovery in the functional output of damaged muscle was independent of the circulating CK and PC response and perceptions of muscle soreness, which did not differ significantly between conditions.

The present study is the first to show that a single oral protein meal administered immediately after exercise-induced DOMS assists the restoration of isometric force and peak power production at 48 h, when the functional consequences of DOMS are often at their greatest (Cheung *et al.* 2003). It can be postulated, in light of previous research, that this effect may be due to effects on increased rates of protein synthesis stimulated by the 40 g of EAAs. Although not measured here, intracellular amino acid availability is a key determining factor regulating protein balance in response to exercise (Tipton and Wolfe 2004). When amino acid availability is increased via exogenous protein administration, net protein balance increases to a net protein gain in the post-exercise period, compared with a net protein loss in the fasted state (Tipton *et al.* 2007). Such an increase in protein synthesis could be expected to lead to greater synthesis of contractile proteins (Esmarck *et al.* 2001; Phillips *et al.* 1997) and potentially a more rapid restoration or enhancement of contractile force, as shown by the present results. Furthermore, an acute protein meal stimulates increased plasma insulin concentrations, which may act to inhibit the rate of protein breakdown in muscle (Anthony *et al.* 2002; Crozier *et al.* 2005; Escobar *et al.* 2005). If recovery is dependent on replacing "damaged muscle" then increased MPB is required for increases in protein turnover and thus more rapid repair via replacement of damaged proteins. However, several authors have reported no inhibition of MPB with provision of AA after exercise (Biolo *et al.* 1997; Borsheim *et al.* 2002; Louis *et al.* 1995; Rassmussen *et al.* 2000; Tipton *et al.* 1999a). Additionally, since the protein fed group showed signs of force reduction and other measures of post-exercise muscle damage (i.e. increased CK / PC concentrations) then the process that leads to the more rapid return of force generation may not depend on the immediate effects on protein metabolism. However, increased MPS following post-exercise

protein feeding may lead to the synthesis of new proteins in order to replace those damaged by exercise without any requirement to break down the “damaged” proteins, thus assisting the restoration of muscle function.

No differences were observed in CK, PC or perceptions of muscle soreness between the protein supplement and placebo at all time points. This is in agreement with observations made by Nosaka *et al.* (2006), i.e., that CK and muscle soreness remained unaffected with only pre- and post-exercise amino acid administration. This finding may not be surprising, since the principle mechanism through which post-exercise muscle soreness is thought to occur is inflammation resulting from damage to the extra-cellular matrix (ECM) (Kjaer, 2004). Muscle collagen protein synthetic rates are unaffected by acute protein ingestion (Babraj *et al.* 2005). Furthermore, whilst intramuscular collagen protein synthesis is augmented after both low and high intensity exercise, this effect is not augmented by the addition of protein feeding (Holm *et al.* 2009). Thus, it may be expected that, in the present study, a single protein bolus would not increase the rate of collagen repair and, therefore, not assist the recovery of soreness following muscle damage.

However, the observed lack of effect of acute protein ingestion on perceived soreness and CK concentrations is in contrast to studies investigating prolonged amino acid supplementation, which consistently showed improvements (reductions) in muscle soreness and CK levels after exercise (Coombes and McNaughton 2000; Knitter et al. 2000; Nosaka et al. 2006; Ohtani et al. 2001). This discrepancy between acute and chronic studies may suggest that, while damage to the ECM is currently thought to be the predominant cause of DOMS, other, as yet unknown mechanisms

may contribute towards increased muscle soreness after eccentric exercise that may be responsive to long-term protein supplementation. Thus, it seems that a single protein meal stimulates intramuscular adaptations that lead to improvements in the functional output of damaged muscle, though chronic supplementation may be required for augmented recovery of systemic markers of cellular damage or stress and perceptions of muscle soreness.

Although subjects' perceptions of muscle soreness were highest at 72 h, suggesting DOMS to be at its most severe at this time point, MVC and PPO were fully recovered by this time in both conditions. While this appears contradictory, it indicates that subjects' perceptions of pain are not a strong indicator of the decline in muscle function following eccentric exercise. This is supported by the finding that CK, a well-established marker of muscle damage, correlated poorly with ratings of perceived pain (R_2 = 0.36 and 0.34 for CON and PRO, respectively). Thus, perceived pain may not accurately reflect muscular damage and (or) may follow a different time course response, which could explain the discrepancy between muscles' functional recovery and peak muscle soreness values.

In conclusion, the present study is the first to report that a single post-exercise protein meal accelerates recovery of static force and dynamic power production during the DOMS period. Although this supplement does not affect the pain response of DOMS, the influence of exogenous protein on functionality may assist athletes in maintaining work output during training. However, although acute recovery from eccentric exercise may be improved, there is some evidence that exercise-induced damage is necessary to facilitate adaptations to training (Trappe *et*

al. 2002). Despite this, Flakoll *et al.* (2004) suggest that chronic protein supplementation during training reduces muscle soreness and injury occurrence. Therefore, if there is damage, the effect of protein supplementation is to the subject's benefit and thus may be encouraged for use during training regimens.

CHAPTER 5. EFFECTS OF MODIFYING O_2 SUPPLY ON PROTEIN SYNTHESIS AND TRANSLATIONAL SIGNALLING AT REST AND IN RESPONSE TO ACUTE RESISTANCE EXERCISE

5.1. Introduction

Prolonged hypoxaemia, whether under environmental or pathological conditions, is associated with reduced muscle mass, altered morphology (e.g. reduced fibre CSA, particularly of Type I fibres) and decrements in muscle function and exercise capacity (see section 1.8. for review). Numerous studies *in vitro* indicate that inhibition of protein synthesis and associated translational signalling, probably via increased levels of the hypoxia-responsive translational repressors REDD1 and / or AMPK phosphorylation, may be responsible for this apparent adaptation to chronic hypoxaemia *in vivo* (see section 1.8.2.3.). This hypothesis remains, however, to be tested directly in humans.

Whilst muscle hypoxia at rest may reduce protein synthesis, the superimposition of exercise may represent an even greater challenge to maintaining muscle protein synthetic capacity. During normoxic conditions, resistance exercise (RE) results in a fall in skeletal muscle O_2 availability at efforts at low as 30% 1-RM (Hicks *et al.* 1999), which is worsened with inspiration of hypoxic (12 – 14% O_2; PO_2 = ~90 -105 mmHg) air during isometric contractions (Hicks *et al.* 1999; Katayama *et al.* 2009). Consequently, such exercise-stimulated muscle hypoxia may exacerbate the effects of environmental / pathological hypoxaemia upon skeletal muscle and, in turn, potentiate any ensuing adaptations or changes. In normoxic conditions, MPS is

increased 200 – 300% at 1-2 h after resistance exercise (Louis *et al.* 2003; Phillips *et al.* 1997), likely through increasing mTOR signalling (Drummond *et al.* 2009; Glover *et al.* 2008; Tannerstedt *et al.* 2009). Over the course of a resistance training program (8 weeks), repeated post-exercise periods of increased protein balance results in net accrual of muscle protein and consequently an 8 – 10% gain in quadriceps cross-sectional area (CSA; Holm *et al.* 2008; Vissing *et al.* 2008). Resistance training during residency at altitude, however, does not appear to result in the same improvements in muscle mass; Narici and Kayser (1995) reported that the hypertrophic response to 4 weeks strength training was completely ablated when performed in hypoxic (5050 m altitude) conditions versus an 11.3% increase in biceps CSA after strength training in normoxia. Furthermore, 75 d at 5250 m altitude induced muscle wasting in both less-active (-15% CSA) and highly-active (-51% CSA) individuals (Mizuno *et al.* 2008; Oelz *et al.* 1986). Resistance and aerobic training in pathological conditions of hypoxaemia (COPD) also do not fully reverse functional abnormalities (e.g. maximal strength) of the quadriceps, and up to a third of patients do not improve their exercise capacity (Bernard *et al.* 1999; Man *et al.* 2009). Additionally, resistance training (12 weeks at 60 – 80% 1-RM) in COPD patients, whilst generally accepted to be an effective countermeasure to loss of muscle mass and function (Whittom *et al.* 1998), leads to only modest increases in quadriceps CSA of 2 – 4% (Casaburi *et al.* 2004; Kongsgaard *et al.* 2004) and in those studies where larger increases in CSA were reported, there was also large variability in responses, i.e. mean CSA increase of 8 ± 13% (Bernard *et al.* 1999). Whether reduced O_2 supply may contribute to this blunted hypertrophic response, or large individual variability, to resistance training is not known.

It may be logical to hypothesise that this blunted response of muscle to exercise training in conditions of hypoxaemia could be due to depressed, or abolished, post-exercise protein synthetic responses. A single study by Imoberdorf *et al.* (2006) measured the protein synthetic response to either passive or active ascent to 4559 m altitude (~12% O_2; PO_2 = ~90 mmHg). They reported that protein synthesis in hypoxia was unchanged in the passive ascent group, and was increased after load-carrying walking exercise in the active group, suggesting exercise was still capable of inducing increases in protein synthesis, even during prolonged hypoxia. A number of flaws in the design of this study, however, render interpretation of these findings problematic (see section 1.8.2.4). As such, rigorously controlled studies are necessary to directly assess the effects of hypoxia on muscle protein synthesis, and the signalling pathways likely to be influential in the regulation of muscle protein mass.

Thus, to date, muscle protein synthetic responses at rest and following acute RE under conditions of environmental hypoxia have not been measured in a controlled manner. Such studies may help us predict putative chronic responses to prolonged hypoxaemia with and without exercise, which would have important implications for those with conditions of pathological hypoxia (such as COPD, peripheral arterial-occlusive disease and obstructive sleep apnoea), and individuals embarking upon high altitude sojourns. The aim of this experiment was, therefore, to determine the acute effects of normobaric hypoxic exposure on muscle protein synthesis and associated molecular signalling events both at rest and following high intensity resistance exercise. It was hypothesised that a) rates of MPS and phosphorylation of anabolic signalling elements would be depressed in the rested state during hypoxic

exposure, b) the increase in MPS and anabolic signalling after RE in normoxia would be abolished during hypoxia and, c) that these responses would be accompanied by increases in phosphorylation of hypoxia-responsive signalling proteins whose increased phosphorylation is involved in the down regulation of anabolic signalling.

5.2. Methods

5.2.1. Participants

Seven males (age 21.4±0.7 years; BMI 24.9±1.5 kg) were recruited for the study. Volunteers were recreationally active (≤ 3 d activity week^{-1}) and were not taking any form of nutritional supplement or medication at the time of testing or for > twelve weeks prior to the study. All subjects were non-asthmatic, had no respiratory ailments and had not been exposed to any form of hypoxic atmosphere for > six months.

5.2.2. Study design

At least two weeks before the first infusion trial subjects reported to the laboratory for familiarisation and explanation of all procedures. The crossover study required subjects to be tested under two experimental conditions: normoxia (normal atmospheric conditions, ~21% O_2) and normobaric hypoxia (reduced O_2 concentration of inspired air, ~12.5% O_2). Trials were ordered and separated by a minimum of 3 months, with the normoxic condition always being performed first to avoid any long lasting effect of the hypoxic bout.

5.2.2.1. Normoxic trial

Participants arrived at the laboratories at ~0800 h in the postabsorptive state (~12 h overnight fast) for insertion of catheters into both arms for blood sampling and tracer infusion (see section 2.4.1.) Participants were then placed in custom built isometric force apparatus (see section 2.6.) for performance of unilateral resistance exercise (RE). MVC was determined as outlined in section 2.6. Following 2 min recovery, RE consisted of 8 sets of 6 repetitions at 70% MVC, with 2 minutes recovery between sets. Immediately after RE a muscle biopsy was taken from the non-exercised leg and a primed, constant intravenous infusion of 1,2-[$^{13}C_2$]leucine was started and maintained for 2.5 h (see section 2.4.1.). Blood samples were collected at 20 min intervals throughout the study. Further muscle biopsies were collected from both legs at 2.5 h post-exercise. Fingertip measure of oxygen saturation (SpO_2) was recorded using pulse oximetry (9500 Onyx, Nonin) every 10 min for the duration of the study, as was the O_2 / CO_2 concentration of inhaled air. A schematic representation of the study protocol can be viewed in Figure 5.1.

5.2.2.2. Hypoxic trial

The experimental conditions for the hypoxic trial were similar to that of the normoxic trial; the difference was that volunteers breathed 12.5% O_2 for 1 h before RE and for the remainder of the study. Hypoxic conditions were delivered using a custom-made face mask, adapted to allow gas sampling of the internal mask environment and connected to a nitrogen generator set to reduce the O_2 concentration of inspired air to 12.4 ± 0.2%.

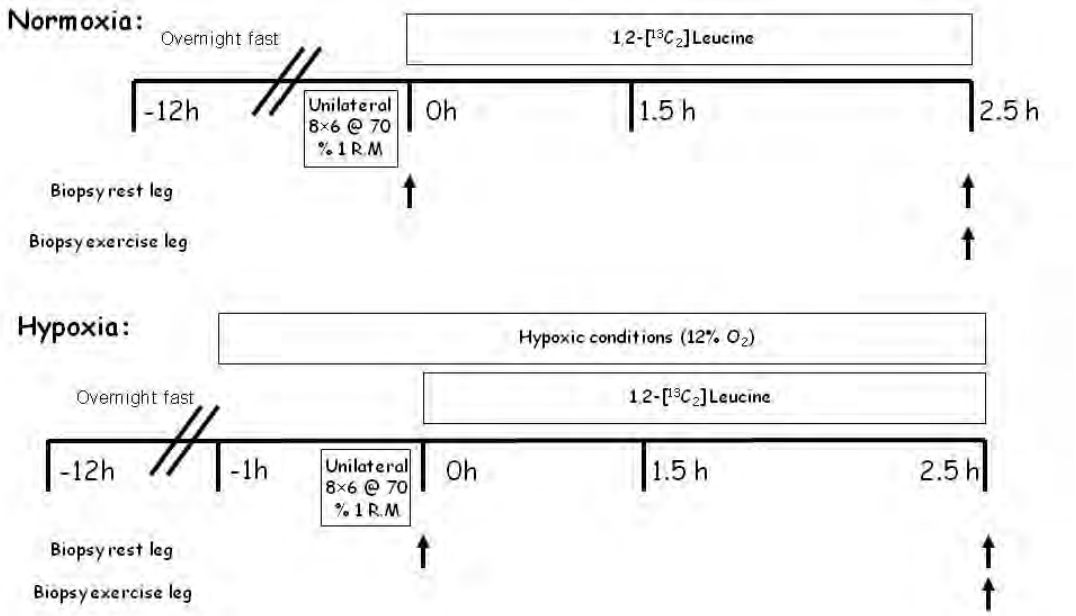

Figure 5.1. Cross over study protocol schematic for investigation of the effects of hypoxia and RE on protein synthesis and translational signalling.

5.2.3. Determination of fractional rates of MPS

Muscle tissue (~25 mg) was prepared as outlined in section 2.4 for evaluation of myofibrillar protein synthesis. Rates of myofibrillar protein synthesis were also analysed and calculated as stated in section 2.4.

5.2.4. Immunoblotting

Western blots were generated and analysed as outlined in section 2.5. Phosphorylation of proteins ACC-β Ser79, p70S6K Thr389, PKB Ser473, mTOR Ser2448, 4EBP1 Thr37/46, eIF4E Ser209, eEF2 Thr56 and ERK1/2 Tyr202-204, and total protein concentrations of REDD1 and β-actin (loading control) were measured using polyclonal antibodies at a concentration 1:2000 (Sigma Aldrich, Poole, UK).

5.2.5. Statistical analysis

Paired sample t-tests were performed on MVC and SpO_2 data to determine any differences in these measures between the normoxic and hypoxic conditions. Data were tested for normality and, if normally distributed, a one-way repeated measures analysis of variance (ANOVA) was used to evaluate possible differences between normoxia and hypoxia in muscle protein FSR, muscle intracellular signalling elements, and mRNA expression between unilateral exercised leg and contralateral control leg. Significant *F* ratios were followed by Tukey's *post hoc* comparisons procedure using GraphPad software (La Jolla, San Diego CA). If data failed the normality test, a one-way ANOVA (Friedman test) was performed and in the event of significance being observed was followed by a Dunn's multiple comparisons test to determine the point of significance. Pearson's product moment correlation was performed to determine relationships between FSR and SpO_2. A *P* value of <0.05 was considered statistically significant. Data in the text are presented as mean ± SEM.

5.3. Results

5.3.1. Mean MVC and SpO_2 responses

Peak MVC, and thus subsequent exercise intensity, was not different between the normoxic and hypoxic conditions (629±63 vs. 613±60 N for normoxic and hypoxic MVC, respectively; $p>0.05$, Figure 5.2A). Throughout the normoxic trial SpO_2 remained unchanged from baseline (98.2±0.3 vs. 97.4±0.5% for SpO_2 at baseline and the average of all subsequent time points, respectively; $p>0.05$). In hypoxia SpO_2 decreased significantly from baseline within 10 min to 86.4±1.7% ($p<0.01$) and the

mean SpO_2 for all subjects of all subsequent time points remained depressed throughout hypoxia (82.7±0.5%; p<0.01, range 75-89%, Figure 5.2B).

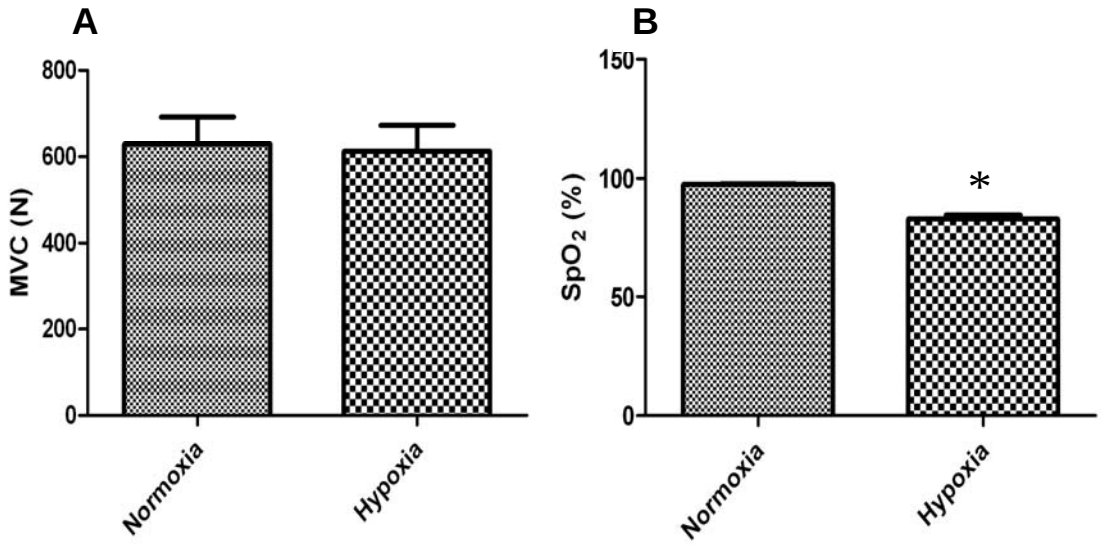

Figure 5.2. Maximal isometric voluntary contraction (A) and mean SpO_2 (B) in hypoxia (12.5% O_2) compared to normoxia. * denotes significant change from normoxic values (p<0.01). Data are presented as means ± SEM; *n* = 7.

5.3.2. Changes in MPS and mTOR signalling following exercise in normoxia

In normoxic conditions myofibrillar FSR was significantly increased from baseline by 2.5 h in the exercised leg (0.033 ± 0.016 vs. 0.104 ± 0.038 $\%h.^{-1}$; p<0.01, Figure 5.3A). After RE, phosphorylation of p70S6K was increased 2.6±1.2-fold (p<0.05, Figure 5.4A), and ACC-β phosphorylation was decreased 0.6±0.1-fold from baseline values (p<0.05, Figure 5.4B). Total REDD1 protein content (Figure 5.5) and phosphorylation of PKB, mTOR, 4EBP1, eIF4E, eEF2 and ERK1/2 (Figures 5.6A-F) were all unchanged after RE in normoxia (p>0.05).

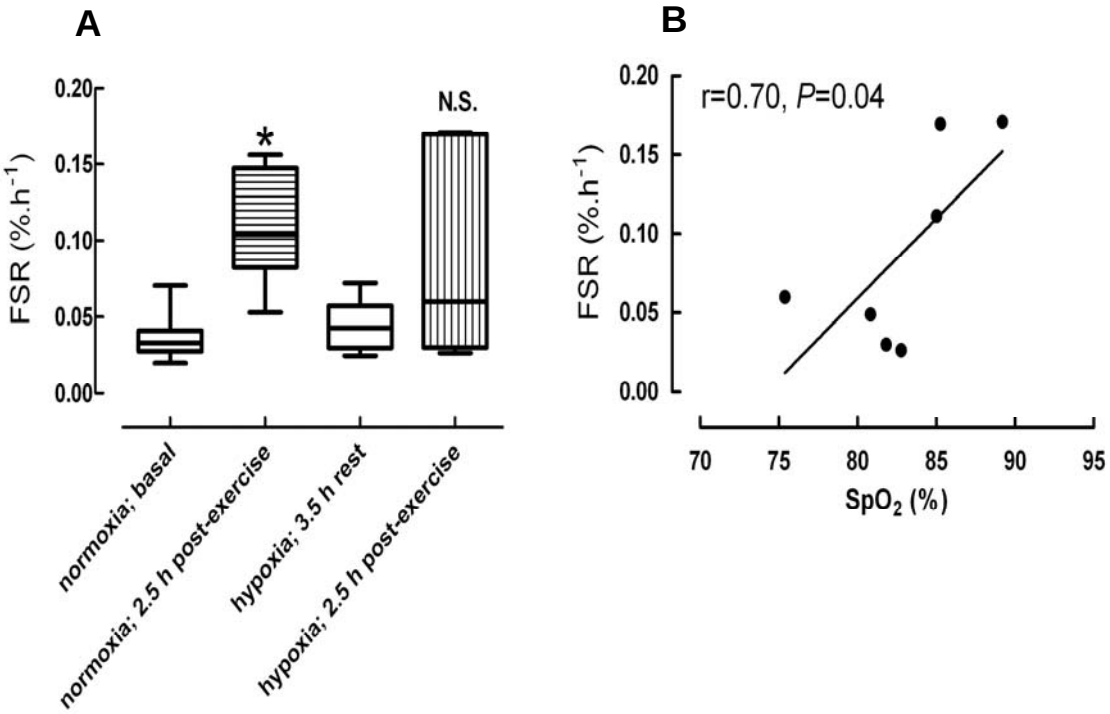

Figure 5.3. A: Changes in myofibrillar fractional synthetic rate (FSR) from normoxic baseline values at 2.5 h post-exercise in normoxia, 3.5 h hypoxic exposure (~12% O_2) in the rested leg, and 2.5 h post-exercise during hypoxic exposure. * denotes significant increase from normoxic postabsorptive FSR ($p<0.01$). N.S. = non-significant change. B: Correlation between individual responses in FSR at 2.5 h post-exercise during hypoxic exposure and individuals' mean SpO_2 of all hypoxic time points.

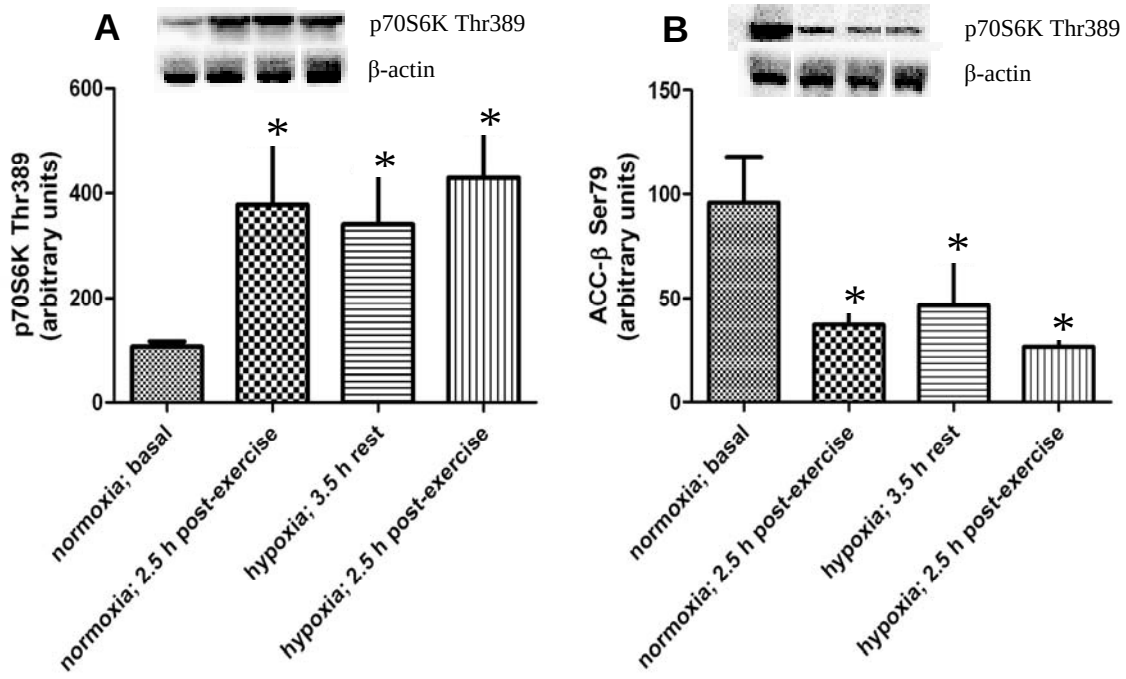

Figure 5.4. Changes in phosphorylation of p70S6K (A) and ACC-β (B) from normoxic baseline values at 2.5 h post-exercise in normoxia, 3.5 h hypoxic exposure (~12% O_2) in the rested leg, and 2.5 h post-exercise during hypoxic exposure. Representative blots show all time points from one subject with corresponding loading controls, both obtained from one Western blot. * denotes significant change from normoxic postabsorptive values ($p<0.05$). Data are presented as means ± SEM; $n = 7$.

5.3.3. MPS and mTOR signalling responses during hypoxia

After 3.5 h hypoxia, postabsorptive myofibrillar FSR was not significantly different from normoxic baseline values in the rested leg (0.033 ± 0.016 vs. 0.043 ± 0.016 %h.$^{-1}$; p>0.05, Figure 5.3A). FSR was also unchanged from normoxic baseline after 2.5 h in the exercised leg (0.06 ± 0.063 %h.$^{-1}$; p>0.05, Figure 5.3A). Pearson's product moment correlation demonstrated a significant relationship between hypoxic FSR 2.5 h post-exercise and mean hypoxic SpO_2 (r^2 = 0.48; p<0.05, Figure 5.3B).

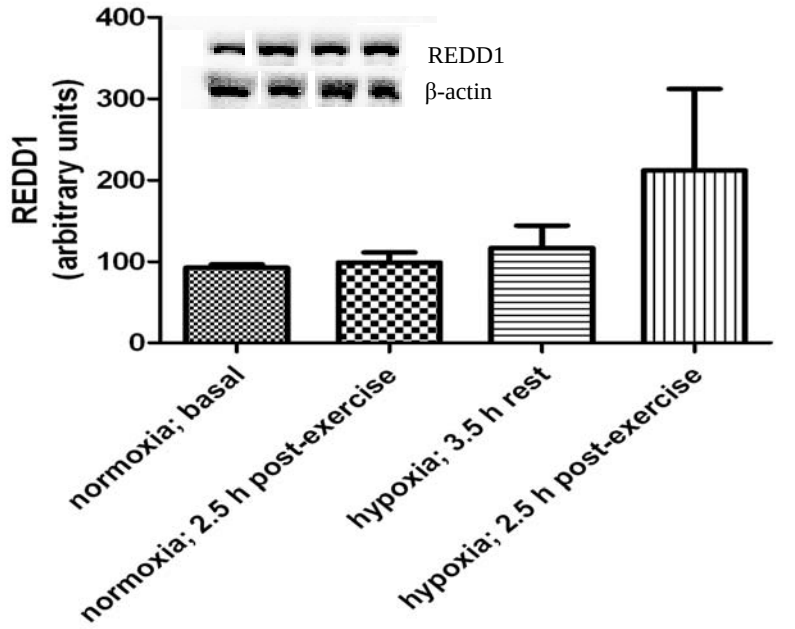

Figure 5.5. Changes in total REDD1 protein content from normoxic baseline values at 2.5 h post-exercise in normoxia, 3.5 h hypoxic exposure (~12% O_2) in the rested leg, and 2.5 h post-exercise during hypoxic exposure. All values were non-significantly different from baseline values (p>0.05). Representative blots show all time points from one subject with corresponding loading controls, both obtained from one Western blot. Data are presented as means ± SEM; *n* = 7.

The phosphorylation of p70S6K was increased 2.3±1.0-fold after 3.5 h hypoxia in the rest leg, and 3.4±1.1-fold 2.5 h after hypoxic exercise (p<0.05), which were not different from values 2.5 h after exercise in normoxia (Figure 5.4A). ACC-β phosphorylation was decreased -0.5±0.2-fold after 3.5 h hypoxia versus baseline values in the rest leg, and -0.7±0.1-fold 2.5 h after exercise in hypoxia (p<0.05, Figure 5.4B), which were also not different from the decrease after normoxic

exercise. Total REDD1 protein concentration (Figure 5.5) and phosphorylation of PKB, mTOR, 4EBP1, eIF4E, eEF2 and ERK1/2 (Figures 5.6A-F) were unchanged in both the rest leg after 3.5 h hypoxia and at 2.5 h after exercise in hypoxia ($p>0.05$). There was no correlation between phosphorylation of any of the signalling proteins measured and SpO_2 ($p>0.05$).

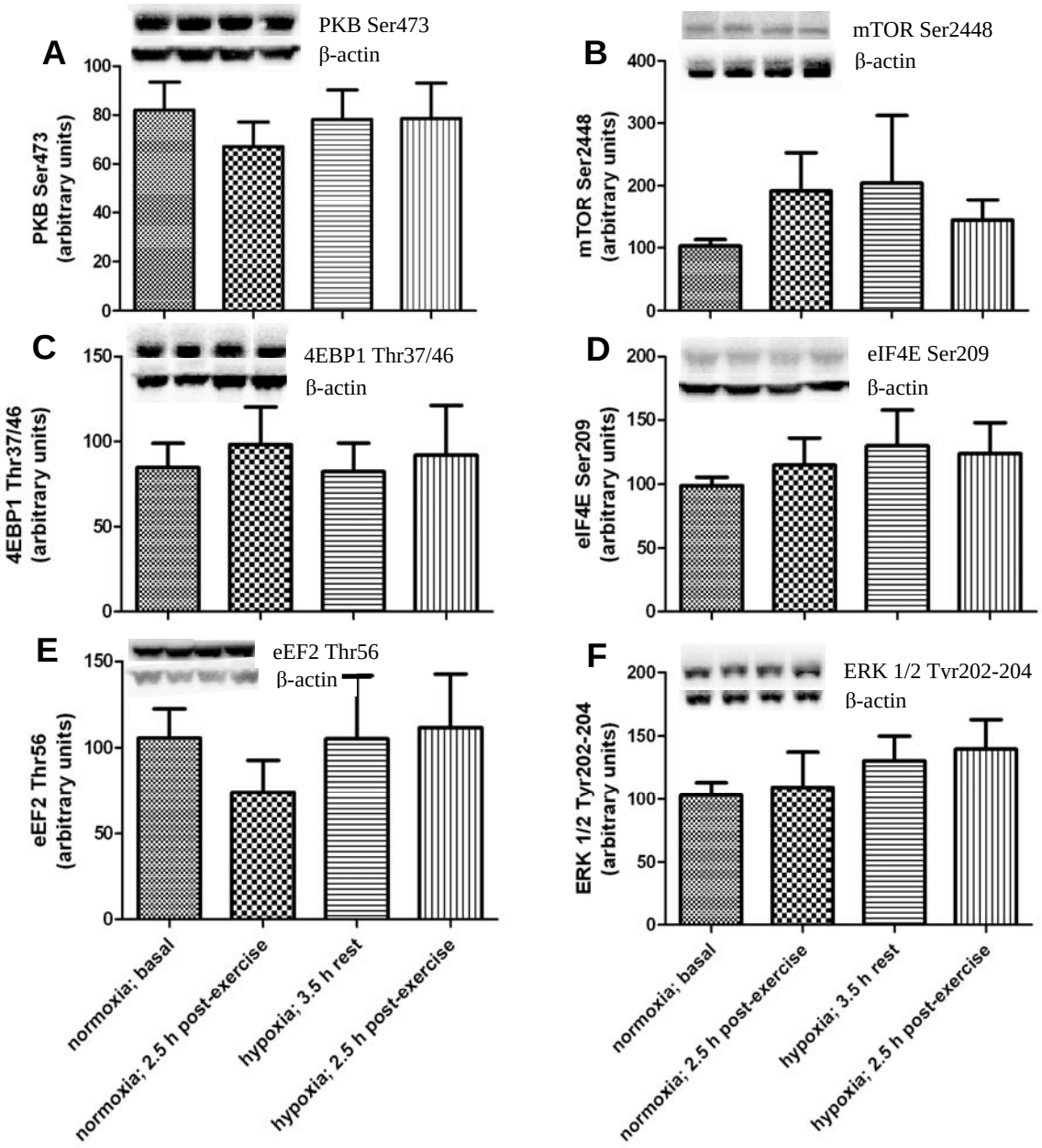

Figure 5.6. Changes in phosphorylation of Akt (A), mTOR (B), 4EBP1 (C), eIF4E (D), eEF2 (E), and ERK1/2 (F) from normoxic baseline values at 2.5 h post-exercise in normoxia, 3.5 h hypoxic exposure (~12% O_2) in the rested leg, and 2.5 h post-exercise during hypoxic exposure. All values were non-significantly different from baseline ($p>0.05$). Representative blots show all time points from one subject with corresponding loading controls, both obtained from one Western blot. Data are presented as means ± SEM; $n = 7$.

5.4. Discussion

This study reports new findings on the response of MPS and associated translational signalling to 3.5 h exposure to hypoxia (12.5% O_2), both at rest and after acute RE. The principle findings of this study are (i) MPS was unchanged following 3.5 h hypoxia in the rested leg, although phosphorylation states of certain proteins thought to be involved in stimulating protein synthesis were altered towards increased translational signalling, (ii) hypoxia abolished the rise in MPS after acute RE, despite increased protein synthetic signalling, and (iii) suppression of MPS after hypoxic exercise correlated with the prevailing SpO_2.

It has recently been hypothesised that the muscle wasting observed in conditions of chronic hypoxaemia may, at least in part, be an effect of reduced O_2 supply to the muscle (Hochachka *et al*, 1996; Hoppeler and Vogt, 2001; Wüst and Degens, 2007); suggesting hypoxia may result in inhibition of rates of protein synthesis whilst in the rested state. The present study is the first to report, under tightly controlled conditions, that MPS is unaffected during acute hypoxaemia at rest. These data during acute hypoxia, therefore, putatively indicate little potential for a role of chronic tissue hypoxia towards muscle atrophy in sedentary individuals, although it cannot be ruled-out that hypoxia-induced increases in muscle protein breakdown contribute towards muscle wasting. This study is also the first to show that phosphorylation of key signalling proteins involved in the regulation of protein synthesis are altered towards increased anabolic signalling (i.e. increased p70S6K and decreased ACC-β phosphorylation) as a result of hypoxia at rest. Though counter-intuitive, given that MPS was unchanged, the indication from this evidence is that low O_2 supply causes a signal for muscle to increase protein translation. It

may be that this response could be a consequence of a likely hypoxia-induced vasodilation and subsequent increased blood flow to muscle, which would increase nutrient and growth hormone supply to the muscle; both of which may independently increase translational signalling. Indeed, during 12% inspired O_2 hypoxia, significant increases in blood flow to the forearm of 70% (Rennie *et al.* 1983) and to the legs of 84% (Barthèlemy *et al.* 2001) occurred in healthy individuals at rest. Thus, though not measured presently, increased blood flow to the quadriceps may have resulted in increased translational signalling, however, muscles' ability to translate this signal into augmented protein synthesis is impaired. It may be speculated that this effect could be a result of reduced intracellular energy levels available for peptide bonding during hypoxia, as has been reported *in vitro* (Boutilier, 2001; Hochachka and Lutz, 2001; Pierce and Czubryt, 1995; Stanley *et al*, 1997), though this seems unlikely since intracellular ATP concentrations remain stable under conditions such as exercise (Karlsson and Saltin, 1970) and hypoxia (Goudemant *et al*, 1995). Alternatively, given the metabolic expense of synthesising proteins other, as yet unknown molecular signals may counteract this anabolic signal; likely as an adaptive process to preserve cellular ATP levels, and thus cell integrity in times of hypoxic stress.

The finding here, that MPS is unaltered during acute hypoxia, is in agreement with Imoberdorf *et al.* (2006) who found no change in rates of mixed-muscle protein synthesis following passive ascent to altitude (4559 m, 12% O_2), but contrary to the results of Rennie *et al.* (1983) who reported reduced forearm leucine uptake (indicative of lowered protein synthesis) during 6.5 h hypoxic exposure (12% O_2). These discrepant results may be a consequence of either a) the techniques utilised to

measure protein synthetic rates, or b) the methods employed to induce hypoxia. Leucine uptake, as used to estimate protein synthesis by Rennie *et al.* (1983) is a less direct measure than the 1,2[$^{13}C_{2]}$leucine incorporation techniques employed in the present study, as leucine taken up into muscle is utilised by both oxidative and protein synthetic pathways. Thus, it cannot be determined whether synthesis actually decreased in Rennie *et al.* (1983), as the reduced leucine uptake reported may equally have been indicative of lowered leucine oxidation, with no change in synthesis. Furthermore, to achieve the same ambient O_2 partial pressure (PO_2; equivalent to ~120 hPa / 91 mmHg, or ~4,500 m altitude) Rennie and colleagues used hypobaric hypoxia (decreased barometric pressure), versus normobaric hypoxia (lowering O_2 fraction of air) employed in the present study. At equivalent PO_2 hypobaric hypoxia induces greater reductions in blood O_2 saturation compared to normobaric hypoxia (Savourey *et al.* 2003). Indeed, Rennie *et al.* (1983) observed much greater falls in O_2 saturation than those reported presently (from ~97 to ~65%, versus 97.4 to 82.7% reported in the present study). As such, a greater hypoxaemic stimulus with hypobaric hypoxia may have been sufficient to inhibit MPS whilst in the rested state, suggesting there may be a threshold in the decline in SpO_2 below which MPS is inhibited. Nevertheless, drops in SpO_2 to as low as 65% are uncommon; requiring rapid ascent to altitudes of 5000 m and above (Anholm *et al.* 1992), and have not been reported in any hypoxaemic pathologies. In contrast, lowered SpO_2 values to between 80-90% are frequently experienced at both moderate altitudes (DeLorey *et al.* 2004; Jones *et al.* 2008; Kawahara *et al.* 2008) and in various pathologies (Chiang, 2006; Clause *et al.* 2008; Nasilowski *et al.* 2008; Noda *et al.* 1998).

Thus, it may be inferred from the findings reported here, that reductions in SpO_2 relevant to altitudes reached frequently by recreational sportspersons / mountaineers and during pathologically relevant clinical situations do not acutely impair basal levels of MPS in sedentary individuals. Whilst it remains possible that perturbations in postabsorptive MPS may occur with longer periods (days to weeks) of hypoxaemia, or with more severe hypoxaemic bouts acutely, the implications of these results are that the muscle wasting observed both at high altitude and in pathological conditions may not be a direct result of limited oxygen supply. However, since rates of muscle protein breakdown were not measured here, the potential role of hypoxia-mediated alterations in protein degradation cannot be excluded as causative in such muscle wasting conditions. As such, the mechanism(s) underlying loss of muscle mass in conditions of hypoxaemia remains to be elucidated.

This study is also the first to show that the rise in protein synthesis after RE is blunted during hypoxaemia. Many scenarios exist whereby the hypertrophic response to RE training is blunted during both environmental (Mizuno *et al.* 2008; Narici and Kayser, 1995; Oelz *et al.* 1986) and pathological (Bernard *et al.* 1999; Casaburi *et al.* 2004; Kongsgaard *et al.* 2004; Man *et al.* 2009) hypoxaemic conditions. The present study provides evidence that this effect may be a direct consequence of attenuated increases in MPS after individual RE bouts which, over the course of a training program may (if protein degradation remains unchanged or is increased) attenuate increased deposition of contractile proteins and thus hypertrophy. The mechanisms regulating this blunted MPS response to RE in hypoxia, however, remain elusive. Total protein content of REDD1, a hypoxia-

responsive element whose increased concentration negatively regulates protein synthesis *in vitro* (Brugarolas *et al.* 2004; DeYoung *et al.* 2008; Schwarzer *et al.* 2005), was unaffected after RE in hypoxia. Furthermore, ACC-β phosphorylation, a downstream effector of AMPK and an indicator of its activity, was depressed in hypoxia after RE. This is opposite to previous reports *in vitro* of increased AMPK activity in response to hypoxic exposure (Liu *et al.* 2006). The lack of changes to PKB, mTOR, 4EBP1, eIF4E, eEF2 and ERK1/2 phosphorylation in all conditions tested is not surprising, given that large variability in individual changes in translational signalling elements after RE has been reported (Kumar *et al.* 2009a), which may contribute to the inter-subject variance in muscle mass and strength gains following resistance training (Hubal *et al.* 2005). This finding does, however, suggest that these components of translational signalling are not involved in the presently observed response to RE in hypoxia. The observation that p70S6K and ACC-β phosphorylation increased and decreased, respectively after hypoxic exercise suggests that, as with the hypoxic signalling response at rest, muscle continues to attempt to signal an increase in protein synthesis in a fashion similar to that after normoxic RE but that muscle is unable to translate this increased signal into elevated protein synthesis. As hypothesised at rest during hypoxia, this may be due to some, as yet unknown, signalling mechanism 'overriding' the exercise-induced signal in order to prevent protein synthesis from increasing. This phenomenon may exist to provide a protective mechanism to help preserve cellular energy homeostasis during low O_2 supply when there is a reduced capacity for oxidative energy production (Arsham *et al*, 2003; Boutilier, 2001). Thus, in times of hypoxic stress when ATP availability is at a premium, increases in protein synthesis may be sacrificed post-RE to spare the ATP necessitated by peptide elongation, therein preventing intracellular

energy imbalance. The net chronic effect of this adaptation would be to promote energy homeostasis and cell survival, at the expense of gains in muscle mass in response to training.

Finally, the magnitude of change in MPS after RE in hypoxia was significantly correlated with measured blood oxygen saturation, i.e. subjects with a smaller degree of hypoxaemia retained their post-RE increase in MPS, whereas those with greater declines in SpO_2 demonstrated inhibited MPS after RE. This indicates the existence of perhaps at least two types of individual; a responder and non-responder. Such a 'responder, non-responder' phenomenon, specifically in falls in SpO_2, has been postulated to exist amongst mountaineers to explain why some experience symptoms of acute mountain sickness whilst others do not (Burtscher *et al.* 2008), and may at least partially explain observations that the efficacy of exercise prescription varies greatly between individuals during conditions of pathological hypoxaemia (Bernard *et al.* 1999; Casaburi *et al.* 2004; Kongsgaard *et al.* 2004; Man *et al.* 2009). Whether a similar pattern is present in healthy humans trained at altitude is unknown, though inter-subject variations in the performance enhancing effects of hypoxic training (Hoppeler *et al.* 2008; Mazzeo, 2008) and in hormonal / transcriptional changes to resistance training in hypoxia (Friedmann *et al.* 2003) has been documented. However, the mechanisms underlying this phenomenon are unknown. No correlation was present between SpO_2 and any of the signalling elements measured. It is possible that individual differences in oxygen carrying capacity of the blood (i.e. haemoglobin concentration and/or hematocrit), or in individual hyperventilatory responses, may explain this effect, though as these variables were not measured here this remains a plausible but as yet untested hypothesis.

In conclusion, the acute onset of hypoxia leads to a significant decline in SpO_2 which does not result in reductions in MPS in the rested state. Thus levels of hypoxaemia that are relevant to both various patient populations and healthy humans visiting moderate altitude are unlikely to be directly responsible for the loss of muscle mass experienced during these situations. After an acute RE bout, however, the increased protein synthetic response present in normoxia is abolished during hypoxic exposure in individuals that experience greater declines in SpO_2 (i.e. 'responders'). It is proposed that this is part of an adaptive process in order to maintain cell energy homeostasis and thus viability during times of hypoxic stress which likely, at least partly, explains the blunted response to resistance training observed in conditions of hypoxaemia.

CHAPTER 6. GENERAL DISCUSSION

In addition to muscles' primary function of force and power development, it is a metabolic tissue central to the control of whole-body metabolism. Indeed, muscle serves as the bodies' largest protein store (Rooyackers and Nair, 1997), which can be catabolised to provide the free amino acids for the synthesis of molecules required by other parts of the body (e.g. Zhang *et al.* 2008b). Additionally, muscle contributes to temperature regulation of the body (MacIntosh *et al.* 2006), and is the major site of whole-body glucose uptake (Horowitz *et al.* 2001; Kraegen *et al.* 1990; Zierler, 1999). Muscle, therefore, not only serves mechanical functions but also contributes to metabolic and energy homeostasis (Smith *et al.* 2008). As such, maintenance of skeletal muscle is crucial to preserve contractile function, health and survival. Moreover, hypertrophy and associated gains in muscle function (i.e. force and power production) are vital for optimising performance in recreational and competitive sportspersons.

As highlighted in section 1 and in the experimental chapters 3 – 5, there are a number of gaps in the knowledge pertaining to how muscle is regulated. Thus, the aim of the studies of this thesis was to investigate interactions between feeding, exercise and environmental conditions on skeletal muscle function and metabolism. Specifically, the present thesis aimed to: 1) determine the temporal relationship between feeding and MPS / translational signalling, 2) investigate the effect of protein feeding on muscle function and recovery after exercise and, 3) investigate how resting MPS and exercise-induced increases in MPS are modulated by decreased O_2 availability.

6.1. Principle findings

Chapter three is the first study to detail the temporal relationship between MPS and anabolic signalling in response to an oral protein feed. It was shown that the rise and subsequent fall in MPS after 48 g whey protein supplement follows a similar pattern to that shown previously in response to constant intravenous infusion of AA (Bohé *et al.* 2001), whereby myofibrillar and sarcoplasmic protein synthesis are approximately tripled and doubled, respectively by 90 min. Furthermore, in accord with the results of Bohé *et al.* (2001), a decline of protein synthesis rates to baseline values by 180 min occurred despite continued elevated plasma and intracellular EAA concentrations. Thus, the decline in MPS after feeding does not appear to be a result of normalised plasma / intracellular AA availability. These data provide new support for the existence of a 'muscle full' phenomenon in response to a single oral feed, whereby muscle can gauge its capacity to synthesise new proteins even when the activating stimulus remains.

Several studies in animals (Wang *et al.* 2005; Peyrollier *et al.* 2000) and humans (Cuthbertson *et al.* 2005; Fujita *et al.* 2007) have implicated the mTORC1 signalling pathway in regulating MPS in response to increasing AA availability, by showing concurrent increases in phosphorylation of mTOR substrates and MPS. The results presented here are also the first to provide temporal data on molecular signals *and* MPS synchronously after feeding. Such information is necessary in order to discriminate regulatory from non-regulatory signals of MPS. It was shown here that the upswing in MPS is preceded by increases in phosphorylation of PKB and eIF4G, though mirrored increased phosphorylation of p70S6K and 4EBP1. These findings

suggest that the activation of PKB and eIF4G may be important in stimulating the initial increase in MPS following a protein meal, and that increased phosphorylation of p70S6K and 4EBP1 may be required for sustaining elevated MPS up to ~90 min. Additionally, increased p70S6K, 4EBP1 and eIF4G phosphorylation outlasted elevated MPS, thus providing the first evidence in humans to support reports in rats that phosphorylation of mTOR signalling (p70S6K, 4EBP1 and eIF4G) remains elevated after MPS has returned to basal values (Norton *et al.* 2009). This lack of agreement between declines in MPS and translational signalling leave the mechanism regulating the postprandial switch-off of MPS in question.

In chapter four the effects of a single oral protein meal on recovery of muscle function and indirect indices of muscle damage after acute eccentric exercise were reported. Post-exercise protein ingestion augmented recovery of muscle force and power production during the DOMS period by 48 h, which was independent of changes in plasma markers of muscle damage (i.e. creatine kinase and protein carbonyl) and perceptions of muscle soreness. This suggests that acute protein feeding may be an effective strategy for minimising the decrements in performance after unfamiliar eccentric exercise bouts, which may be hypothesised to be a result of increases in MPS and thus a faster rate of repair of contractile protein damage. Recently, numerous studies have been published on this topic with conflicting results. In accordance with the present findings, acute whey protein (25 g) intake was shown to attenuate the loss of muscle force 6 h after acute resistance exercise (Buckley *et al.* 2008). Similarly, isokinetic performance, plasma CK and muscle soreness had recovered 48 h after exercise-induced muscle damage with acute post-exercise CHO and protein ingestion (Cockburn *et al.* 2008).

Conversely, White *et al.* (2008) showed no improvement in MVC, and no reduction in CK concentrations or muscle soreness with acute CHO and protein ingestion after eccentric leg exercise, though the supplement contained only 9 g whole protein (~4.5 g EAA), which is insufficient for maximal stimulation of MPS (Cuthbertson *et al.* 2005) and may not, therefore, induce full repair of contractile proteins (and thus muscle function) following muscle-damage. Furthermore, Betts *et al.* (2009) reported no improvement in isometric performance or reductions in CK concentrations after 90 min shuttle-running with CHO and protein intake, though this was only measured up to 24 h post-exercise which, as indicated by the present results, is likely insufficient to observe any effect. Finally, Green *et al.* (2008) also found reductions in MVC and increases in CK and muscle soreness were not improved at 24, 48 and 72 h after eccentric-exercise when combined with CHO and protein supplementation, although the lack of a cross-over study design plus small subject numbers (n = 6 per group) may have masked any effect of protein on recovery in this study. As such the precise effects of protein intake on muscle recovery remain controversial.

However, despite the recent increase in studies investigating recovery from muscle-damaging exercise with acute protein administration, this remains the only study to have detailed the recovery of muscle power, as opposed to simple isometric performance measures, during the DOMS period, which may have greater applicability to activities of daily living / sporting events than isometric contractions alone. Furthermore, without exception, studies investigating muscle recovery during the 72 h DOMS period have administered combined CHO-protein supplements. As such, this is the only study to date that allows discrimination of the effects of protein

ingestion alone on recovery of muscle up to 72 h after exercise-induced damage, independent of the potential influence of CHO.

Lastly, chapter five is the only study to have investigated the effects of reduced O_2 supply on MPS and anabolic signalling at rest and after RE in humans. Numerous authors have hypothesised that lowered O_2 supply may contribute to the muscle wasting observed in conditions of pathological and environmental hypoxia, via hypoxia-mediated inhibition of protein synthesis (Hochachka *et al.* 1996; Hoppeler and Vogt, 2001; Wüst and Degens, 2007). This notion has been supported by studies *in vitro* demonstrating suppression of protein synthesis in mammalian cells under hypoxic (<0.2% O_2) conditions (Caron *et al.* 2009). Furthermore, a single study in humans using indirect measures of MPS (i.e. leucine uptake into forearm muscle) found MPS to be depressed during hypobaric hypoxia (Rennie *et al.* 1983). However, the present results contradict these earlier studies in demonstrating that at rest MPS is unaltered as a consequence of reduced muscle O_2 supply alone, despite increases in anabolic signalling (i.e. increased p70S6K and decreased ACC-β phosphorylation). This discrepancy may be due to the severity of induced-hypoxia; *in vitro* studies exclusively utilised either anoxia (Koumenis *et al.* 2002) or severe hypoxia (Caron *et al.* 2009; Jibb and Richards, 2008; Lefebvre *et al.* 1993), and Rennie and colleagues employed hypobaric hypoxia which stimulates significantly greater reductions in muscle O_2 supply than the normobaric hypoxia used presently (Rennie *et al.* 1983; Savourey *et al.* 2003). It may, therefore, be postulated that a greater degree of hypoxia is necessary to repress MPS, which would comply with observations that muscle wasting worsens with increasing altitudes (see section 1.4.2.1.), and with findings that leucine absorption and cumulative oxidation are

reduced in Bolivian children at high altitude (altitude and % oxygen) when compared with a lower altitude (altitude and % oxygen) group (San Miguel *et al.* 2002). Nevertheless, the results presented in chapter five demonstrate that levels of hypoxaemia relevant to both pathological hypoxia and altitudes frequently visited by alpine athletes do not lead to acute perturbations in MPS, therein dismissing earlier hypotheses that reduced O_2 supply may underlie muscle wasting in these conditions. Thus, wasting in hypoxaemic conditions may arise from changes in either delivery, digestion or absorption of nutrients, or from other metabolic consequences of high altitude, for example changes in MPB to rates exceeding those of MPS.

Despite the absence of a change in MPS at rest, after acute RE in hypoxia the increases in MPS observed after RE in normoxia are abolished, despite comparable increases in anabolic signalling. Thus, reduced O_2 supply may underlie observations of blunted hypertrophic responses to resistance training in pathological (Casaburi *et al.* 2004; Kongsgaard *et al.* 2004) and environmental (Mizuno *et al.* 2008; Narici and Kayser, 1995; Oelz *et al.* 1986) conditions of hypoxia. Interestingly, the MPS response after RE in hypoxia correlated with the magnitude of decrease in SpO_2, suggesting the existence of a 'responder, non-responder' phenomenon whereby those that experienced greater declines in SpO_2 demonstrated blunted increases in MPS post-RE compared to those who experience lower levels of hypoxaemia that maintain the capacity to increase MPS after RE. Though speculative, these observations provide evidence that hypoxia may account for the variable efficacy of exercise training programs as a countermeasure to muscle wasting and loss of functional ability in clinical conditions of hypoxia (Bernard *et al.* 1999; Casaburi *et al.* 2004; Kongsgaard *et al.* 2004; Man *et al.* 2009), and why hypoxic training

programs do not consistently lead to improvements in muscle performance in athletic populations (Hoppeler *et al.* 2008; Mazzeo, 2008). However, given the lack of correlation between SpO_2 and phosphorylation of any of the signalling proteins measured, the mechanisms underlying this response remain to be determined.

6.2. Practical applications of findings

6.2.1. Chapter three

The results reported in chapter three may have important implications for designing optimal nutritional strategies in hospitalised patients and in prescribing dietary recommendations to the elderly; the effectiveness of which depends on maximising the anabolic effects of food. It is clear from the present results and those reported previously (Bohé *et al.* 2001) that there is a limit to the extent that MPS can be stimulated following the onset of AA provision i.e., muscle becomes 'full' of protein. This finding calls into question the efficacy of administering constant infusion of nutrients (e.g. in patients receiving total parenteral nutrition; TPN), since after ~90 min MPS is switched-off despite continued AA availability. As such, while it remains unclear how long the latent period is before MPS can be re-stimulated by food, 'pulse feeding' (i.e. intermittent nutrient boluses throughout the diurnal cycle) may represent a more effective strategy of feeding those for whom nutrition need to be optimised. Similarly, dietary prescriptions for the elderly who display blunted MPS responses to feeding (Cuthbertson *et al.* 2005) may benefit from repeated maximal stimulation of MPS by pulse feeding.

6.2.2. Chapter four

The efficacy of exercise training for increasing muscle mass and function is well documented in healthy individuals (reviewed in Phillips, 2009; Roig *et al.* 2009), the elderly (Evans and Campbell, 1993; Koopman and van Loon, 2009; Little and Phillips, 2009) and in numerous patient groups such as diabetes and obesity (Tresierras and Balady, 2009; Willey and Singh, 2003). The resulting increases in mobility are associated with improved quality of life and maintained independence (Yeom *et al.* 2009). In order to maximise these benefits, strategies may be employed that aim to optimise the muscle mass and functional gains of training. In accordance with the results of chapter four, supplemental dietary protein ingested immediately after exercise may attenuate the functional declines commonly experienced during the 72 h DOMS period, which are similar between young (~19 yr) and elderly (~70 yr) individuals (Lavender and Nosaka, 2006). Consequently, higher workloads may be performed during subsequent exercise bouts, thus maximising the positive effects on muscle of exercise training. Moreover, adherence to prescribed physical activity programs is low, due in part to the occurrence of negative symptoms associated with DOMS (Ary *et al.* 1986; Jones *et al.* 2007). Thus, by minimising reductions in the ability to perform activities of daily living, negative perceptions of exercise would be lowered; potentially increasing rates of adherence to training programs. Additionally, athletes training for competition are required to maintain optimised levels of work output during training in order to maximise improvements in performance. Protein supplementation may, therefore, be an effective strategy for minimising functional declines after exercise, particularly following exercise bouts containing a large eccentric component such as plyometric or heavy resistance exercise.

6.2.3. Chapter five

The blunted MPS response to RE in hypoxia reported in chapter five suggest that reduced O_2 supply is an important contributor to blunted hypertrophic responses to RE training observed in sportspersons training at altitude, as well as in the many clinical conditions characterised by chronic reductions in muscle O_2 supply. As such, provision of supplemental O_2 during RE in these conditions may be beneficial in reinstating muscles' ability to synthesise new proteins after RE. If effective, such a strategy may, over time, allow attainment of the muscle mass and functional benefits of resistance training seen under normoxic conditions. Such an effect would lead to improvements in performance for athletes training and competing at altitude, thus providing an advantage over rival competitors that do not utilise supplemental O_2. It may also lead to improvements in adaptations to training in pathologically hypoxaemic patients. Furthermore, the observation that the level of blunting of MPS is correlated with extant SpO_2, suggests that exercise prescription and intervention strategies (e.g. supplemental O_2 provision) should be tailored to the individual.

6.3. Future research directions

In order to further our understanding of the regulation of MPS by feeding future studies should aim to, a) determine how quickly MPS can be re-stimulated following the decline in MPS after an initial protein feed so that nutritional strategies can be optimised, b) investigate the functional significance of increases in anabolic signalling outlasting elevated MPS, which may include allowing quicker MPS responses to subsequent feeding, or conversely a de-sensitisation of the protein synthetic machinery to subsequent feeds, c) investigate how this relates to the

method employed for administration of AA, i.e. the chronic effects of ‘pulse’ feeding versus TPN on muscle mass and function, d) investigate additional molecular mechanisms regulating the increase in MPS after feeding, such as eIF4F assembly and / or polyribosome clustering and, e) investigate which mechanisms control the decline in MPS after 90 min, which may include metabolite accumulation resulting from deamination of excess amino acids in the cell.

Following from the findings reported in chapter four, future work should focus on measuring performance and muscle soreness / damage with protein ingestion over the course of a more extensive training program in order to determine the efficacy of chronic supplemental protein. Similarly, the acute and chronic effects of post-exercise protein ingestion in those more likely to experience accelerated muscle wasting (for example, elderly and / or immobilised individuals) should be studied. The mechanism by which protein intake improves recovery of PPO and MVC during DOMS, focusing on the role of augmented amino acid availability on myofibrillar protein synthesis and / or protein breakdown and anabolic signalling should also be examined. Furthermore, future studies should aim to define whether there exist any specific effects of CHO versus protein intake on recovery of muscle function, perceptions of soreness and muscle damage markers.

Lastly, as an adjunct to the findings reported in chapter five, future studies should focus on, a) understanding the mechanisms underlying the apparent ‘responder non-responder’ phenomenon of MPS to environmental hypoxia, which may include variations in haematocrit, haemoglobin concentrations or hyperventilatory responses to hypoxia, b) investigating the intracellular mechanisms that appear to prevent MPS

from increasing despite augmented mTOR signalling, both at rest and after acute RE (for example, reduced ATP availability for peptide bonding, or alternative molecular signals that 'override' that maintained increases in mTOR signalling), c) detailing the chronic adaptations of protein synthesis, anabolic signalling and ultimately muscle mass and function to hypoxia at rest and with resistance training under tightly controlled conditions, in both conditions of environmental and pathological hypoxia, d) the response of protein breakdown to acute and chronic hypoxia in order to determine the role of protein degradation in maintaining / losing muscle mass during hypoxia and, e) determining, both acutely and chronically, the effects of supplemental O_2 in hypoxaemic / ischaemic conditions administered during and / or after RE on MPS, MPB and ultimately muscle mass and function.

6.4. Conclusion

Collectively, work in this thesis has increased current understanding of how skeletal muscle is regulated by key modulators of protein metabolism i.e., nutrition, exercise and hypoxia. First, a single oral protein bolus stimulates a biphasic response to food before returning to basal values after ~90 min, despite continued AA availability and maintained increased anabolic signalling. Temporal evidence is also provided as to the molecular signals that may be regulating the increase in MPS after feeding. Second, the functional applicability of protein ingestion was demonstrated after an acute muscle-damaging exercise bout, in the form of increased recovery of MVC and PPO 48 h post-exercise. Thirdly, reductions is O_2 supply has been demonstrated as an important regulator of MPS following acute RE, whereby RE-mediated increases in MPS are abolished over 2.5 h. Taken together, these findings have important

implications in the development and application of interventions aimed at maintaining / increasing muscle mass and function in both athletic and clinical populations.

CHAPTER 7. REFERENCES

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