

**EFFECTS OF RESISTANCE EXERCISE (INTENSITY AND VOLUME)
WITH OR WITHOUT LEUCINE ENRICHED PROTEIN
SUPPLEMENTATION ON HUMAN MYOFIBRILLAR PROTEIN
SYNTHESIS AND CELL ANABOLIC SIGNALLING**

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Abstract

Sarcopenia or the involuntary age associated muscle wasting starts in the fourth decade of life and accelerates markedly from the fifth decade. This gradual loss of muscle mass eventually results in an inability of older people to carry out simple daily tasks, instability, is associated with an increased risk of falls and fractures, loss of independence, and reduced quality of life. As the number of older people is growing steadily in our society, this in turn places an increasing burden on health care resources, making the topic of sarcopenia and its consequences an important area for research.

Resistance exercise and protein enriched feeding are potent stimulators of MPS and act synergistically to increase the MPS; however, the muscle protein synthetic responses to amino acids are blunted in the elderly in the resting state. Leucine has been shown to be the most potent branched-chain amino acid acting as a signal for accelerating MPS in the resting state.

How intensity and duration of resistance exercise can affect MPS and anabolic signalling in the elderly is less well understood. Can leucine enriched protein supplementation coupled with resistance exercise rejuvenate the MPS responses in the elderly? We aimed to answer these questions.

The results revealed a sigmoidal dose-response relationship between exercise intensity and the stimulation of MPS in the post absorptive state, with little increase from 20-40% 1RM, then a bigger rise at 60 % of 1 RM with no significant further

increase up to 90% 1RM in both the young and the elderly. Both groups showed quantitatively similar increases in phosphorylation of both p70s6K and 4E-BP1, which were maximal for exercise at 60-90% 1 RM at 1 h post exercise, i.e. just before the maximal increase in MPS. However, older men demonstrated a blunted rise in MPS and anabolic signalling activity after exercise, suggesting a general pattern of a reduced protein synthetic response to exercise in the elderly. This may explain, in part the mechanisms through which muscle is lost gradually with ageing.

Increasing exercise volume from 3 to 6 sets at 40% and 75% 1RM produced no additional MPS responses in post absorptive young men; however, in older men, it resulted in enhanced MPS and p70S6K responses at both intensities, suggesting that the muscle of older men requires a greater volume of exercise to activate the protein synthetic machinery sufficiently to achieve synthetic responses comparable to those seen in younger men.

Exercise, irrespective of intensity and volume caused only short term stimulation in MPS (returned to basal level at 4h post exercise) in the post absorptive state.

Leucine supplementation to protein feeding after resistance exercise appeared to overcome age-related anabolic blunting of responses of myofibrillar protein synthesis and p70S6K phosphorylation in skeletal muscle of older men by rejuvenating their synthetic responses.

In summary, the results gave a clear indication as to the likely optimal exercise intensity and volume of acute resistance exercise (6 sets of 8-10 reps at 75% 1RM)

coupled with optimal amino acid supplementation (leucine supplemented drink containing about 20 g of protein) required to effectively stimulate MPS and anabolic signalling in the elderly for maintenance of muscle mass. This work helps shed light on the pathophysiology of sarcopenia and suggests strategies that could be used to develop effective countermeasures to counteract sarcopenia.

Publications

- 1) **Kumar V**, Atherton P, Smith K, & Rennie MJ (2009). Human muscle protein synthesis and breakdown during and after exercise. *J Appl Physiol.* **106**, 2026-39.
- 2) **Kumar V**, Selby A, Rankin D, Patel R, Atherton P, Hildebrandt W, Williams J, Smith K, Seynnes O, Hiscock N, & Rennie MJ (2009). Age-related differences in the dose-response relationship of muscle protein synthesis to resistance exercise in young and old men. *J Physiol*, **587**, 211-7
- 3) Rennie MJ, Selby A, Atherton P, Smith K, **Kumar V**, Glover EL, & Philips SM (2010). Facts, noise and wishful thinking: muscle protein turnover in aging and human disuse atrophy. *Scand J Med Sci Sports.* **20**, 5-9.

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Involvement in work

1) Study Design

The study protocol including exercise protocol, number of muscle biopsies from both legs, timing of nutrition supplementation is designed by myself, Olivier Seynnes (Manchester Metropolitan University), Dr. Ken Smith (University of Nottingham), Dr. Natalie Hiscock (Unilever Discover R & D) and Professor Michael Rennie (University of Nottingham).

2) Volunteer Recruitment and Acute Clinical Studies

All subjects were recruited by me via advertisements. I was solely responsible for carrying out all of the screening visits including obtaining informed written consents, physical examination, blood tests, electrocardiograms, DXA and 1RM measurement and acute clinical studies.

3) Tracer Infusion and Capsules Containing Amino Acids Or Tracer

I was solely responsible for writing up prescriptions for tracer and capsules, arranging and collecting from the pharmacy on the day before the study day.

4) Plasma Samples

I was solely responsible for separating plasma samples from whole blood, the meticulous labelling and storage of plasma samples in refrigerators. The preparation of plasma for amino acid concentration analysis and amino acid enrichment determination was carried out by myself, Dr. Rekha Patel (University of Nottingham), Dr. Anna Selby (University of Nottingham), Dr. Kenneth Smith (University of Nottingham).

5) Muscle Samples

I was solely responsible for the labelling and storage of muscle samples in refrigerators. Muscle extraction was carried out by myself, Dr. Rekha Patel (University of Nottingham), Dr. Anna Selby (University of Nottingham), Dr. Kenneth Smith (University of Nottingham), Ms. Debbie Rankin (University of Nottingham). The isolated myofibrillar protein fractions were derivatized and run on the GC-C-RMS and analysed by Dr. Rekha Patel (University of Nottingham), Dr. Anna Selby (University of Nottingham) or, Dr. Kenneth Smith (University of Nottingham). Western blotting was carried out and analyzed by Ms. Debbie Rankin (University of Nottingham) and Dr. Philip Atherton (University of Nottingham).

6) Interpretation and Analysis of Data

The interpretation and analysis of data were carried out by myself and Professor M J Rennie.

7) Drafting and Final Approval of Manuscripts

I was responsible for initial drafting and revision of the manuscripts. Critical revision and final approval of manuscripts were carried out by Dr Philip Atherton (University of Nottingham), Dr. Ken Smith (University of Nottingham) and Professor Michael Rennie (University of Nottingham).

8) Presentation To The Research Community

I was responsible for initial preparation and communication of oral as well as poster presentations in the research meetings (see appendix) and final approval was given by Professor Michael Rennie (University of Nottingham)

Statement by Supervisor (Professor Michael Rennie)

I declare that Dr. Vinod Kumar's thesis is his own work and the result of work done while registered for the degree

Signature.....

Abbreviations

4E-BP1	Eukaryotic Initiation Factor 4E-Binding Protein 1
5'TOP	5'-Terminal Oligopyrimidine Tract
AA	Amino Acids
Akt	Protein Kinase B
AMPK	Adenosine Monophosphate-Activated Protein Kinase
AUC	Area Under The Curve
BCCA	Branch Chain Amino Acid
CT	Computerised Tomography
DNA	Deoxyribonucleic Acid
DXA	Dual Energy X-Ray Absorptiometry
EAA	Essential Amino Acid
EDTA	Ethylenediaminetetraacetate
EE	Endurance Exercise
Eef2	Eukaryotic Elongation Factor 2
EIF2B	Eukaryotic Initiation Factor 2b
EIF4F	Eukaryotic Initiation Factor Complex 4F
FBR	Fractional Breakdown Rate (%.H ⁻¹)
FSR	Fractional Synthesis Rate (%.H ⁻¹)
GSK-3	Glycogen Synthase Kinase 3
IGF-1	Insulin-Like Growth Factor
KIC	α -Ketoisocaproic Acid
LBM	Lean Body Mass
LC	Lengthening Contractions

LE	Leg Extension
LP	Leg Press
MAPK	Mitogen Activated Protein Kinase
MHC	Myosin Heavy Chain
MPB	Muscle Protein Breakdown
MPS	Muscle Protein Synthesis
MRF4	Myogenic Regulatory Factor 4
mTOR	Mammalian Target Of Rapamycin
P70s6k	P70 S6 Kinase
Pex	Post Exercise
PKB	Protein Kinase B
PVDF	Polyvinylidene Difluoride
Ra	Rate of Appearance of Phenylalanine (Nmol.Min ⁻¹ .100g Leg ⁻¹)
Rd	Rate of Disappearance of Phenylalanine (Nmol.Min ⁻¹ .100gLeg ⁻¹)
RE	Resistance Exercise
RM	Repetitions Maximum
RNA	Ribonucleic Acid
rps6	Ribosomal Protein S6 Kinase
SC	Shortening Contractions
SD	Standard Deviation
SEM	Standard Error of Mean
SFO	SlimFast Optima

CHAPTER 1: INTRODUCTION AND REVIEW OF THE LITERATURE

1.1 INTRODUCTION

Skeletal muscle demonstrates extraordinary plasticity in its responses to exercise of different modes, intensity, and duration, which must involve alterations of muscle protein turnover, both acutely and chronically. In this chapter, I bring together information on the alterations in the rates of synthesis and degradation of human muscle protein by different types of exercise and the influences of nutrition, age, and sexual dimorphism on muscle protein turnover. I have included four tables in this chapter, where I have summarized the pertinent data from studies carried out over past 2-3 decades in this field, highlighting specific variables i.e. age and sex, type of exercise performed, nutritional intervention and changes in protein synthesis and breakdown, and alterations in the major cell signalling molecules involved in regulating protein synthesis in response to exercise and feeding, thereby providing the reader an overview of work in this area. I have also included two figures describing a general scheme of alterations in the major cell signalling pathways involved in regulation of protein synthesis in response to exercise and feeding, so where possible I will describe only briefly associated changes in the activity of regulatory pathways, as inferred from changes in phosphorylation status.

Exercise of both the resistance and non-resistance types appears to depress muscle protein synthesis (MPS), whereas muscle protein breakdown (MPB) probably remains unchanged during exercise. However, both MPS and MPB are elevated after exercise in the fasted state, when net muscle protein balance remains negative. Positive net balance is achieved only when amino acid availability is increased, thereby raising MPS markedly. However, postexercise-increased amino acid availability is less important for inhibiting MPB than insulin, the secretion of which is stimulated by hyperglycemia, without itself stimulating MPS. Exercise training

appears to increase rates of basal muscle protein turnover, with differential responses of the myofibrillar and mitochondrial protein fractions to acute exercise in the trained state. Ageing reduces the responses of myofibrillar protein and anabolic signaling to feeding and possibly to exercise. The acute response of elderly muscle to exercise of varying intensity and volume remains to be investigated in both the fed and the postabsorptive states.

There appear to be few, if any, differences in the response of young women and young men to acute exercise, although there are indications that, in older women, the responses may be blunted more than in older men.

The chapter concludes with highlighting a number of gaps that exist within the literature with regard to alterations in human skeletal muscle protein synthesis and its translational control following resistance exercise with or without feeding as well as explaining the objectives of the research work.

1.2 SKELETAL MUSCLE: STRUCTURE AND FUNCTION RELATED TO PROTEIN METABOLISM

Muscle fibres are multinucleated cylindrical contractile cells that are the functional units of skeletal muscle. Total volume of muscle in an individual depends upon number and the size of these muscle fibres (Wackerhage & Rennie, 2006). The number of muscle fibres appears to be genetically determined whereas the size of muscle fibre is maintained by continuous remodelling of muscle protein secondary to constant protein synthesis and breakdown in response to various stimuli such as exercise, immobilization, feeding or fasting.

Skeletal muscle makes up about 40% of the total body weight of a healthy adult male (Wackerhage & Rennie, 2006) and protein contents in muscle accounts for approximately 20% of the total skeletal muscle mass. 65% of the muscle protein contents are made up of actin, myosin and collagen and account for about 25% (Nair et al, 1992) of all protein turnover in the body. Actin and myosin constitute the contractile machinery of the myofibril, muscle contraction being the result of a "sliding mechanism" of the myosin-rich thick filament over the actin-rich thin filament after neuronal activation. Muscle collagen protein is found in extracellular connective tissue matrix of skeletal muscle and accounts for 1-9% of skeletal muscle mass. Collagen constitutes a connective tissue framework in skeletal muscle which joins the contractile myofibres into a functional unit and aids the transfer of contractile force to tendon and bone.

Skeletal muscle fibres differ significantly in their biochemical, morphological and physiological properties from other human body cells. Skeletal muscle fibres contain

multiple elliptical peripheral nuclei. The endoplasmic reticulum expands between the myofibrils and the surface of endoplasmic reticulum is not studded with protein manufacturing ribosomes. Although lysosomes in muscle cells contain the full complement of protein degradation enzymes, they are not morphologically well-defined as in other body cells. Skeletal muscle contains a population of undifferentiated mononuclear myogenic cells termed cells, which regulate muscle regeneration (Charge & Rudnicki, 2004).

The individual skeletal muscle is composed of a blend of different types of myofibres, designated largely by myosin heavy chain isoforms, Type I (slow twitch, red with smallest cross-sectional area (CSA) in men), Type IIa (fast twitch, red with largest CSA in men) and Type IIx (fast twitch, white with medium CSA in men and smallest CSA in women). The cross-sectional areas of all three major fiber types (I, IIA, and IIB) are significantly larger for the men compared to the women (Staron *et al*, 2000). Muscle fibre type composition in an individual depends on the genotype (Simoneau & Bouchard, 1995) and environmental factors such as long-term resistance or non-resistance exercise. The relative amount of each fibre type within a muscle affects its general contractile property e.g. knee and hip extensors (anti-gravity muscles) contain more oxidative type I fibres, whereas triceps and plantaris (for prolonged or short-lived anaerobic activities) contain more type II fibres (Spangenburg & Booth, 2003). In human muscle, these different myofibres are found to have similar sarcoplasmic and myofibrillar synthesis rates (Mittendorfer *et al*, 2005; Dickinson *et al*, 2010), in contrary to the earlier reports of higher basal rate of protein turnover in Type I fibres in animal muscles (Laurent *et al*, 1978). Therefore,

loss or increase of a particular fibre type in muscle doesn't affect the rate of muscle or whole body protein metabolism.

The primary function of skeletal muscle is to provide a rigid contractile structure to the body for the maintenance of posture and locomotion. In addition, it also has significant metabolic functions (Christensen, 1986; Daniel, 1977; Felig, 1975) as a store of amino acids and glycogen. Muscle plays a fundamental role in whole-body protein metabolism as it has a considerable store of protein-bound amino acids (more than 80%) to maintain protein synthesis in vital organs and tissues such as heart, liver, skin and brain (Wolfe, 2006). In the absence of nutritional intake, these essential organs maintain their mass and protein synthesis due to the steady supply of required amino acids from muscle (Cahill, Jr., 1970; Felig *et al*, 1969; Biolo *et al*, 1995). In addition, muscle continually supplies carbon in the form of alanine and nitrogen in the form of alanine and glutamine as a substrate for hepatic gluconeogenesis (Consoli *et al*, 1990; Felig, 1973) and ureagenesis (Nurjhan *et al*, 1995) throughout the diurnal (fasting and feeding) cycle. Skeletal muscle also stores glycogen (1% of muscle wet weight) and is considered as the principal insulin-sensitive tissue in the human body and is the leading tissue for post-prandial insulin-stimulated glucose utilization (Wojtaszewski *et al*, 2002). Skeletal muscle cell also contains intramyocellular lipid, which seems to provide some fuel for muscle during exercise (Schrauwen-Hinderling *et al*, 2003), thus accounting as a site for fatty acid oxidation in the body and largest sole contributor to daily energy expenditure.

Thus, skeletal muscle has diverse range of metabolic as well as thermogenic (i.e. energy consuming and heat producing) functions and maintenance of adequate

muscle mass is vital not only for locomotion but also for glucose, fat as well as protein homeostasis.

1.3 THE MECHANISMS OF MUSCLE PROTEIN SYNTHESIS (TRANSCRIPTION AND TRANSLATION)

Muscle protein synthesis is initiated by various anabolic stimuli such as exercise or feeding. Once stimulated MPS involves two main steps at molecular levels:

1) **Transcription** of DNA (the nuclear code) to form messenger RNA (mRNA) in the nucleus.

2) **Translation** of mRNA in the sarcoplasm to create polypeptides for a specific protein synthesis. This step of protein synthesis further includes three phases: initiation, elongation and termination. These three phases are regulated by proteins called eukaryotic initiation, elongation and release factors, respectively (Proud, 2007). The increase in the rate of mRNA translation increases the rate of muscle protein synthesis in the short term after an anabolic stimulus such as exercise or feeding (Welle *et al*, 1999).

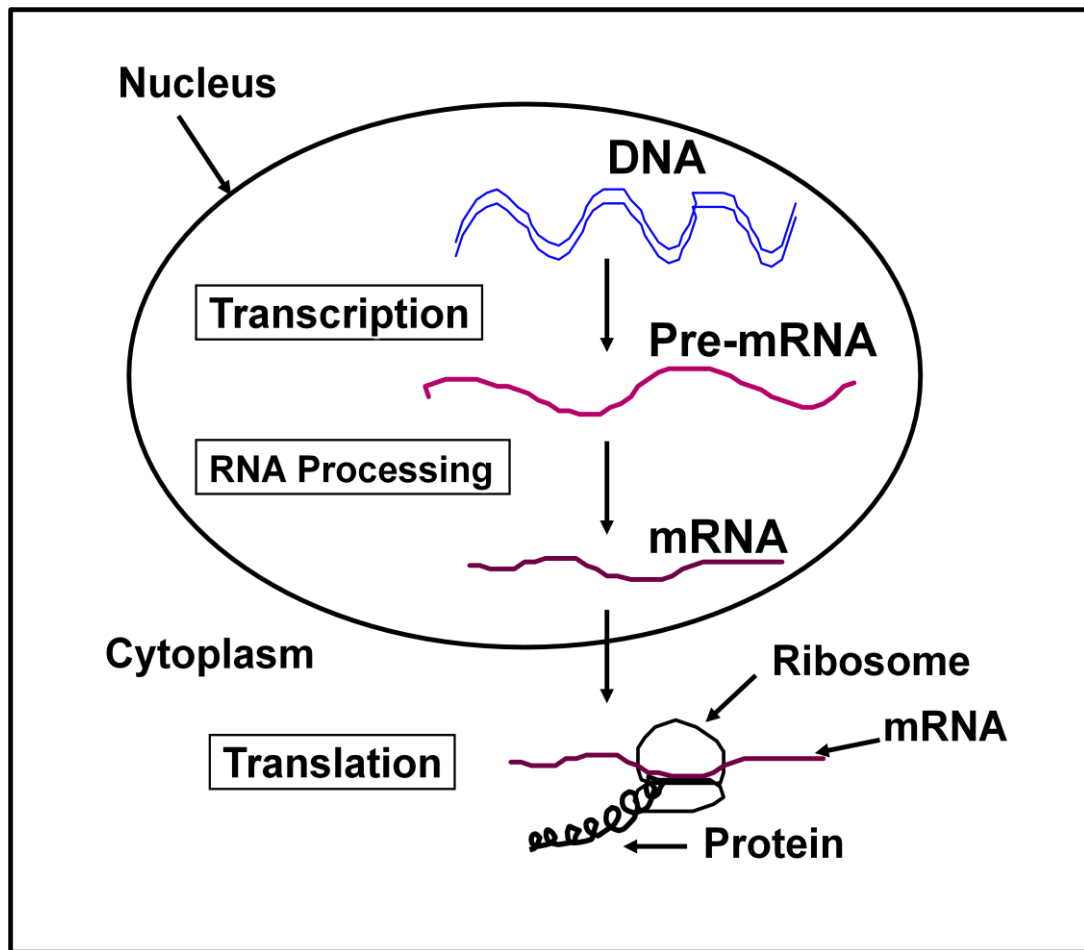


Figure 1.1 General schemes of steps of muscle protein synthesis.

1) **Transcription:** Transcription is a process by which information transcribed from DNA to RNA when complementary mRNA is synthesized from the replication of DNA by RNA polymerase. RNA polymerase attaches to a promoter sequence within the segment of DNA upon which it moves along the nucleotide in a 5' to 3' direction. As a result, the primary transcript or pre-mRNA is created which then goes through modifications such as methylation, acetylation, cleavage or splicing before migrating to the sarcoplasm. A 7-methylguanosine cap is added to the 5' end which prevent degradation by phosphatases or nucleases and a poly-adenylation segment (Poly-A tail) is inserted to the 3' end of mRNA sequences. Transcription is regulated by several transcription factors such as calmodulin and calcineurin.

2) **Translation:** Translation is the step of muscle protein synthesis during which the codons (sequence of bases) on a particular mRNA are translated to generate a chain of amino acids in forming a particular protein (Lewis, 2006). Polypeptide synthesis is commenced by attachment of the mRNA to ribosomal RNA (rRNA) (Pain, 1986), specifically to 80S ribosome creating an active 80S initiation complex. The AA-charged transfer RNA (tRNA) then brings the subsequent amino acids to the ribosome complex and commences the elongation step. During elongation tRNA bound AA are lined up and bonded together through peptide bonds in accordance with the mRNA code. The order in which amino acids are combined through peptide bond formation is regulated by the triple base sequence (codon) of the mRNA which complements a sequence of opposite bases (anti-codon) on the tRNA. Each amino acid has its own specific tRNA molecule, representing the immediate precursors for protein synthesis. The amino-acyl tRNA makes up only 15% of the total RNA but their turnover is rapid (<10s) (Watt *et al*, 1991). During elongation a given mRNA

can attach to several ribosomes forming a polyribosome (or polysome). There are two separate pools of ribosomes, one free cytosolic form and the other membrane bound form, attached to the endoplasmic reticulum. These different ribosomes regulate the type of protein (structural or secretory) being synthesized in the cell (Palade, 1975).

Upon completion of translation of the complete message, elongation is terminated and the polypeptide chain is released from the ribosome-protein complex. The polypeptide chain then undergoes further post translational modification including folding into its tertiary structure before being integrated into the cell structure or stored intracellularly in vesicles before secretion.

1.4 CELL SIGNALLING PATHWAYS REGULATING MUSCLE PROTEIN SYNTHESIS

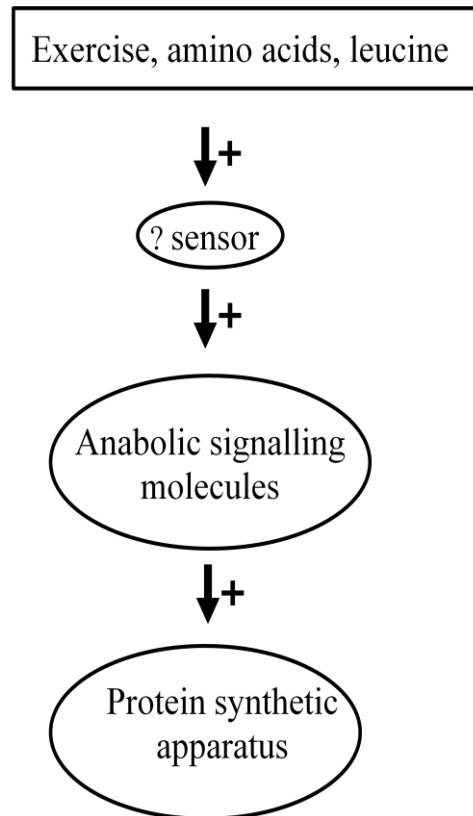


Figure 1.2 General schemes of muscle protein synthetic machinery; + means stimulation

The protein synthetic machinery is controlled by a number of transcriptional and translational signalling proteins and intracellular signaling pathways, involving feedback loops and inhibitory proteins (Hershey, 1989; Hershey, 1991; Pain, 1986; Bennet & Rennie, 1991). The rise in MPS in response to an acute anabolic stimulus has been shown to be brought about by increased mRNA translational efficiency, primarily at the stage of initiation (Welle *et al*, 1999; Holz *et al*, 2005; Hornberger *et al*, 2006). Further muscle growth or hypertrophy seems to be dependent on satellite cell proliferation and differentiation (Wackerhage & Rennie, 2006).

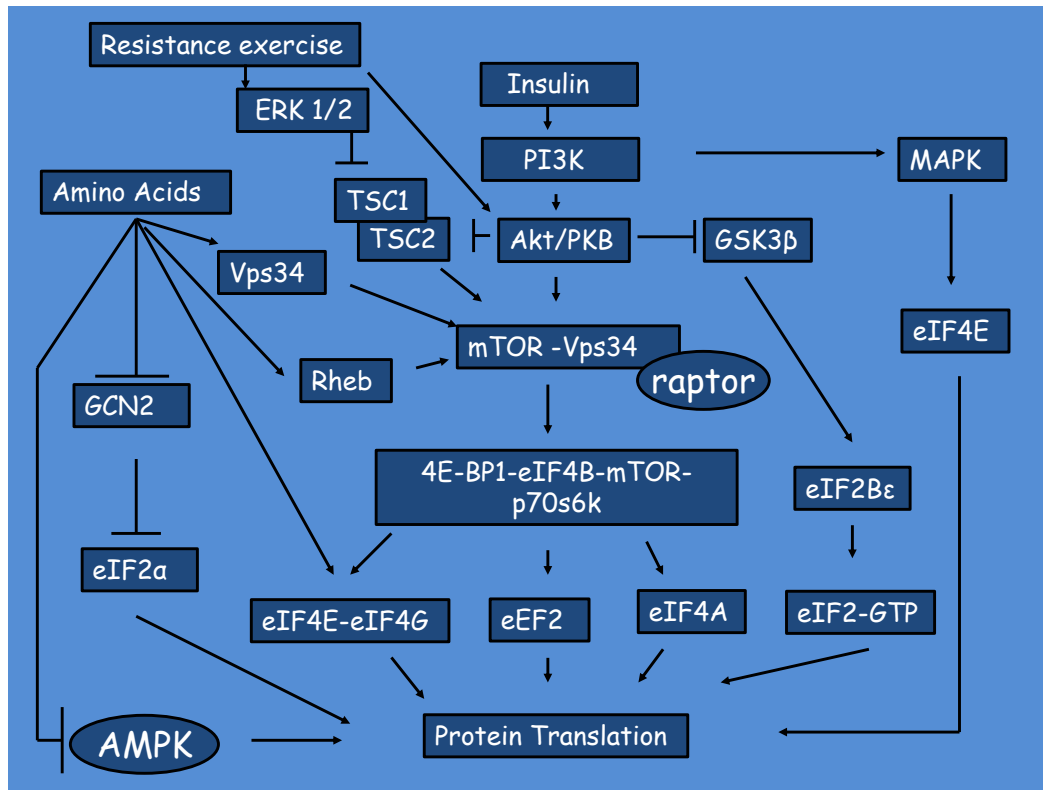


Figure 1.3 Schematic representations of intracellular signalling pathways for activation of mammalian target of rapamycin (mTOR), leading to increased muscle protein synthesis by resistance exercise, amino acids and insulin. Arrows indicate activation; solid lines indicate inhibition. Figure kindly provided by Professor MJ Rennie. MAPK, mitogen-activated protein kinase; AMPK, AMP-activated protein kinase; Akt protein kinase B; mTORC1, mammalian target of rapamycin complex 1; eIF4E-BP1 eukaryotic initiation factor 4E binding protein 1; p70S6k1, p70S6 kinase; eIF2Bε, eukaryotic initiation factor 2Bε; eEF2, eukaryotic elongation factor 2; eIF4F, eukaryotic initiation factor complex 4F; ERK, extracellular-related kinase; PI3K, phosphatidylinositol 3 kinase; TSC, tuberous sclerosis complex; Rheb, rat homologue enriched in brain; Vps34, vacuolar protein sorting subunit 34; GSK3β, glycogen synthase kinase 3β.

There is still much to learn about how the MPS is regulated by signalling proteins, however at this point, it is known that the protein translation-regulated signaling pathways include (figure 1.3):

1) **The mTOR dependent pathway:** phosphatidylinositol 3-kinase (PI3K)-protein kinase B (PKB)-mammalian target of rapamycin (mTOR) pathway involving eukaryotic initiation factor 4E-binding protein 1 (4EBP-1) and ribosomal protein S6 kinases (p70S6K) (Rommel *et al*, 2001; Bodine *et al*, 2001; Hay & Sonenberg, 2004).

2) **The mTOR independent pathway:** PKB/GSK-3 β (Welsh *et al*, 1997), involving eIF2B.

1) **The mTOR dependent pathway:** PKB/Akt is located upstream from mTOR (Glass, 2005; Raught *et al*, 2004) and is activated by a variety of growth factors (e.g. IGF-1) and hormones (insulin) through phosphorylation on two regulatory sites, Thr308 and Ser473, which further activates mTOR (Nave *et al*, 1999). Activated mTOR induces the phosphorylation of p70S6K-induced phosphorylation of the small ribosomal protein 6 (rpS6) and the eukaryotic translation initiation factor 4B (eIF4B), which promotes the initiation of protein translation (Raught *et al*, 2004; Wang *et al*, 2003; Parsa & Holland, 2004). Phosphorylation of p70S6K promotes translation of a specific subset of mRNAs containing an oligopyrimidine tract at the 5' terminus (5'TOP mRNAs), which encode ribosomal proteins and elongation factors (eEF1A and eEF2) (Terada *et al*, 1994). The eIF4E protein plays a rate-limiting role in translation initiation, and the eIF4E-binding protein (4E-BP) prevents

eIF4E from engaging in the active translation initiation process (Holz *et al*, 2005; Parsa & Holland, 2004; Wang *et al*, 2003). mTOR also phosphorylates 4E-BP1, reducing its affinity for eIF4E and releasing eIF4E to facilitate translation initiation (Parsa & Holland, 2004; Wang *et al*, 2003). eIF4E initiates translation by binding to the 5' cap structure of mRNA. Thus mTOR activation is an important determinant of the cell's ability to stimulate protein synthesis through translation initiation (through 4E-BP1) and ribosomal biogenesis (through p70S6K) (Kawasome *et al*, 1998).

An increase in circulating insulin secondary to feeding activates phosphatidylinositol 3 kinase, leading to the phosphorylation of Akt-protein kinase B, which in turn activates mTOR, ultimately leading to translation initiation and increased protein synthesis (Anthony *et al*, 2002; Bodine, 2006).

Recent work suggests a role for extracellular-related kinase (ERK) 1/2 in post-exercise MPS stimulation, in that its activation in response to an acute bout of exercise activates the mTOR by phosphorylating and inactivating the negative regulator of mTOR, tuberin-tuberous sclerosis complex (TSC 1/2) (Ma *et al*, 2005). Furthermore, ERK1/2 is shown to directly activate both eukaryotic initiation factor (eIF) 4E and ribosomal protein S6 (Atherton & Rennie, 2006).

Amino acids have shown to stimulate mTOR and its downstream effectors, p70S6K and 4E-BP1 through a different upstream input than insulin such as Ras homolog enriched in brain and the human vacuolar protein sorting 34 (hVps34) (Bolster *et al*, 2004; Byfield *et al*, 2005; Nobukuni *et al*, 2005).

- 2) **The mTOR independent pathway:** PKB phosphorylates and inhibits GSK-3 β , relieving its inhibition on the guanine nucleotide exchange factor, eIF2B, which then initiates protein translation (Welsh *et al*, 1997).

Table 1.1 Effect of feeding and resistance or non-resistance exercise on human muscle cell anabolic signalling

Subjects	Exercise or Feeding Protocol	Alteration in Phosphorylation Status		References
		Increased (↑)	Decreased (↓)	
5M 2W Fasted	BCAA infusion × 6 h: 1.66 μmol/min·kg after priming with 5.0 μmol/kg·min × 30 min; measurement at 6 h	↑ 4E-BP1, ↑ p70S6K		<i>Liu et al. 2001</i>
8M 8EM Fasted	Acute session of 3 x 10 reps of LE at 70% 1RM; measurement immediately after exercise	Young: ↑ ERK 1/2, ↑ MnK1 Old: Higher basal ERK and MnK1 phosphorylation Both groups: ↔ 4E-BP1	Old: ↓ ERK 1/2, ↓ MnK1, ↓ MAPK	<i>Williamson et al. 2003</i>
6M 8EM Fasted	Euglycemic hyperinsulinemic hyperaminoacidemic clamp; Measurement at 4 h post infusion	Young: ↑↑ p70S6K Both groups: Similar ↑ activity of PKB, mTOR, & 4E-BP1		<i>Guillet et al. 2004</i>
7M Fasted	4 x 10 reps of LE at 80% 1RM ± BCAA (45% leu, 30% valine, and 25% isoleu) during and after exercise; Biopsy at 0-, 1- and 2 h post exercise	Ex only: ↑ p70S6K (0-2 h) EX + BCCA: ↑↑ p70S6K (0-2 h) BCAA only: ↑ rpS6 only		<i>Karlsson et al. 2004</i>
20M 24EM Fasted	Oral 10 g EAA; Biopsy at 3 h post intervention	Both groups: ↑ mTOR, ↑ p70S6K and ↑ 4EBP1	Blunted response in the elderly	<i>Cuthbertson et al. 2005</i>
7M, 4F Fasted	10 x 10 reps at 80%; Measurements at 0-, 1- and 2 h post exercise	↑ PKB (1 h), ↑ mTOR (1-2 h), ↑ p70S6K (2 h)	↓ TSC2 (1 h), ↓ 4E-BP1 (0 h), ↓ eEf2 (1-2 h)	<i>Dreyer et al. 2006</i>
10M Fasted	4 x 6 concentric or eccentric contractions in fasted conditions; Measurements at 0-, 1- and 2 h post ex	↑ P70S6K (0-2 h), ↑ rpS6 after maximal eccentric only; No change: Akt (0-2 h), mTOR (0-2 h)		<i>Eliasson et al. 2006</i>
13M Fasted	One bout of 1 h cycling at 70% VO ₂ peak or 8 x 5 maximal repetitions of isokinetic LE in 7 endurance and 6 strength trained men; Biopsy at 0- and 3 h post exercise	Strength training: ↑ AMPK (0 h), ↑ p70S6K (3 h), ↑ rpS6 only in endurance trained men, ↔ Akt and eIF2B Endurance training: ↑ AMPK (0 h), ↑ Akt (0 h), ↔ TSC2, p70S6K and rpS6	Strength: ↓ TSC2 (0-3 h), Endurance: ↓ eIF2B (0-3 h) only in strength trained, ↓ Akt (3 h) in EE trained	<i>Coffey et al. 2006</i>
8M Fasted	Stepping exercise (+25% body weight) till fatigued with 45 g EAA + 135 g CHO administered 2 h post exercise; Measurements at 3-, 6- and 24 h post ex	↑ Akt (3 -24 h) and ↑ p70S6K (3-24 h), no effect of mode of contraction		<i>Cuthbertson 2006 et al.</i>
8M Fasted	Single session of 8 x 10 reps 75% LP, 8 x 10 reps 75% LE; Measurements at 0-, 0.5-, and 2 h post ex	↑ AMPK (0 h), ↑ p70S6K (0-2 h), ↔ rpS6, ↑↑ p70S6K in type II fibres	↓ 4E-BP1 (0 h)	<i>Koopman et al. 2006</i>
14M Fasted	Leu EAA+CHO (0.35 g+0.5 g.kg.FM ⁻¹); Biopsy at 1h	↑Akt, ↑mTOR, ↑p70S6K and ↑4EBP1	↓AMPK, ↓TSC2 and ↓eEf2	<i>Fujita et al. 2007</i>
7M Fasted	8 x 10 reps 75% LP, 8 x 10 reps 75% LE followed by 0.3 g/kg CHO ± 0.3 g /kg protein (PRO) at 0 and 1 h; Measurements at 0-, 1- and 4 h post exercise	Exercise + CHO: ↑ 4EBP1 (1-4 h), ↑ p70S6K (0-4 h), Exercise + CHO + PRO: ↑ 4EBP1 (1-4 h), ↑↑ p70S6K (0-4 h), ↑↑ rpS6 (0-4 h)	Ex + CHO: ↓↓ 4E-BP1 (0 h), ↓ rpS6 (0 h) Ex + PRO: ↓ 4E-BP1 (0 h)	<i>Koopman et al. 2007</i>
6M	Cycling at 75% VO ₂ max for 1 h; Measurements at	↑ Akt (1-2 h), ↑ p70S6K (0-3 h), ↑ mTOR (0-3 h),	↓ eEF2 (30 min – 3 h)	<i>Mascher et</i>

Fasted	0-, 0.5-, 1-, 2- and 3 h post exercise	↑ GSK-3 (0-3 h)		<i>al. 2007</i>
16M Fasted	10 x 10 reps at 70% ± Leu EAA + CHO administered 1 h post exercise; Measurements at 0-, 1- and 2 h post exercise	Exercise alone: ↑ Akt (1 h), ↑ TSC2 (0-2 h), ↑ mTOR (0-2 h), ↑ 4EBP1 (0 h), ↑ p70S6K (0-2 h) Ex + feeding: ↑ Akt (2h), ↑ TSC2 (2 h), ↑↑ mTOR (2 h), ↑ 4EBP1 (0 h), ↑↑ p70S6K (2 h),	Ex alone: ↓ eEf2 (1-2h) Ex + feeding: ↓ eEf2 (2h)	<i>Dreyer et al. 2008</i>
8M Fasted	400 ml of 7.8% dextrose and 1.8% protein or placebo after 45 min cycling at 75% VO ₂ max + sprints; measurement at 45 min post exercise	Exercise alone: ↑ p70S6K, ↑ rpS6 Ex + Feeding: ↑ Akt, ↑ mTOR, ↑ p70S6K, ↑ rpS6	Exercise alone or with feeding: ↓ GS3β	<i>Ivy et al. 2008</i>
9M Fasted	10 x 10 reps unilateral LE at 80% 1RM ± creatine administration for 5 days; Measurements at 0-, 24- and 72 h post exercise		↓ Akt and ↓ 4E-BP1 at 0 h in both groups and 24 h post-ex with creatinine	<i>Deldicque et al 2008</i>
9M Fasted and fed	Single unilateral session of 4 x 8 at 10RM of LP and LE, biopsies from exercised and non-exercised leg ± 3 x 10 g protein, 41 g carbohydrate, 4 g fat at 90, 180, and 270 min postex; Biopsy at 6 h post ex	Feeding rest: ↑ PKB/Akt Feeding ex: ↑↑ p70S6K, ↑ rpS6 above exercise alone Fasted ex: ↑ Akt, ↑ p70S6K, ↑ rpS6 No effect on mTOR, FAK, GSK-3β	Fasted ex: ↓ eIF2B	<i>Glover et al. 2008</i>
7M 6EM Fasted	20 g of EAA 1 h following leg extension 8 x 10 reps at 70% 1RM; Measurements at 1-, 3- and 6 h post exercise	Young: : ↑↑ ERK1/2 (1 h), ↑ Akt (3 h), ↑↑ MNK1 (1-3 h) Old: : ↑↑ AMPK (1-3 h) Both: ↑ mTOR (1-6 h), ↑ S6K1 (1-6 h), ↑ 4E-BP1 (3-6 h)	Young: : ↓↓ EIF2α (1 h), ↓ eEF2 (3-6 h) similar between both groups	<i>Drummond et al. 2008</i>
8M Fed	6 sets of LP at 6RM – 14 weeks of training vs. no training; Measurement at 30 min post exercise	Untrained: ↑ p70S6K (30 min), ↑ mTOR (30 min) Trained: ↑ p70S6K correlated with ↑ in muscle mass	Untrained: ↓ Akt (30 min)	<i>Terzis et al. 2008</i>
10 M Fed	5 x 10 reps 80% LE or 45min 75% VO ₂ max, 1.1 g prot.kg ⁻¹ ; 10 weeks of resistance or endurance training vs. untrained, measurements at 4 h post exercise following acute unilateral RE and EE	Untrained: ↑Akt, ↑mTOR, ↑p70S6K, no difference in RE or EE legs Trained: ↑ Akt, ↑ mTOR, ↑ p70S6K only in RE trained group		<i>Wilkinson et al 2008</i>
10M Fed	Single unilateral LE, biopsies from exercised and rest leg; Measurements at 0- and 6 h post exercise	Feeding only: ↑ 4E-BP1 (6 h), ↔ mTOR, p70S6K (0 h) Ex + feeding: ↔ AMPK, ↑↑ 4E-BP1 (6 h), ↔ mTOR, ↑↑ p70S6K (0 h)	Feeding: ↓ 4E-BP1 (0 h) Ex+feeding: ↓↓ 4E-BP1 (0h)	<i>Witard et al. 2009</i>
8M 8F Fasted	Hyperinsulinemic-hyperaminoacidemic-euglycemic clamp x 3 h, 105 mg AA·kg FFM ⁻¹ ·h ⁻¹	↔ Akt, ↔ mTOR, and ↔ p70s6k between both men and women at 3 h post clamp		<i>Smith et al. 2009</i>

↑ increase; ↓ decrease; ↔ no difference; M, young male; EM, elderly male; F, female; MAPK, mitogen-activated protein kinase; AMPK, AMP-activated protein kinase; Akt, protein kinase B; mTORC1, mammalian target of rapamycin complex 1; eIF4E-BP1, eukaryotic initiation factor 4E binding protein 1; p70S6k1, p70S6 kinase; eIF2B, eukaryotic initiation factor 2B; rps6, ribosomal protein s6 kinase; eEF2, eukaryotic elongation factor 2; eIF4F, eukaryotic initiation factor complex 4F; TSC, tuberous sclerosis complex; LP, leg press; LE, leg extension; CHO, carbohydrate; BCAA, branched chain amino acids; RE, resistance exercise; EE, endurance exercise.

1.5 METHODS TO STUDY HUMAN SKELETAL MUSCLE PROTEIN METABOLISM

Skeletal muscle has comparatively low fractional synthetic rates, being five times lower than the synthetic rates of heart and thirteen times lower than liver. Therefore, although muscle makes up about 40% of the total protein in an adult male, it contributes only about 27% to the whole body protein synthesis (Baumann *et al*, 1994; Nair *et al*, 1988). Thus, measurement of whole body protein synthesis using stable isotope labelled amino acid tracer (Matthews *et al*, 1980) does not accurately mirror muscle protein synthesis. Additionally, muscle protein is made up of different protein fractions i.e. myofibrillar, sarcoplasmic and mitochondrial and these different protein fractions react differently to the nature of exercise (Wilkinson *et al*, 2008) and likely to certain disease states also. Thus, measurement of mixed muscle protein synthesis may not accurately reflect alterations in the synthetic rates of the different protein fractions (Louis *et al*, 2003; Rooyackers *et al*, 1996) in response to anabolic intervention.

However, since 1975, when human myofibrillar and sarcoplasmic protein synthesis were first measured (Halliday & McKeran, 1975), advances in techniques have led to a set of methods that are able to reliably measure the effects of physiological changes to different muscle protein fractions over periods as short as 1h. Improvements in the sensitivity and precision of gas chromatography mass spectrometry (including combustion mass spectrometry) and, more recently, the use of proteomic techniques have allowed the measurement of rates of synthesis of individual proteins (Nair *et al*, 2004) over relatively short periods and can now be applied to measure the acute response of MPS to exercise.

There are two methods commonly used for the measurement of muscle protein synthesis from the incorporation of labelled amino acid tracers, the 'constant infusion method' and flooding method'.

Generally, constant infusion method involves a primed, constant infusion of, among others, use of [1-¹³C]leucine (Rennie *et al*, 1982), [1-¹³C]α-ketoisocaproate (Chinkes *et al*, 1996), d₅ phenylalanine (Phillips *et al*, 1997; Phillips *et al*, 1999), or [ring-¹³C₆]phenylalanine (Glover *et al*, 2008) as tracers, to achieve a steady state of tracer labeling in plasma. Muscle protein synthesis is measured by calculating the plateau labelling of the tracee as well as the increase in tissue incorporation of the tracer at a single time point at the end of the infusion (Smith & Rennie, 1996).

The flooding method involves administering the tracer as a large "flooding" dose, to equilibrate the tracer in all the intra- and extracellular amino acid pools, thereby minimizing the uncertainty in the labeling of the immediate precursor for protein synthesis, i.e., the amino-acyl tRNA. However, the demonstration that both leucine and phenylalanine stimulate MPS when administered as a large bolus (>3 g) has led to the use of this approach being questioned (Smith *et al*, 1992). Nevertheless, our laboratory has recently obtained MPS rates identical to those seen with constant infusion of labeled leucine when using a flooding dose of ¹³C- or ¹⁵N-labeled proline, (Miller *et al*, 2005) probably linked to the fact that proline is a nonessential amino acid and only essential amino acids appear to stimulate MPS in the flooding method.

Regarding muscle protein breakdown, methods for discerning dilution of free intracellular amino acids as measures of fractional protein breakdown (FBR) offer the possibility of measuring both arms of mixed muscle turnover, i.e., synthesis and breakdown in a single study, but they are technically demanding and so far have been

applied successfully only in a few studies (Phillips *et al*, 2002; Zhang *et al*, 2002). The arteriovenous (A-V) tracer dilution method (Cheng *et al*, 1985), and its later modifications, produces values of limb (including skin, fat and bone) rather than muscle protein turnover and, if a carbon tracer of a branched chain amino acid is used, amino acid oxidation; it has proved to be very useful (Biolo *et al*, 1995; Biolo *et al*, 1997; Tipton *et al*, 1999), but it should be used selectively, i.e., only when there is confidence of the existence of steady states of blood flow, unlabeled and labeled amino acid, and hormone concentrations. This is mostly due to the fact that changes in blood flow, as a result of exercise, would change the transit time of tracers (and, as a consequence, their uptake and release from muscle tissue) that are not co-temporal and could not be accounted for without exact knowledge of arterial-venous and amino acid pool transit times. Studies that violate these conditions produce less than ideal results and are often only qualitatively indicative. Further explanation of the methodologies involved in measuring protein turnover is outside the remit of this thesis, the interested reader is directed to the following review articles (Rennie, 1999; Wagenmakers, 1999).

3-Methyl histidine (3 MeH) is produced by posttranslational methylation of histidine residues on actin and myosin and is not subject to reincorporation into protein after proteolysis. Therefore, its appearance has been suggested as an index of the rate of myofibrillar proteolysis when assayed in either biopsied muscle or in muscle dialysate. However, the method, in my opinion, is unreliable without coincident measures of tracer dilution, muscle blood flow, and particularly muscle microvascular blood flow. A good example of the unreliability of this approach is demonstrated when, against almost all other findings in the literature (Biolo *et al*,

1995a; Phillips *et al*, 1997; Phillips *et al*, 1999), it delivered results of no change in muscle proteolysis after intense exercise (Haus *et al*, 2007).

1.6 REGULATION OF MUSCLE MASS

Muscle mass is maintained through the regulated balance between MPS and MPB. A net gain of muscle mass is only possible if MPS exceeds MPB, i.e., protein net balance is positive, whereas the converse occurs when MPB exceeds MPS. In the resting, fasted state (more accurately known as the postabsorptive condition), muscle protein net balance is negative, and positive balance is usually achieved only via feeding, with the result that muscle protein lost between meals is replaced, thereby maintaining a stable muscle mass. After exercise in the fasting state, despite the rise in MPS (see below), net muscle protein balance, although becoming less negative, does not achieve a positive value, because the rate of MPB, which exceeded that of MPS before exercise, also rises (Biolo *et al*, 1995). However, when amino acids or protein is ingested after exercise, the net muscle protein balance becomes positive as the rate of MPS surpasses the rate of MPB, which itself may be suppressed (Tipton *et al*, 2003).

1.7 EFFECTS OF EXERCISE (RESISTANCE AND NON-RESISTANCE) ON MUSCLE PROTEIN SYNTHESIS

(See tables 1.2 and 1.3)

1.7.1 Changes In Muscle Protein Synthesis During Resistance Exercise

Measures of human MPS made during resistance exercise are uncommon, as most studies involve exercise of a duration that is shorter than the minimum time period (~

1 h) current methods require to achieve robust measurements with stable isotope tracers. Also, as the exercise is discontinuous, using sets of contraction repetitions with rest periods between, the muscle is not in a steady state, and this complicates the interpretation of data obtained, especially with techniques relying on A-V sampling and blood flow. Data from studies in both rodent muscle (Bylund-Fellenius *et al*, 1984) and human muscle (Dreyer *et al*, 2006; Fujita *et al*, 2008) confirm that MPS is depressed during resistance-type exercise. In contrast, other work using the A-V tracer dilution method suggests no alteration of the rate of uptake of tracer, i.e., leg protein synthesis is unchanged (Durham *et al*, 2004). The contradiction between findings from earlier studies (Dreyer *et al*, 2006) and latter report (Durham *et al*, 2004) may be the result of methodological differences (i.e., the use of direct incorporation method vs. A-V tracer dilution method) or may possibly be the result of difference in volume of work (see table 1.2). This fall in MPS has been shown to be mediated by a decrease in mRNA translation initiation and elongation steps (Kimball *et al*, 2002) via reduced phosphorylation of 4E binding protein 1 (4EBP-1), and a tendency for a rise in phosphorylation of eukaryotic elongation factor 2 (eEF2), a negative regulator of peptide-chain elongation (Dreyer *et al*, 2006) (Fig. 1.4, see below).

Bylund-Fellenius and colleagues (Bylund-Fellenius *et al*, 1984) attributed the contraction-induced fall in MPS in perfused, electrically stimulated rat muscle to an increase in the AMP-to-ATP ratio as a result of myosin ATPase activity, which might indeed have possible stimulatory effects on AMP-activated protein kinase (AMPK) activity (Hardie, 2008), leading to inhibition of the signaling effect of tuberous sclerosis complex 2 on mTOR and reduced 4EBP-1 phosphorylation (Bolster *et al*, 2002). Indeed, AMPK- α activity rises by >30% as a result of

resistance exercise (Dreyer *et al*, 2006), but the importance of the intraexercise inhibition of mTOR is uncertain, as protein synthesis proceeded to rise in the postexercise period, despite continued elevation of AMPK phosphorylation (Dreyer *et al*, 2006).

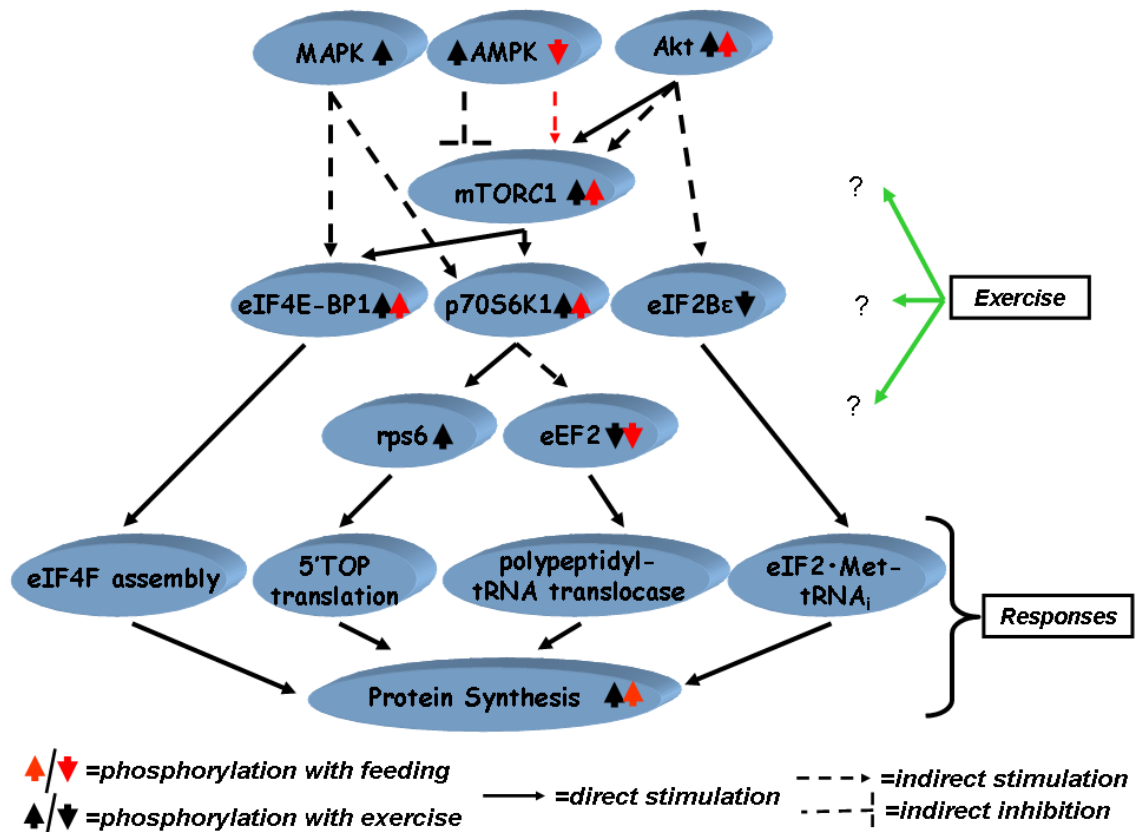


Figure 1.4 Alterations in intracellular signalling molecules following resistance exercise and feeding. MAPK (mitogen-activated protein kinase), AMPK (AMP-activated protein kinase), Akt (protein kinase B), mTORC1 (mammalian target of rapamycin complex 1), eIF4E-BP1 (eukaryotic initiation factor 4E binding protein 1), p70S6k1 (p70S6 kinase), eIF2Bε (eukaryotic initiation factor 2Bε), rps6 (ribosomal protein s6 kinase), eEF2 (eukaryotic elongation factor 2), eIF4F eukaryotic initiation factor complex 4F), 5'TOP (5'-terminal oligopyrimidine tract), eIF2•met-tRNA_i (eukaryotic initiation factor 2•initiator methionyl-transferRNA).

Recent work has also highlighted the efficacy of prior amino acid feeding on MPS during exercise. In overnight fasted subjects, fed with 0.35 g/kg fat free mass of essential amino acids and 0.5 g/kg fat free mass of sucrose 1 h before exercise [10 x 10 at 70% one repetition maximum (1 RM)], exercise still suppressed MPS during exercise, but not below basal rates, as seen with the controlled, fasted group (Fujita *et al*, 2008). Similarly, protein feeding before and during a 2-h intermittent, whole body resistance exercise session improved whole body net protein balance and increased MPS during the exercise (Beelen *et al*, 2008).

Table 1.2 Effect of resistance and non-resistance exercise on human muscle protein synthesis and breakdown in the postabsorptive state

Subjects	Fasted/ Fed	Exercise protocol	FSR period	Muscle fraction	Synthesis (FSR or [Rd])		Net change	Breakdown (FBR or [Ra])		Net change	Comments	Reference
					Basal	PEx		Basal	PEx			
6 M	Fasted	4 x 6-12 reps 80% 1RM x 3 types of curl	4 h	Mixed	0.067	0.100	↑				FSR ↑ at 4 h and 24 h PEx	<i>Chesley, 1992</i>
5 M	Fasted	5 x 10 reps 12RM, 4 x 8 10RM (x LP, LE & LC)	3 h	Mixed	0.044 [32]	0.104 [62]	↑	[48]	[69]	↑	4 h PEx	<i>Biolo, 1995</i>
4 M 4 F	Fasted	8 x 8 reps 80% 1RM either LC or SC	3 h	Mixed	0.05	0.12	↑	0.11	0.15	↑	FBR ↑ up to 24 h & FSR ↑ up to 48 h	<i>Phillips, 1997</i>
6 M, 6 F	Fasted	8 x 10 flexions 120%	3-4 h	Mixed	0.036	0.08	↑	0.075	0.105	↑	FBR immediately PEx	<i>Phillips, 1999</i>
7 F	Fasted	Leg/arm exercises over 1 h	5 h	Mixed	0.045	0.048	↔				Leg ex performed 1st	<i>Tipton, 1996</i>
6 M	Fasted	6 x 8 reps at 80%	10min- 3 h	Mixed	basal	+0.030	↑				Increase at 180 min PEx	<i>Sheffield- Moore, 2005</i>
6 EM	Fasted	6 x 8 reps at 80%	10 min- 3 h	Mixed	basal	+0.044	↑				Transient increase over 10 min	<i>Sheffield- Moore, 2005</i>
5 M, 2 F	Fasted	8 x 10 reps 75% LP, 8 x 8 reps 80% LE	During Ex	Mixed	[22]	[30]	↔	[43]	[52]	↔	During Ex	<i>Durham, 2004</i>
7 M, 4 F	Fasted	10 x 10 reps at 80%	hourly	Mixed	0.06	0.009	↑				1 and 2 h PEx, 0.04 during Ex	<i>Dreyer, 2006</i>
8 M	Fasted	4 x 10 reps 80% LP, 4 x 10 reps 80% LE	4 h	Mixed	0.04	0.094	↑				Difference in mixed FSR, no change in Myofibrillar FSR	<i>Kim, 2005</i>
7 F	Fasted	Swim 1.5h	5 h	Mixed	0.045	0.064	↑					<i>Tipton, 1996</i>
7 F	Fasted	Combined RE and Swim over 2.7h	5 h	Mixed	0.045	0.082	↑					<i>Tipton, 1996</i>
6 M	Fasted	45min at 45% VO ₂ max	10 min- 3 h	Mixed	basal	+0.036	↑	basal	(+80)	↑ at 10 min	FSR increased at 60 min of basal by 180 min	<i>Sheffield- Moore, 2005</i>
6 EM	Fasted	45min at 45% VO ₂ max	10 min- 3 h	Mixed	basal	+0.083	↑	basal	(+75)	↑ at 10 min only	FSR increased at 10 min basal at 60 and 180 min	<i>Sheffield- Moore, 2005</i>

FSR, fractional synthetic rate (%.h⁻¹); FBR, fractional breakdown rate (%.h⁻¹); PEx, post exercise; M, male; reps, repetitions; RM, repetition maximum; LP, leg press; LE, leg extension; LC, lengthening contractions; F, female; SC, shortening contractions; Ex exercise; RE, resistance exercise; EM, elderly male.

Table 1.3 Effect of resistance and non-resistance exercise on human muscle protein synthesis and breakdown in the fed state

Subjects	Fasted/Fed	Exercise protocol	FSR period	Muscle fraction	Synthesis (FSR or [Rd])		Net change	Breakdown (FBR or [Ra])		Net change	Comments	Reference
					Basal	PEx		Basal	PEx			
6 M	I/V AA (10 g AA.h ⁻¹)	5 x 10 reps 12RM, 4 x 8 reps 10RM (x squat, curl & LE)	3 h	Mixed	0.065	0.144	↑	[38]	[50]	↔		<i>Biolo, 1997</i>
3 M, 3 F	Oral 40 g AA	5 x 10 reps 75% 1RM, 4 x 8 reps 75% 1RM (x squat, curl & LE)	4.5h	Mixed	[50]	[85]	↔	[75]	[74]	↔	With 40 g EAA similar response, only net balance significant	<i>Tipton, 1999</i>
3 M, 3 F	Oral 6 g EAA	10 x 8 reps 80% LP, 8 x 8 reps 80% LE	3 h	Mixed	[50]	[170]	↑	[60]	[75]	↔	Similar response at 1 and 3 h	<i>Rasmussen, 2000</i>
3 M, 3 F	Oral 6 g EAA (PRE-EX)	10 x 8 reps 80% LP, 8 x 8 reps 80% LE	2 h	Mixed	[65]	[190]	↑	[80]	[90]	↔	↑ Rd during exercise and 1h PEx	<i>Tipton, 2001</i>
3 M, 3 F	Oral 6g EAA	10 x 8 reps 80% LP, 8 x 8 reps 80% LE	2 h	Mixed	[45]	[50]	↔	[60]	[45]	↔	↔ throughout Ex and PEx period	<i>Tipton, 2001</i>
3 M, 3 F	Oral 6 g EAA (at 1 and 2 h PEx)	10 x 8 reps 80% LP, 8 x 8 reps 80% LE	3 h	Mixed	[25]	[120]	↑	[38]	[36]	↔	Ra only ↑ at 3h, Rd return to basal 3h	<i>Borsheim, 2002</i>
3 M, 3 F	Oral 15 g EAA (x2) 1 h apart	8 x 8 reps at 80%	3 h	Mixed	basal	0.188	↑				Exercise alone 0.076 %.h ⁻¹	<i>Tipton, 2003</i>
8 M	Oral 10 g whey + CHO	4 x 10 reps 80% LE, 4 x 10 reps 80% LP	2 h	Mixed	0.05	0.115	↑				CHO alone 0.08 %.h ⁻¹	<i>Tang, 2007</i>
10 M	Oral 7g protein.h ⁻¹	6 x 10 reps at 80%	3 h	Mixed	0.045	0.09	↑					<i>Tang, 2008</i>
16 M	Leu EAA + CHO	10 x 10 reps at 70%	1 h	Mixed	0.06	0.16	↑				1h PEx, during exercise 0.045%.h ⁻¹	<i>Dreyer, 2008</i>
6 M, 5 F	Leu EAA +CHO (0.35 g + 0.5 g.kg.FM ⁻¹)	10 x 10 reps at 70%	1 h	Mixed	0.06	0.12	↑				Prior feeding FSR elevated only at 2 h PEx, FSR AUC over 4 h similar to control group	<i>Fujita, 2008</i>
10 M	Oral 10 g prot. h ⁻¹	RE over 2 h	2 h	Mixed	0.06	0.085	↑				Feeding throughout exercise	<i>Beelen, 2008</i>
20 M	Oral 10 g prot. h ⁻¹ + 10 g CHO	Resistance & cycle exercise over 2 h	2 h	Mixed	0.056	0.083	↑				Feeding throughout exercise	<i>Beelen, 2008</i>

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10 M	Oral 1.1 g prot.kg ⁻¹	5 x 10 reps at 80%	4 h	Myo	0.06	0.11	↑				Mitochondrial FSR increased from 0.075 to 0.15 %·h ⁻¹	<i>Wilkinson, 2008</i>
10 M	Oral 1.1 g prot.kg ⁻¹	45 min 75% VO ₂ max	4 h	Myo	0.055	0.055	↔				Mitochondrial FSR increased from 0.075 to 0.18 %·h ⁻¹	<i>Wilkinson, 2008</i>
7 M	Oral 6 g prot.h ⁻¹	20 x 10 reps at 75%	3 h	Myo	0.057	0.164	↑	[29]	[26]	↔	Sarcoplasmic FSR ↑ 3× to 0.22 %·h ⁻¹	<i>Louis, 2003</i>
8 M	Oral 7-8 g prot.h ⁻¹	6 x 10 reps LC, 6 x 10 reps SC	3+4 h	Myo	0.07	0.13	↑				Similar FSR at 4 and 8 h PEx	<i>Moore, 2005</i>
8 M	45 g EAA + CHO	Stepping exercise (+25% body wt) till fatigued	3 h	Myo	0.042	0.135	↑				0.05 at 3 h PEx, ↑ at 6 and 24 h	<i>Cuthbertson, 2006</i>
8 M	1.4 x BMR, 15% Protein	1 leg kicking 67% VO ₂ max for 1h	3.5 h	Myo	0.04	0.1	↑				0.12 at 24 h, 0.08 at 48 h, ↔ by 72 h	<i>Miller, 2005</i>
6M	0, 5, 10, 20 or 40 g of whole egg protein	4 x 8 – 10 reps (x LP, curl & LE)	4 h	Mixed/ Albumin	0.055/ 0.21	0.105/ 0.41	↑				Linear dose response up to 20 g protein	<i>Moore, 2008</i>

FSR, fractional synthetic rate (%·h⁻¹); FBR, fractional breakdown rate (%·h⁻¹); PEx, post exercise; M, male; AA, amino acids; reps, repetitions; RM, repetition maximum; LE, leg extension; F, female; EAA, essential amino acids; LP, leg press; PRE-EX, pre exercise; Ex exercise; CHO, carbohydrate; Leu EAA, leucine enriched essential amino acids; AUC, area under the curve; Myo, myofibrillar; LC, lengthening contractions; SC, shortening contraction.

1.7.2 Changes in Muscle Protein Synthesis During Non-Resistance Exercise

Previously it has been shown that during running exercise in rodents, MPS is depressed (Dohm *et al*, 1982). This has been confirmed by more recent studies in which a fall of 26% in MPS was observed during a 2-h treadmill run by rats (Gautsch *et al*, 1998). This type of exercise also increased activity of AMPK and suppressed both mTOR signaling and the overall rate of mRNA translation in mice during running on a treadmill for 30 min, which might underlie the changes in MPS (Williamson *et al*, 2006). In human subjects, a fall in whole body protein synthesis was described during walking uphill at 40% of maximum O₂ uptake ($\dot{V}_{O_{2max}}$) (Rennie *et al*, 1980), and similar changes were observed during 2 h of walking at 60% of $\dot{V}_{O_{2max}}$ (Bowtell *et al*, 1998). As MPS comprises a significant portion of whole body protein synthesis, and it is known that the ATP-to-ADP ratio falls markedly during non-resistance exercise (Bylund-Fellenius *et al*, 1984), it is reasonable to assume that, during walking or running exercise, human MPS falls, but there is little documented evidence for this. In fact, during treadmill walking at 40% of $\dot{V}_{O_{2max}}$ (Carraro *et al*, 1990), no significant change in MPS was detected from the resting period; however, the basal values may have been uncharacteristically low compared with those on the nonexercise day, so this may be a false negative result. It may also be that an insufficiently intense rate of exercise was chosen in the study. Cycle ergometer exercise for 1 h at 70% of $\dot{V}_{O_{2max}}$, in young healthy human subjects, increased activation of muscle AMPK alpha 2 (Fujii *et al*, 2000) measured in quadriceps biopsies taken immediately after exercise; in comparable studies of exercise for 90 min, there was marked Ca²⁺-induced activation of the calmodulin-dependent protein kinase eEF2 kinase, with accompanying (and probably resultant) inhibition of eEF2 activity and (by inference) protein chain elongation in healthy

postabsorptive men (Rose *et al*, 2005). These results are consonant with the hypothesis that there is a fall in MPS during cycling and running; however, it is difficult technically to design a study in which the subjects exercise for sufficient time at a high load to satisfactorily observe the effects on protein turnover during exercise, but it should not be impossible. This is a gap waiting to be filled.

1.7.3 Changes in Muscle Protein Synthesis Post Resistance Exercise

It is generally agreed that resistance exercise results in increased MPS in the postexercise recovery period (Chesley *et al*, 1992; MacDougall *et al*, 1995; Yarasheski *et al*, 1993). Indeed, an acute bout of resistance exercise can increase the rate of MPS about two- to fivefold after exercise and this effect can persist for up to 48 h in subjects (Phillips *et al*, 1997). Reports differ (Dreyer *et al*, 2006; Sheffield-Moore *et al*, 2005) as to whether there is inhibition of MPS immediately after strenuous contractile activity. In our laboratory, we routinely observe no change above basal in measured incorporation of tracer into protein for ~1–1.5 h, but others do not (Dreyer *et al*, 2006; Sheffield-Moore *et al*, 2005); nevertheless, most workers who have examined it suggest that any postexercise rise is usually small initially and is maximized later (Drummond *et al*, 2008). The stimulation of protein synthesis after resistance exercise occurs in both myofibrillar and mitochondrial pools in untrained subjects (Wilkinson *et al*, 2008). The effect of intensity and volume of work and adaptive responses of muscle protein turnover to resistance training at different intensities or volume remains to be investigated.

The underlying molecular mechanisms associated with stimulatory effect of resistance exercise have been extensively studied in recent years, initially using

rodents (Atherton *et al*, 2005; Baar & Esser, 1999; Bolster *et al*, 2003) and then transferring the techniques for analysis to human muscle (Aronson *et al*, 1997; Deldicque *et al*, 2008; Dreyer *et al*, 2006; Dreyer *et al*, 2008; Karlsson *et al*, 2004) (see table 1.1). Sufficient reports have now emerged to provide what is likely to be a reliable description of the extent and time course of signaling during and immediately after resistance exercise in the fed and fasted states (figure 1.4). A detailed description of alterations of phosphorylation or activity of the cell signaling molecules regulating MPS in response to resistance exercise has already been explained. However, briefly, the activation of signaling molecules regulating translation initiation and protein synthesis, such as Akt (protein kinase B), mitogen-activated protein kinase, mTOR, and its downstream effectors, such as eukaryotic initiation factor 4EBP-1, p70S6k1 (70-kDa S6 protein kinase), and ribosomal protein s6 kinase, have been shown to be associated with increased MPS in the postexercise period (Aronson *et al*, 1997; Dreyer *et al*, 2006; Dreyer *et al*, 2008; Karlsson *et al*, 2004). Nevertheless, the temporal relationship and longevity of these responses and the dose-response characteristics remain to be elucidated, as does any potential role in the adaptive response of muscle to both acute and chronic exercise.

With regard to cell signaling, we believe that presently we lack a good understanding of the precise relationship between the extent of the changes in signaling and consequent changes in MPS and MPB. In particular, it is not clear if the molecules in the signaling pathways act as simple on-off switches, or if they act as amplifiers to modulate the resulting metabolic action. Results from studies of insulin action on human muscle suggest that it is too simplistic to assume a particular physiological effect on protein synthesis or breakdown from alterations in the degree of phosphorylation of any given molecule (Greenhaff *et al.*, 2008). This information

may be most elegantly obtained by time course and dose-response data of the kind our laboratory has been endeavoring to collect (Bohe *et al*, 2001; Bohe *et al*, 2003).

1.7.4 Changes in Muscle Protein Synthesis Post Non-Resistance Exercise

After treadmill walking at 40% of $\dot{V}O_{2\max}$ in the postabsorptive state, there was an increase in mixed muscle MPS of ~45% (Carraro *et al*, 1990); a similar change was reported by Sheffield-Moore *et al*. (Sheffield-Moore *et al*, 2004). Even larger increases in the myofibrillar fractional synthetic rate can be produced by more intense exercise; in fed young men, 1 h of one-legged kicking exercise at ~70% of 1 RM doubled the quadriceps myofibrillar protein synthetic rate by 24 h postexercise, an effect lasting for up to 72 h (Miller *et al*, 2005). These results might have been considered surprising before they started to accumulate beyond any doubt, because it was generally assumed that exercise of this type (which would be likely to increase mitochondrial biogenesis) would not result in hypertrophy and thus would be unlikely to stimulate myofibrillar protein synthesis. In fact, it has been recently shown in untrained subjects, bouts of either resistance or bicycling exercise stimulate both myofibrillar and mitochondrial protein synthesis, possibly the results of a general postexercise anabolic signal, whereas, in the trained state, no increase of myofibrillar synthesis is occasioned by bicycling exercise, and no increase of mitochondrial protein synthesis by acute resistance exercise (Wilkinson *et al*, 2008).

The degree of change in MPS in response to exercise may depend on whether or not the exercise produces significant impact force, or create a strong enough effect on muscle fibers, which exerts during a short time interval but is amply large to cause an considerable activation of muscle protein synthetic machinery, as it has been observed that there were no significant changes in MPS in healthy subjects after

high-intensity swimming (Tipton *et al*, 1996). However, it is important to note that these measurements were made under fasting conditions after a prolonged training session. The subjects were also highly trained, and chronic training has been shown to increase the basal MPS rate and diminish MPS responses to acute bouts of exercise (Phillips *et al*, 1999; Picosky *et al*, 2006; Short *et al*, 2004).

We now have many descriptions of the alterations of phosphorylation of signaling, which might underlie possible changes in MPS after an acute bout of non-resistance exercise as for changes in MPS itself (e.g., increases in mTOR signaling, decreases in eEF2, MAPK etc.) (Aronson *et al*, 1997; Benziane *et al*, 2008; Mascher *et al*, 2007; Sakamoto *et al*, 2004); However, quantitatively, the changes observed are similar to those reported for resistance-type exercise, and indeed there is little difference in the extent of the responses after acute exercise in muscles of legs working in different modes, i.e., "resistance" and "endurance" in the same individual in the untrained state (Wilkinson *et al*, 2008). This suggests that any major increase in contractile activity or possibly fuel utilization in untrained muscle will result in the same global anabolic response. However, after training, the acute anabolic response of MPS becomes more directed to the specific mode of exercise, resulting in synthesis of specific subcellular muscle protein fraction (mitochondrial or myofibrillar), subsequently leading to the phenotypic changes seen with the different training modes (Wilkinson *et al*, 2008). In addition, phenotypic changes probably only result from repeated bouts of either resistance or dynamic types of exercise. We remain puzzled about the significance to the training effect of alterations in signaling protein phosphorylation as only limited data exist to date, certainly not enough to be able to predict alterations in protein turnover from the phosphorylation changes.

1.8 EFFECTS OF EXERCISE (RESISTANCE AND NON-RESISTANCE) ON MUSCLE PROTEIN BREAKDOWN

(See table 1.2 and 1.3)

1.8.1 Changes in Muscle Protein Breakdown During Resistance And Non-Resistance Exercise

The only feasible techniques for measuring protein breakdown during exercise are those based on A-V dilution of tracer amino acids, although, as discussed previously, the reliability of this approach during non-steady-state conditions is questionable. So far as we have been able to discover, there are only two studies in which the rate of dilution of a tracer has been measured "during" exercise (actually during rest periods between sets) in the postabsorptive state; in these studies, phenylalanine rate of appearance, indicative of protein breakdown, was not elevated above rest (Durham *et al*, 2004; Tipton *et al*, 2001). However, it may be that, if the major process of muscle proteolysis is via the ATP-dependent ubiquitin proteasome system (Attaix *et al*, 2005), and, as discussed previously, AMP-to-ATP ratio increases during resistance exercise, then it too might be depressed during exercise as for protein synthesis (see above).

There is also uncertainty regarding the changes in MPB during non-resistance exercise period. In many studies of cycling exercise, the increase in net amino acid efflux from the leg is reported to be large (MacLean *et al*, 1994; MacLean *et al*, 1996), and it has been assumed that this was due to an increase in proteolysis. However, the efflux of amino acids during exercise could easily arise from a greater

inhibition of protein synthesis relative to breakdown, the result of which would still be an expansion of the free amino acid pool and a greater net efflux of amino acids.

1.8.2 Changes in Muscle Protein Breakdown Post Resistance and Non-Resistance Exercise

1.8.2.1 Post Resistance Exercise

Whatever the uncertainty regarding the exercise period itself there is no doubt that in the postabsorptive state after exercise human muscle proteolysis is elevated, as shown both by tracer leg dilution (Biolo *et al*, 1995; Biolo *et al*, 1999) and the fractional breakdown rate (FBR) method (Phillips *et al*, 1997). Before, exercise muscle is in net negative amino acid balance, and this situation is only marginally improved by strenuous resistance exercise alone, because, although MPS increases about twofold, the FBR, which is significantly higher than FSR (a measure of MPS) in the postabsorptive state, also increases by 30–50% by 3 h afterwards, thereby maintaining the negative balance (Biolo *et al*, 1995; Phillips *et al*, 1997; Phillips *et al*, 1999). However, the elevation in muscle breakdown appears to be more short-lived than that of FSR (24 rather than 48 h) (Phillips *et al*, 1997).

1.8.2.2 Post non-resistance exercise

There is no doubt that, in the postabsorptive state after non-resistance exercise, human muscle proteolysis is elevated, as shown in both untrained fasted young and older men after 45 min of treadmill walking at 40% of $\dot{V}_{O_{2max}}$. Leg proteolysis was increased 10 min postexercise, but the increase disappeared by 60 min in the young but not the older men (Sheffield-Moore *et al*, 2004).

In contrast, results obtained using the microdialysis technique suggested an unchanged concentration of 3 MeH in dialysis fluid from 6 to 72 after 1 h of one-legged kicking exercise at ~70% of 1 RM (Haus *et al*, 2007). There is a possibility that the major part of postexercise proteolysis is of non-myofibrillar protein, which would not show up as an increase in 3 MeH, but it seems more likely that this result probably speaks more for the unreliability for the method used than a lack of any muscle proteolysis (Biolo *et al*, 1995; Phillips *et al*, 1997; Rennie *et al*, 2008).

1.8.3 Signalling and muscle protein breakdown

The signalling pathways controlling MPB and the proteolytic pathways involved in human muscle remain poorly defined, especially during exercise. The different proteolytic pathways (including lysosomal, the calcium-activated and the ubiquitin-proteasome-dependent systems, caspases and metalloproteinases, as well as nonspecific di- and tripeptidases) must be involved in the remodeling of skeletal muscle in response to exercise, but the part played by each is not clear.

In rat muscle, an elevated activity of calcium-activated proteases and metalloproteinases has been reported during/after treadmill running (Belcastro, 1993; Carmeli *et al*, 2005). However, there are few reports of measurement of acute changes in capacity or control of human muscle proteolytic pathways. Two muscle-specific ubiquitin ligases, muscle atrophy F-box (MAFbx) and muscle-specific really interesting novel gene finger protein 1 (MuRF1), have been shown to play regulatory roles in muscle proteolysis (Attaix *et al*, 2005) in rodent muscle. In human muscle, studies of proteolytic gene expression, specifically ubiquitin proteasome-related gene expression, in response to resistance exercise showed upregulation of MAFbx and MuRF1 messenger RNA (mRNA), but with no significant changes in forkhead box

3A mRNA, a transcription factor involved in protein degradation and apoptosis, in young subjects 4 h after resistance exercise (Raue *et al*, 2007). Paradoxically, studies carried out by our group showed a downregulation of MAFbx mRNA up to 24 h after resistance exercise, which was unexpected considering resistance exercise increases MPB (Kostek *et al*, 2007) in the postexercise state. This may be related to the volume of exercise carried out in the latter study, since subjects performed exercise involving stepping up and down, carrying 25% of their body weight, to complete exhaustion, and also the timing of the measurement. Alternatively, there may not be a direct relationship between MAFbx expression and MPB, as our laboratory has previously observed (Greenhaff *et al*, 2008). In all likelihood, multiple pathways are activated and one pathway is likely contributing a considerable amount to overall proteolysis; however, it is intriguing to imagine how intact myofibrillar proteins might be "dismantled" or remodeled so as to make room for newly synthesized proteins, which, according to several reports (Sheffield-Moore *et al*, 2004; Tipton *et al*, 1996), are made within hours of an exercise stimulus.

1.9 THE EFFECT OF NUTRITION ON MUSCLE PROTEIN SYNTHESIS

1.9.1 The role of amino acids

Feeding a mixed meal doubles mixed MPS (Rennie *et al*, 1982); the effect seems, according to the evidence in our hands, to be mostly due to the actions of amino acids alone (Bennet *et al*, 1989; Smith *et al*, 1992) and particularly leucine (Smith *et al*, 1992) without much influence of insulin (Bohe *et al*, 2003; Cuthbertson *et al*, 2005). Amino acids increase the synthesis of myofibrillar, sarcoplasmic, as well as

mitochondrial proteins in skeletal muscle (Bohe *et al*, 2001), probably in a dose-dependent manner (Bohe *et al*, 2003; Cuthbertson *et al*, 2005).

1.9.2 Dose-response relationship

The dose response of MPS to exercise and increasing amounts of protein (Moore *et al*, 2008) appear to be similar in shape to that obtained at rest (Bohe *et al*, 2001), albeit shifted upward and to the left somewhat, as the result of exercise. Although the work demonstrates the synergy between exercise and feeding, it also suggests that there is no benefit of ingesting large amounts of protein (>20 g, which is actually a relatively small amount) in an attempt to increase protein accretion in muscle; the maximum effective dose is probably 15–20 g of high-quality protein, such as beef, egg, or soy.

1.9.3 Combined effects of exercise and feeding on muscle protein synthesis (see table 1.3)

Feeding and resistance exercise act synergistically to increase MPS and lead to positive net muscle protein balance after exercise, greater than that achieved by food alone (Biolo *et al*, 1997). Several groups have reported that protein or amino acid ingestion, with or without ingested or infused carbohydrate, after an acute bout of resistance (Biolo *et al*, 1997; Borsheim *et al*, 2002; Cuthbertson *et al*, 2006; Koopman *et al*, 2005; Miller *et al*, 2003; Moore *et al*, 2005; Rasmussen *et al*, 2000; Tang *et al*, 2008; Tipton *et al*, 1999) or non-resistance exercises (Miller *et al*, 2005) further enhanced MPS. For example, a 145% rise in MPS above baseline occurred when a leucine-enriched essential amino acid solution with carbohydrate was taken after a single bout of resistance exercise, whereas, without the provision of nutrition,

only a 41% rise in MPS occurred (Dreyer *et al*, 2008). This increase in the rate of MPS remains/persists for a longer period (72 h) (Miller *et al*, 2005) than with feeding (Bohe *et al*, 2001) or probably exercises alone. This enhanced effect of feeding postexercise seems to be due to the presence of increased amounts of amino acids and not glucose in the blood (Borsheim *et al*, 2004; Miller *et al*, 2003).

1.9.4 The effects of timing of nutrition intake on muscle protein synthesis

There is disagreement as to whether amino acid feeding before or after resistance exercise promotes MPS to a greater extent. It has been reported (Tipton *et al*, 2001) that ingestion of essential amino acids taken with a carbohydrate supplement immediately before resistance exercise resulted in greater leg uptake of amino acids, but the results are quantitatively difficult to believe, given that they were made under non-steady-state conditions, and the increases in uptake were physiologically unlikely to represent increases in MPS, given their size (20-fold!), but, more likely, some artifact, such as pooling of amino acids within muscle or an abnormally elevated blood flow (4 times great in pre-exercise feeding). Furthermore, it has been recently shown by direct measurement of FSR in humans that feeding 1 h before an acute bout of high-intensity resistance exercise did not further enhance MPS during the 2-h postexercise period (Fujita *et al*, 2008). Thus, once again, the leg tracer dilution method appears to yield qualitatively and quantitatively different results to those obtained by incorporation of tracer amino acids.

While there is still a disagreement with regard to the appropriate timing of protein feeding required to maximize the muscle protein synthetic response to an acute bout of exercise, there are some reports with respect to chronic exercise training showing

that the stimulation in MPS, indicated by indirect measures, such as muscle fiber hypertrophy, lean mass accretion, and muscle strength gain in young and old men, is enhanced when protein is consumed immediately after the exercise rather than some hours later (Esmarck *et al*, 2001; Hartman *et al*, 2007; Levenhagen *et al*, 2001).

1.9.5 Role of quality of protein

There has been considerable interest in the proposition that proteins of different biological quality and digestibility might be more or less efficient at supplying amino acids to muscle after exercise. Recent work by Phillips and colleagues (Hartman *et al*, 2007; Wilkinson *et al*, 2007) seem to show that whey proteins are superior to casein and soy and that whole milk supplies all that is required for net muscle protein accretion. Although not yet proven, it seems likely to us that any high-quality protein source, such as beef, egg, or soy, will be as good as milk for muscle protein accretion (Millward & Jackson, 2004).

1.10 THE EFFECT OF NUTRITION ON MUSCLE PROTEIN BREAKDOWN

Amino acids per se have, at most, a small (Greenhaff *et al*, 2008), inhibitory effect on human limb protein breakdown, especially in the presence of insulin, but the effects are less than seen in animals. Suppression of protein breakdown in human forearm occurs after infusions of mixed or branched chain amino acids (Louard *et al*, 1990; Louard *et al*, 1995). Much of the physiological effect of amino acids on MPB at rest is likely to be mediated through increased insulin secretion. However, several workers have reported that increased availability of amino acids after exercise does not significantly inhibit human MPB, but does prevent the increase seen in the post-

absorptive state (Biolo *et al*, 1997; Borsheim *et al*, 2002; Louis *et al*, 2003; Rasmussen *et al*, 2000; Tipton *et al*, 1999).

1.11 MUSCLE CELL SIGNALING RESPONSES TO FEEDING

The underlying molecular mechanisms associated with this enhanced stimulatory effect of feeding after exercise appear to be associated with the enhanced phosphorylation of mTOR, p70S6K1, and 4EBP-1, greater than that achieved by exercise alone (Atherton *et al*, 2010; Dreyer *et al*, 2008; Karlsson *et al*, 2004; Koopman *et al*, 2005) (see figure 1.4 and table 1.1).

Table 1.4 Effect of resistance and non-resistance exercise training on human muscle protein synthesis and breakdown in the postabsorptive or fed states.

Subjects	Fasted/ Fed	Exercise protocol	FSR period	Muscle fraction	Synthesis (FSR or [Rd])		Net change	Breakdown (FBR or [Ra])		Net change	Comments	Ref
					Basal	Post Training		Basal	PEx			
2 M, 4 F	Fasted	2 weeks RE training	4 h	Mixed	0.049	0.075	↑				Studied 3 h after last bout of exercise	<i>Yarasheski, 1993</i>
4 EM, 2 EF	Fasted	2 weeks RE training	4 h	Mixed	0.03	0.076	↑				Studied 3 h after last bout of exercise	<i>Yarasheski, 1993</i>
4 M, 3 F	Fasted	2 weeks RE training	12-13 h	Mixed/ MHC ⁱ	0.048/ 0.038	0.10/ 0.072	↑				16 h PEx, may be temporal effect not training effect	<i>Hasten, 2000</i>
3 EM, 4 EF	Fasted	2 weeks RE training	12-13 h	Mixed/ MHC	0.037/ 0.024	0.102/ 0.050	↑					<i>Hasten, 2000</i>
4 EM	Fasted	12 weeks RE training	12 h	Mixed	105	170	↑					<i>Yarasheski, 1999</i>
8 EF	Fasted	12 weeks RE training	12 h	Mixed	95	150	↑				~17 h PEx	<i>Yarasheski, 1999</i>
19 M, 20 EM	Fasted	10 weeks RE training	5 h	Mixed/ MHC	0.041/ 0.028	0.066/ 0.042	↑				Similar effect in MHC, Study 4 days PEx	<i>Balagopal, 2001</i>
16 M	1/12 daily intake/30 min	12 weeks RE training	6 h	Mixed	0.048	0.066	↑				Studied 18-20 h after last bout of ex	<i>Yarasheski, 1992</i>
6 M, 6 F	Fasted	8 x 10 flexion 120%	3-4 h	Mixed	0.045	0.067	↑				Acute Ex response only in untrained	<i>Phillips, 1999</i>
6 M, 6 F	Fasted	8 x 10 flexion 120%, regular RE training	3-4 h	Mixed	0.073	0.082	↔				Basal FSR higher in trained	<i>Phillips, 1999</i>
8 M	Fasted	4 x 10 reps 80% LP, 4 x 10 reps 80% LE, 8 weeks training	4 h	Mixed/ Myo	0.061	0.075	↑				Difference in mixed muscle fraction not in myofibrillar fraction	<i>Kim, 2005</i>
10 M	7g prot.h ⁻¹	6 x 10 reps ⁿ 80% LE, 8 weeks training	3 h	Mixed	0.048	0.123	↑				Basal not high despite feeding and training	<i>Tang, 2008</i>
10 M	1.1g prot.kg ⁻¹	5 x 10 reps 80% LE, 10 weeks training	4 h	Myo	0.08	0.12	↑					<i>Wilkinson, 2008</i>
10 M	1.1g prot.kg ⁻¹	45min 75% VO ₂ max, 10 weeks cycling	4 h	Myo	0.05	0.075	↔				Mitochondrial FSR increased only in cycle	<i>Wilkinson, 2008</i>

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											group in response to acute exercise	
4 M, 4 F	Fasted	Running/walking at 65-85% maximal heart rate, 4 weeks training	5 h	Mixed	0.077	0.089	↑	0.105	0.143	↑	Studied ~40 h after last bout of exercise, NET balance decreased	<i>Pikosky, 2006</i>
38 M, 40 W	Fasted	Bicycle training at 80% maximal heart rate, 4 months training	10 h	Mixed	0.04	0.05	↑				Studied 5 days after last bout of exercise	<i>Short, 2004</i>

FSR, fractional synthetic rate (%.h⁻¹); FBR, fractional breakdown rate (%.h⁻¹); PEx, post exercise; M, male; ; F, female; RE, resistance exercise; EM, elderly male; EF, elderly female; MHC, myosin heavy chain; Ex, exercise; LP, leg press; LE, leg extension; Myo, myofibrillar; reps, repetitions.

1.12 THE EFFECT OF EXERCISE TRAINING ON MUSCLE PROTEIN METABOLISM (See table 1.4)

Chronic resistance exercise increases mean muscle fiber cross-sectional area and induces muscle hypertrophy. Although we are largely ignorant of the time course of the changes, and the exact mechanism(s) involved, they must involve alterations in both MPS and, for remodelling and to achieve destruction of obsolete or damaged proteins, MPB. Several workers have reported that resistance training increases the basal rate of MPS (Balagopal *et al*, 2001; Phillips *et al*, 1999). It has also been reported that even short-term (2-wk) resistance exercise training increases resting MPS, but the data are difficult to interpret, since MPS was measured shortly (between 3 and 18 h) after the last bout of exercise, and MPS may have been increased due to the acute effect of the exercise and not the training per se (Hasten *et al*, 2000; Yarasheski *et al*, 1993; Yarasheski *et al*, 1999). In support of an increase in the rates of resting MPS after training, phosphorylation of Akt-mTOR-p70S6k is reportedly elevated compared with pretraining (Wilkinson *et al*, 2008). However, a study from the same laboratory failed to confirm this increase in basal MPS in response to chronic training (Tang *et al*, 2008). Nevertheless, on investigating the effects of acute resistance or non-resistance (cycling) exercise in legs of the same individual before and after 10-wk training on the synthesis of myofibrillar and mitochondrial proteins: in the resistance-trained leg, there was an increase in the basal synthesis rate of myofibrillar protein, whereas the non-resistance exercise increased only basal mitochondrial protein synthesis (Wilkinson *et al*, 2008). These results point to the likelihood that repeated bouts of one particular mode of exercise induces increases in the synthesis of different subcellular fractions, not as a result of short-term modulation of translational activity, but the activation of specific

programs of gene transcription and subsequent protein translation (Kostek *et al*, 2007; Raue *et al*, 2007). It has also been reported that chronic resistance training inhibited the muscle protein synthetic response to an acute bout of resistance exercise (Phillips *et al*, 1999). However, the same laboratory recently reported a ~48% increase in MPS in response to an acute bout of resistance exercise following 12 wk of chronic resistance exercise training (Kim *et al*, 2005). This difference was suggested to be due to the relatively lower stimulus in the trained state, since resistance exercise was performed at the same absolute intensity before and after training in the previous study (Kim *et al*, 2005). However, our group has also observed a reduced acute myofibrillar synthetic response (~30%) to an acute bout of resistance exercise in the resistance-trained leg at the same relative intensity (Wilkinson *et al*, 2008).

It has also become evident that not only does the magnitude of response change, but also the temporal response of MPS to acute resistance exercise is mutable; chronic resistance exercise has been shown to cause a more rapid but more short-lived rise in MPS than an acute bout in the untrained individuals (Tang *et al*, 2008). Therefore, it appears that training status is an important variable when assessing the response of muscle to acute resistance exercise.

Regarding the effects of non-resistance exercise training, an elevated resting MPS of vastus lateralis by 22% has been reported after 16 wk of bicycle training (45 min at 80% peak heart rate, 3–4 days/wk) (Short *et al*, 2004). It is likely that the modest increase in mixed muscle FSR was the result of much higher increase in the mitochondrial and/or sarcoplasmic protein fractions. Even 4 wk following a running/walking program, exercising at 65–85% of maximum heart rate modestly elevated basal mixed muscle FSR (~17%); however, basal FBR was, somewhat

paradoxically, also reportedly increased (~40%), resulting in a more negative protein balance (Pikosky *et al*, 2006).

If there are increases in MPS after non-resistance exercise training, then why do muscles not hypertrophy? The increase in MPS after dynamic exercise training may be partially related to an increase in synthesis of proteins that are responsible for bringing about adaptations associated with this type of exercise, i.e., increased mitochondrial volume, mitochondrial enzyme activity, and mitochondrial protein synthesis (Gollnick & Saltin, 1982; Hoppeler, 1986). In support of this, Short and colleagues reported increased synthesis of glucose transport proteins, mitochondrial proteins, mitochondrial enzymes levels, and a 22% increase in resting mixed MPS following a 16-wk "aerobic" exercise training program (Short *et al*, 2003; Short *et al*, 2004). Recently, Wilkinson *et al*. (Wilkinson *et al*, 2008) reported that chronic dynamic exercise over 10 wk enhanced only mitochondrial protein synthesis and had no effect on myofibrillar protein synthesis or on the basal phosphorylation of Akt-mTOR-p70S6k in young, healthy men. On a transcriptional level, dynamic exercise (30 min of treadmill running at 75% of $\dot{V}O_{2max}$) increased the mRNA abundance and transcription of a variety of myogenic and metabolic genes (for myogenic differentiation, hexokinase II, and pyruvate dehydrogenase kinase 4) after exercise, peaking 4–8 h postexercise and returning to basal within 24 h (Yang *et al*, 2005). The cumulative effects of this transient elevation following repeated dynamic training seem likely to induce the above-mentioned muscle adaptation associated with non-resistance exercise (Coffey & Hawley, 2007).

Adaptive changes to dynamic training have recently been shown, with downregulation of AMPK, extracellular signal-regulated kinase-1/2, and mTOR signaling activity following 10 daily intense cycling bouts for 45–60 min at 75–90%

in healthy men (Benziane *et al*, 2008). Increased expression of the muscle-specific transcriptional coactivator, peroxisome proliferator-activated receptor- γ coactivator-1 α suggests it may also be associated with the adaptive responses of muscle to regular dynamic exercise, leading to mitochondrial biogenesis and increased oxidative capacity (Pilegaard *et al*, 2003; Pilegaard & Richter, 2008). However, the physiological role of muscle peroxisome proliferator-activated receptor- γ coactivator-1 α in adaptive responses to exercise training still needs to be explored fully.

1.13 EFFECT OF SEX DIFFERENCES ON MUSCLE PROTEIN TURNOVER

Unfortunately, little is known about the mechanisms that lead to sexual dimorphism in body composition, with men having greater muscle mass than women. Testosterone is well known to have an anabolic effect on muscle (Forbes, 1985; Isidori *et al*, 2005), and hypertestosteronaemia during puberty is highly likely to be responsible for the increase in muscle mass during early adulthood. Testosterone also increases the basal rate of MPS in both young and old men (Brodsky *et al*, 1996; Urban *et al*, 1995), but this effect is unlikely to be due to acute changes in protein synthesis, but instead to gene-dependent changes and possibly increased satellite cell activity driven by nuclear androgen receptors. Female sex hormones may inhibit MPS and muscle growth in rats (Toth *et al*, 2001), but there are no detectable differences between young men and women in basal mixed muscle FSR or the response to intravenous amino acid feeding at moderate insulin availability (Smith *et al*, 2009). Similarly, there have been no reported differences in the basal or postexercise rates of MPS or MPB between young adult men and women (Fujita *et al*, 2007; Jahn *et al*, 1999; Miller *et al*, 2006; Parise *et al*, 2001).

However, it has been recently reported that postmenopausal women have ~20–30% higher basal rates of MPS than men (Henderson *et al*, 2009; Smith *et al*, 2008) and a smaller response to feeding (Smith *et al*, 2008), so sex differences in muscle protein metabolism do appear to occur with age and probably as a result of changes in hormonal status. These differences appear to occur irrespective of body composition, i.e., subjects in Smith *et al* study were obese (body mass index 36–38) (Smith *et al*, 2008) compared with the subjects studied by Nair's group (Henderson *et al*, 2009), who reported the similar sex differences in basal MPS. It is known that older women have a lower hypertrophic response than men (~33% less) following a resistance exercise training program (3 days/wk, 26 wk) (Bamman *et al*, 2003), possibly as a result of their inability to maintain adaptive responses to chronic resistance training, since elderly men increased the basal rate of MPS by ~50% after 3-mo training, whereas, in the elderly women, the increase was only ~15% (Smith G *et al*, 2008).

1.14 EFFECTS OF AGEING ON MUSCLE PROTEIN TURNOVER

Sarcopenia is the term for loss of lean body mass seen with ageing (Rosenberg & Moore, 1997; Roubenoff, 1999; Roubenoff, 2000). Using the DXA as a body composition measurement technique in a cross-sectional study in a population aged 19-87 y, it has been shown that lean mass was lost at a rate of 3.5% per decade (Short *et al*, 2004). In addition, it was shown, using CT scans, that this rate of muscle loss was higher (1-2% per year) in those >65 y (Frontera *et al*, 2000).

Sarcopenia is a multifactorial problem, including reduced appetite and food intake in elderly, reduced physical activity, hormonal changes specifically lower testosterone levels (Boirie *et al*, 2001; Herbst & Bhasin, 2004), loss of type 2 fibers secondary

degeneration of the lower motor neuron (Lexell, 1995), increased production of interleukin-6 (IL-6) (Mysliwska *et al*, 1998) as well as tumour necrosis factor- α (TNF- α) (Mooradian *et al*, 1991) and imbalance between MPS and MPB.

There is some controversy regarding the rates of basal MPS in the elderly, with some earlier studies reporting reduced basal muscle protein synthetic rate in the elderly compared with the young subjects (Rooyackers *et al*, 1996; Welle *et al*, 1993; Yarasheski, 2003). However, if the magnitude of this fall is correct, then the rate of muscle wasting in the elderly would be expected to be much greater than commonly seen, and most workers now agree that, in healthy men, ageing has no effect on the basal rate of MPS, and net protein balance is not reduced in healthy elderly people (Cuthbertson *et al*, 2005; Volpi *et al*, 1998; Volpi *et al*, 2001).

A moderate increase in physical activity has been shown to prevent the age-associated loss of muscle strength and also the age-associated increase in the muscle fat infiltration in the elderly people (Goodpaster *et al*, 2008). In addition, it has been shown that MPS can be stimulated by resistance exercise in older people, (Balagopal *et al*, 2001; Hasten *et al*, 2000; Yarasheski *et al*, 1993; Yarasheski *et al*, 1999). However, there is one study, which reported that 3 months of resistance training had no stimulatory effect on resting myofibrillar protein synthesis (Welle *et al*, 1995). Drummond *et al*. (Drummond *et al*, 2008) recently reported a delayed muscle anabolic response to an acute bout of resistance exercise and food intake in the elderly over a 5-h recovery period in young vs. elderly subjects, which was thought to be attributed to a more pronounced activation of AMPK and reduced activation of ERK1/2 in the elderly during exercise. Studies in rodents have revealed that the activation of mTOR signaling after resistance exercise is reduced and the activity of

AMPK is elevated in old rats compared with young rats (Funai *et al*, 2006; Parkington *et al*, 2004; Tipton *et al*, 2003).

It has been shown in a number of studies that feeding stimulated MPS in the elderly (Welle *et al*, 1994; Volpi *et al*, 1998; Volpi *et al*, 1999). However, older men showed anabolic resistance of MPS to feeding, revealing a reduced sensitivity and capacity of response to the anabolic effects of amino acids alone (Cuthbertson *et al*, 2005; Volpi *et al*, 2000), or with amino acids plus glucose mixture (Volpi *et al*, 2000). The poorer anabolic response of MPS to feeding in the older muscle seems to be related to a reduced activation of upstream of mTOR signaling activity compared with young muscle. The stimulation of MPS by resistance exercise is significantly increased when amino acids are given immediately after exercise in the elderly (Esmarck *et al*, 2001; Tipton *et al*, 2001).

There is a paucity of data regarding the measurement of MPB in response to exercise in the elderly. Using the A-V tracer dilution method, resting leg protein breakdown is suggested to be increased slightly in older men (Volpi *et al*, 2001). However, we have data that reveal no difference in basal MPB but that the normal inhibition of MPB by insulin is significantly less in elderly (Wilkes *et al*, 2008). It appears that "anabolic blunting" is a widespread feature of ageing muscle.

Clearly, the goal in ageing is to minimize muscle wasting and attempt to maintain muscle mass and function; for that to be achievable, we need to understand the synergy between exercise and feeding and develop appropriate exercise and feeding strategies for the elderly.

1.15 GAPS IN OUR UNDERSTANDING

This review emphasizes a number of gaps that are present within the literature with regards to effects of resistance exercise and feeding on physiological and molecular changes in muscle protein synthesis in human beings.

First, although resistance exercise is well established to stimulate muscle protein synthesis in both young and old subjects, the age related differential effects of resistance exercise on MPS and cell anabolic signaling in young and old men in postabsorptive state are not clearly defined.

Secondly, a comparison of the effects of resistance exercise between postabsorptive young and old across a full spectrum of exercise intensity on MPS and associated changes in signalling activity is unavailable.

Thirdly, we remain ignorant of how increasing the duration of resistance exercise influences MPS and associated anabolic signalling molecules in the post absorptive state in either young or old muscle.

Finally, Others have already shown benefits of leucine in stimulating MPS at rest in older people (Katsanos *et al*, 2006) or of giving essential amino acids (including leucine) after resistance exercise (Drummond *et al*, 2008) but the effect in elderly persons of supplementation of protein drinks with leucine alone following an acute bout of resistance exercise remained to be investigated.

Furthermore, to date, the interaction between feeding/exercise and changes in MPS/cell anabolic signalling has been addressed in terms of the magnitude or time

course using the study designs with too long a period between muscle sampling (e.g. 6, 12 or 24h) to detect the rapid effects of exercise or feeding on muscle synthetic responses, thereby missing the rapid changes in muscle synthetic responses following exercise or feeding. Therefore, short term changes in protein synthetic responses following an intervention are less well understood.

1.16 STUDY OBJECTIVES:

- 1) To characterize the dose response relationship between resistance exercise intensity and muscle protein synthesis in lean young and old men in postabsorptive conditions (Chapter 3).

- 2) To compare the results of the dose-response curve in the young with those found in the old to assess whether sarcopenia is partly due to a defect in MPS responses of elderly to RE when compare to those seen in young (Chapter 3).

- 3) If any age related differences in stimulation of protein synthesis with resistance exercise do exist, to determine whether they may be explained by anabolic signaling defects (Chapter 3).

- 4) To determine the effect of doubling the exercise duration on the anabolic response of muscle protein synthesis in postabsorptive, lean young and old men and if any increased responses in the subjects may be explained by signaling responses (Chapter 4).

- 5) To optimise the exercise regime (chapter 3 and 4), which would be adopted for studies investigating the possible synergy between exercise and protein/amino acid feeding (chapter 5).

- 6) To assess the impact of leucine enriched protein ingestion after an acute bout of RE on magnitude and time course of the muscle synthetic responses and cell anabolic signaling in the young and the elderly men to determine whether exercise + nutritional intervention can restore muscle protein synthesis in older lean men to rates similar to those in young men (Chapter 5).

- 7) To assess the time course and magnitude of responses of MPS and cell anabolic signalling after exercise or nutritional intervention in young and lean men with a sufficiently sensitive time-discrimination by using an intensive study protocol with frequent muscle biopsies to detect the likely rapid effects of exercise or feeding on muscle synthetic responses (Chapter 3, 4, 5).

CHAPTER 2: MATERIALS AND METHODS

2.1 GENERAL DESCRIPTION OF THE PROTOCOLS

Overall this work involved 3 studies:

Study 1: To determine the independent effect of resistance exercise intensity on changes in myofibrillar protein synthesis and anabolic signaling proteins in healthy, postabsorptive, lean young and older men (Chapter 3).

Study 2: To determine the independent effect of resistance exercise duration on changes in myofibrillar protein synthesis and anabolic signaling proteins in healthy, postabsorptive, lean young and older men (Chapter 4).

Study 3: To determine the effect of combined resistance exercise of optimal intensity and duration (data from study 1 and 2) and nutritional intervention on myofibrillar protein synthesis and anabolic signalling proteins in healthy, postabsorptive lean young and older men (Chapter 5).

To carry out the work we studied 125 young (20-25 y) and older (65-75 y) men in groups of 6-9 each in the postabsorptive state i.e. following an overnight fast. All subjects underwent health screening checkups, and those determined to be healthy underwent measurement of their body compositions and 1RM during their screening visits.

Each subject performed resistance exercise involving unilateral full cycle, isotonic leg extension exercises with his dominant leg. The single leg knee extension exercise protocol was chosen so that each subject could exercise optimally after having 2 muscle biopsies from the contra lateral leg without worrying about the biopsy sites.

We used the 'constant infusion method' involving measuring the incorporation of stable isotopically labelled [1, 2-¹³C₂]leucine tracer into vastus lateralis quadriceps muscle, biopsied at regular intervals, to measure the protein synthesis changes in the myofibrillar fraction of skeletal muscle after resistance exercise ± nutritional intervention (Rennie *et al*, 1982; Halliday *et al*, 1975, Watt *et al*, 1991, Chinkes *et al*, 1996). Western blotting using specific antibodies was used to measure the changes in phosphorylation of muscle signaling proteins (Wilkinson *et al*, 2008).

As tracer takes about 150 min to achieve a steady state of tracer labelling in plasma (Rennie *et al*, 1999; Smith *et al*, 1996), we obtained two basal muscle biopsies from the rest leg (0 h and then 2.5 h) before the exercise/ nutritional intervention to measure basal muscle synthetic rates. After exercise, we obtained muscle biopsies from the exercise leg at about 10 min to measure the changes in muscle synthetic responses during exercise. On the basis of a previous limited pilot study (Bowtell *et al*, 2003), we hypothesized that there would be a latent period of ~1 h before any rise in MPS and this rise would peak between 1- 2 h after exercise. Therefore, muscle tissue was obtained at 1 h to measure any signaling changes preempting MPS changes, and at 2 h to measure the additional MPS and signaling changes. We took a further biopsy at 4 h to measure the time course and general trend of these synthetic responses. Regular blood (every 30 min) samples were obtained throughout the study period to calculate labelling of venous plasma α -KIC as a surrogate for true precursor labelling (Watt *et al*, 1991). The tracer infusion was stopped and cannulas removed after the last (sixth) biopsy and the final blood draw.

2.2 VOLUNTEERS

Healthy young (aged 18-30 years) and elderly men (aged 65-75 y) were recruited by advertisement in the local and university press, local radio, display posters and informal presentations at local social clubs. Written study information sheets were sent to all volunteers who enquired about the study via e-mail or phone to make them fully aware of benefits and risks of participating in the study prior to their screening visits. The subjects were informed of the experimental protocols, both verbally and in writing, before giving their informed consent during the screening visit. Prior to participation in the study, all volunteers filled out a health questionnaire and took a physical examination, including a blood test for full blood count, clotting screen, kidney and liver function, fasting blood glucose, thyroid function and an electrocardiogram. Exclusion criteria included frailty, active or previous malignancy, a history of cardiac, pulmonary, liver, kidney, vascular or autoimmune disease, clotting disorders, uncontrolled hypertension ($>160/90$ mmHg), diabetes, thyroid disorders, obesity, anaemia, cancer, alcohol abuse, visually obvious muscle wasting, corticosteroid use or joint pain that restricted movement. Subjects with mild controlled hypertension ($<140/90$ mmHg without medication) were not excluded from the study. Volunteers who were taking prophylactic low dose aspirin were advised to stop it 1 week before the study day to reduce the risk of bleeding following muscle biopsy. All subjects were recreationally active and independent in activities of daily living. A total of 125 healthy men completed the study protocols, all of which were approved by the Nottingham University Ethics Committee (Reference number C/5/2007) and according to the Helsinki Declaration.

2.3 INFUSION AND CAPSULES CONTAINING STABLE ISOTOPE LABELED TRACER

The stable isotope labeled [1,2-¹³C₂]leucine tracer was purchased from Cambridge Isotope Laboratories, Massachusetts, USA. The capsules and infusions containing [1,2-¹³C₂]leucine were prepared aseptically under sterile conditions by the Clinical Trials Sterile Production Unit, Pharmacy, Queens Medical Centre, Nottingham, UK.

The prescription for the infusion was calculated according to the subject's weight during the screening visit. These infusions were made a day before the study day in the sterile unit by weighing the tracer as per prescription, then dissolving it in 500 ml of 0.9% normal saline and then filtering through a 0.45µ filter to remove pathogens. The infusions were transported to the pharmacy at Royal Derby Hospital in cool boxes via a taxi and kept there in fridges until collection by myself or one of the team members.

On the study day, the tracer infusion was administered via an infusion pump (Volumed µVP 5005 Arcomed Ag, Switzerland) at 0.75mg/kg (priming dose) and 1mg/kg/h (constant infusion rate, maintained throughout the period of the study) through 18G intravenous cannulae.

2.4 ORAL AMINO ACID SOLUTION (SLIMFAST) AND CAPSULES

One of the aims of the study (Chapter 5) was to assess the effect of leucine enriched protein supplementation after resistance exercise on myofibrillar

protein synthesis and cell anabolic signalling in healthy young and elderly subjects. For this, both young and old subjects were fed either a protein carbohydrate drink (SlimFast Optima: equivalent to 1g leucine) or SFO + 4.2 g of oral leucine or alanine (isonitrogenous), as control, capsules after resistance exercise.

The protein-carbohydrate drink (SlimFast) and the capsules containing unlabelled leucine and alanine were a gift from Unilever, Englewood Cliffs, NJ 07632, USA.

One can (325 ml) of SlimFast Optima contained 10 g protein, 24 g carbohydrate and 6 g fat. The protein composition of the drink was: 8 g casein, 2 g whey protein, and 0.05 g soy protein, of which 1.05 g leucine, 0.36 g alanine, 0.64 g isoleucine and 0.73 g valine. The total energy of SlimFast Optima was 180 kCal.

A capsule containing [1,2-¹³C₂]leucine tracer was given orally to maintain the isotopic enrichment and to prevent the dilution of plasma [1,2-¹³C₂]leucine as a result of ingestion of unlabelled leucine (Rasmussen *et al*, 2000). Subjects who took only Slim-Fast Optima (1 g leucine) was given a capsule containing 40mg [1,2-¹³C₂]leucine tracer and who took 5.2 g of leucine was given 200 mg of [1,2-¹³C₂]leucine tracer.

2.5 CLINICAL AND ANTHROPOMETRIC MEASUREMENTS

The clinical studies were carried out in the Clinical study room, Department of Clinical Physiology, School of Graduate Entry Medicine & Health, Royal

Derby Hospital, Derby, UK. Subjects' height and weight were determined during their initial screening visit several days prior to the study, using weighing scales with a stadiometer (Seca, Leicester). From these measurements, body mass index, and the quantity of stable isotope infusion required for the acute study were determined.

2.5.1 1 Repetition Maximum (1RM)

The maximal strength of the subjects' dominant leg or 1 repetition maximum (1RM) was measured on a free weight leg extension machine (ISO leg extension, Leisure Lines (GB) Ltd).

To measure the 1RM, each subject was initially familiarized with the machine, and then asked to sit with his hips at 90 deg and his back against a backrest inclined at 30 deg from horizontal. The pivot of the machine was aligned with the lateral aspect of the midline of the subject's knee. The leg pad of the machine was positioned above the subject's ankle and the rotation arm was positioned so that the subject's knee was bent to 90 deg. A full repetition using light weight was considered when the subject was able to move the weight through an arc of ~80 deg (from 90 deg to ~170 deg) using his dominant leg. The sitting position and height of the lift was recorded and kept constant using the headpiece of stadiometer (Seca, Leicester) during the subsequent study period. Each subject warmed up with 8–10 repetitions lifting light weights with his dominant leg before performing a single best effort at a weight estimated to be the subject's 1RM based on his height and body weight.



Fig 2.1 Measurement of 1 repetition maximum (1RM) of dominant leg on a free weight leg extension machine

The weight was then increased or decreased depending on whether the subject could just manage to perform the full repetition and hold for a 1-s count. The 1 repetition maximum was rechecked by asking the subject to perform a 1RM at the previously determined weight following an interval of 30 minutes during the screening visit or sometime 2-3 days following the screening visit.

2.5.2 *Body Composition Analysis*

Dual-energy photon X-ray absorptiometry (DXA; GE Lunar Prodigy II, GE Healthcare) was used to measure total skeletal muscle mass, leg lean mass and fat mass of each postabsorptive subject during the screening visit. During the scans, the body positions were recorded with the use of bony landmarks and scan table references to ensure that body position was maintained as constant as possible. All participants were scanned with their hands by their sides in a supinated position with their feet about 30 cm apart.

Graduate Entry Medical School
University of Nottingham
Derby City General Hospital

Patient:		Patient ID:	MB01C
Birth Date:		Attendant:	
Height / Weight:	164.0 cm 77.3 kg	Measured:	19/03/2009 09:11:24 (8.80)
Sex / Ethnic:	Female White	Analyzed:	19/03/2009 09:12:50 (8.80)

BODY COMPOSITION

Region	Tissue (%Fat)	Region (%Fat)	Tissue (g)	Fat (g)	Lean (g)	BMC (g)	Total Mass (kg)
Left Arm	39.4	38.1	3,031	1,193	1,838	102	-
Left Leg	41.9	40.6	13,073	5,480	7,592	439	-
Left Trunk	42.0	41.3	18,513	7,779	10,734	300	-
Left Total	40.1	39.0	37,485	15,047	22,438	1,090	-
Right Arm	39.3	38.0	3,157	1,242	1,915	112	-
Right Leg	41.9	40.5	12,540	5,257	7,283	456	-
Right Trunk	42.0	41.3	18,650	7,838	10,812	343	-
Right Total	40.8	39.6	36,038	14,699	21,339	1,051	-
Arms	39.4	38.0	6,188	2,435	3,753	214	-
Legs	41.9	40.5	25,612	10,737	14,876	895	-
Trunk	42.0	41.3	37,163	15,617	21,546	642	-
Android	45.8	45.4	4,966	2,273	2,693	38	-
Gynoid	47.9	47.0	11,385	5,455	5,929	222	-
Total	40.5	39.3	73,523	29,746	43,777	2,141	75.7

FAT MASS RATIOS

Trunk/Total	Legs/Total	(Arms+Legs)/Trunk
0.53	0.36	0.84

2 -UK Total Body Reference Population (v102)
 3 -Matched for Age, Weight (females 25-100 kg), Ethnic

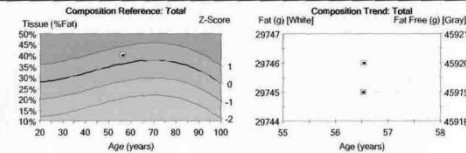
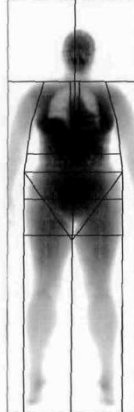
GE Medical Systems
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DF+14824

Graduate Entry Medical School
University of Nottingham
Derby City General Hospital

Patient:		Patient ID:	MB01C
Birth Date:		Attendant:	
Height / Weight:	164.0 cm 77.3 kg	Measured:	19/03/2009 09:11:24 (8.80)
Sex / Ethnic:	Female White	Analyzed:	19/03/2009 09:12:50 (8.80)

Total Body Tissue Quantitation



Measured Date	Age (years)	Android (%Fat)	Gynoid (%Fat)	A/G Ratio	Total Body (%Fat)
19/03/2009	56.5	45.8	47.9	0.96	40.5

COMMENTS:

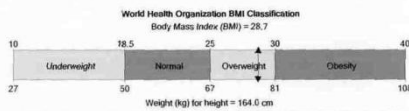


Image not for diagnosis
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 Filename: m809k0fs.dft
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Prology
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Fig 2.2 Measurement of body composition using Dual-energy photon X-ray absorptiometry

2.6 CONDUCT OF THE STUDY

All subjects were studied following an overnight fast. They were asked to keep to their usual diet and activity but to refrain from any heavy exercise for 72 h before the study day. They attended the clinical study room, Department of Clinical Physiology, School of Graduate Entry Medicine and Health, Derby at 08.00 am, having been picked up from home by a taxi.

Each subject had 18 G polyethylene catheters inserted in both antecubital veins, one for tracer infusion and the other for blood sampling. Each study day lasted about 7 h. The first baseline blood was taken before obtaining the muscle biopsy (see below). Subsequently, a primed, continuous infusion (0.7 mg.kg^{-1} , 1 mg.kg.h^{-1}) of [1, 2- $^{13}\text{C}_2$]leucine (99 Atoms %, Cambridge Isotopes, Cambridge, MA, USA) was started (at 0 h) immediately after the first biopsy and maintained until the end of the study, according to the study protocol. Subsequent blood samples were taken at 20-30 min intervals. The blood samples were stored on ice and plasma was separated from whole blood by centrifugation immediately after the study. After the study, both catheters were removed and subjects were fed and monitored for half an hour before being escorted home.

2.6.1 *Muscle Biopsies*

Muscle biopsies were taken from the m. vastus lateralis of quadriceps muscle of both legs. The skin was sterilized with iodine solution (Videne, Ecolab Ltd, Leeds, UK) and approximately 5 ml of 1% lignocaine (Taro Pharmaceuticals Ltd., UK) was injected under the skin and into subcutaneous tissue as local anaesthetic. After 1-2 min, a 0.5 – 1cm incision was made in the skin and

subcutaneous tissue at about 15 cm above the knee from the lateral aspect of thigh. Muscle biopsies were obtained using the conchotome biopsy technique (Dietrichson *et al.*, 1987). Samples of 100-150 mg of muscle tissue were removed; the muscle tissue was washed in ice cold saline before blood, visible fat and connective tissue were removed, then the biopsy was immediately frozen in liquid nitrogen then stored at -80°C until further analysis.



Fig 2.3 Obtaining muscle biopsy from m. vastus lateralis of quadriceps muscle

2.7 ANALYTICAL METHODS

2.7.1 Myofibrillar Protein Extraction (Bohe *et al*, 2001; Cuthbertson *et al*, 2006; Louis *et al*, 2003; Moore *et al*, 2005)

The fractional synthesis rate (FSR) of mixed muscle protein was measured from the incorporation of ^{13}C leucine, using the labelling of muscle free leucine as precursor and defining the average value of this both from the muscle biopsies and the plasma KIC.

Aliquots of frozen muscle sample (~ 25 mg) were ground in liquid nitrogen, and the frozen powder was then added to homogenization buffer containing protease and phosphatase inhibitors (0.15 M NaCl, 0.1% Triton, 0.02 M Tris, 50 μM DTT, 0.1 M EDTA, and 1 mM PMSF).

The resulting homogenate was subjected to centrifugation at $1,600 \times g$ for 20 min to precipitate the myofibrillar and collagen fractions. The supernatant was subjected to high-speed centrifugation (7,000 g, 15 min), and the sarcoplasmic fraction was removed and precipitated by bringing the supernatant to 70% with ethanol and collected by centrifugation ($1,600 \times g$).

The pellet containing myofibrillar and collagen protein fractions was incubated in 0.3 M NaOH to dissolve the myofibrillar proteins, and then centrifuged at $3000 \times g$ for 20 min to pellet the collagen. Myofibrillar protein was precipitated by bringing the supernatant to 70% with ethanol and collected by centrifugation.

Protein-bound amino acids were released by acid hydrolysis (0.05 M HCl in dowex slurry) at 110°C overnight and the amino acids were purified by ion exchange chromatography on Dowex H⁺ resin. Incorporation of [1, 2-¹³C₂] leucine into protein was determined by gas chromatography combustion-isotope ratio mass spectrometry.

2.7.2 Calculations of fractional synthetic rates (FSR)

The FSR of myofibrillar protein was calculated by measuring the increase in tracer [1, 2-¹³C₂]leucine enrichment in myofibrillar protein, in the interval between two chronological muscle biopsies, with the plasma [1, 2-¹³C₂]KIC enrichment used as the surrogate for the precursor pool enrichment, using the standard precursor-product method:

Fractional protein synthesis (k_s , %·h⁻¹) = $\Delta E_m / E_p \times 1/t \times 100$, where ΔE_m is the change in protein enrichment between two biopsy samples, E_p is the mean enrichment over time of venous α -KIC enrichment, and t is the time between biopsies.

2.7.3 WESTERN BLOTTING (Smith G *et al*, 2008; Wilkinson *et al*, 2008)

After mincing with scissors about 25 mg of muscle tissue and homogenization in ice-cold buffer (10 μ l/mg⁻¹) containing 50 mM Tris-HCl (pH 7.4), 0.1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.1% 2-mercaptoethanol, 10 mM β -glycerophosphate, 50 mM NaF, 0.5 mM activated sodium orthovanadate (all Sigma Aldrich, Poole, UK) and a complete protease inhibitor cocktail tablet (Roche, West Sussex, UK), soluble proteins were separated from myofibrillar proteins by rotating on a Vibramax for 10 min at 4°C and

centrifugation at 10,000 g for 10min at 4°C. Bradford assays were used to determine sarcoplasmic protein concentrations after which samples were standardized to 1 mg.ml⁻¹ by dilution with 3 × Laemmli loading buffer in order to measure relative phosphorylated protein concentrations of Akt Ser473, mTOR Ser2448, p70S6K1 Thr389, 4EBP1 Thr37/46, eEF2α Ser51 and pan-actin (Sigma-Aldrich, Poole, UK). Samples were mixed and heated at 95°C for 7 min before 15 µg of protein/lane was loaded on to Criterion XT Bis-Tris 12% SDS-PAGE gels (Bio-Rad, Hemel Hempstead, UK) for electrophoresis at 200 V for ~60 min. At this point, where possible, membranes were cut allowing for probing at multiple targets of different sizes on the same membrane to minimise cost and maximise output. Gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol) for 30 min before proteins were electroblotted on to 0.2 µm PVDF membranes (Bio-Rad) at 100 V for 30 min. After blocking with 5% low-fat milk in TBS-T (Tris Buffered Saline and 0.1% Tween-20; both Sigma-Aldrich, Poole, UK) for 1 h, membranes were rotated overnight with primary antibody (all AbCam, Cambridge, UK) against the aforementioned targets at a concentration of 1:2000 at 4°C. Membranes were washed (3×5 min) with TBS-T and incubated for 1 h at room temperature with HRP-conjugated anti-rabbit secondary antibody (New England Biolabs, UK), before further washing (3×5 min) with TBS-T and incubation for 5 min with ECL reagents (enhanced chemiluminescence kit, Immunstar; Bio-Rad). Blots were imaged and quantified by assessing peak density after ensuring bands were within the linear range of detection using the Chemidoc XRS system (Bio-Rad, Hemel

Hempstead, UK). Phosphorylation of signalling proteins was corrected for loading anomalies to α -actin.

2.8 STATISTICAL ANALYSIS

All data are reported as means \pm standard error of mean (SEM). All analysis was made using Graph pad version 5.0 software (Graph Pad Software, La Jolla, CA, USA). Where appropriate, correlations were tested by assessing the existence of a linear fit between variables. The MPS and p70S6K responses were calculated as area under the curve above baseline values by calculating the sum of the areas of $\Delta\text{time} \times \Delta\text{FSR}$ (or p70S6K), i.e. a rectangle, and assuming linear changes in FSR between time points, $0.5 \times (\Delta\text{time} \times \Delta\text{FSR})$, to calculate the area of triangle. Comparisons between means of two groups were made with Student's t-test (two-tailed) and between-and within-group differences were tested by repeated measures ANOVA. Where ANOVA measured significance, a Tukey's post hoc procedure was used to identify pairwise differences. Significance was accepted as <0.05 .

**CHAPTER 3: AGE-RELATED DIFFERENCES IN DOSE RESPONSE
OF MUSCLE PROTEIN SYNTHESIS TO RESISTANCE EXERCISE IN
YOUNG AND OLD MEN**

3.1 SUMMARY

We investigated how myofibrillar protein synthesis (MPS) and muscle anabolic signalling were affected by resistance exercise at intensities from 20-90% of 1 repetition maximum (1 RM) in two groups (25 each) of post-absorptive, healthy, young (24 +/- 6 years) and old (70 +/- 5 years) men with identical body mass indices (24 +/- 2 kg.m⁻²). We hypothesized that, in response to exercise intensity, anabolic signalling molecule phosphorylation and MPS would be modified in a dose-dependant fashion, but to a lesser extent in older men. Vastus lateralis muscle was sampled before, immediately after, and 1, 2 and 4 h post-exercise. MPS was measured by incorporation of [1, 2-¹³C₂]leucine (gas chromatography-combustion-mass spectrometry using plasma [1,2-(13)C]alpha-ketoisocaproate as surrogate precursor); the phosphorylation of p70 ribosomal S6 kinase (p70s6K) and eukaryotic initiation factor 4E binding protein 1 (4EBP1) was measured using Western analysis with anti-phosphoantibodies. In each group, there was a sigmoidal dose-response relationship between MPS at 1-2 h post-exercise and exercise intensity, which was blunted ($P < 0.05$) in the older men. At all intensities, MPS fell in both groups to near-basal values by 2-4 h post-exercise. The phosphorylation of p70s6K and 4EBP1 at 60-90% 1 RM was blunted in older men. At 1 h post-exercise at 60-90% 1 RM, p70s6K phosphorylation predicted the rate of MPS at 1-2 h post-exercise in the young but not in the old. The results suggest that in the post-absorptive state: (i) MPS is dose dependant on intensity rising to a plateau at 60-90% 1 RM; (ii) older men show anabolic resistance of signalling and MPS to resistance exercise.

3.2 INTRODUCTION

Exercise is known to stimulate the rate of post-exercise myofibrillar protein synthesis (MPS) in healthy young people, the extent of which probably depends upon several factors including nutritional state, mode, intensity and duration of exercise. (Biolo *et al*, 1995; Chesley *et al*, 1992; Dreyer *et al*, 2006; Drummond *et al*, 2008; Miller *et al*, 2005; Wilkinson *et al*, 2008). However, little or no information is available in respect of the dose–response regarding exercise intensity. Muscles of older people are also capable of increases in MPS after resistance exercise (Drummond *et al*, 2008; Sheffield-Moore *et al*, 2005) but again dose–response information is lacking.

Normal ageing is associated with a loss of skeletal muscle mass at 0.5–2% per annum, causing sarcopenia with an incidence rate of 13–24% in those aged 50–70 years and up to 50% for those in their eighties (Baumgartner *et al*, 1998). However, maintaining physical activity appears to preserve muscle mass (Raguso *et al*, 2006) and even nonagenarians can benefit from resistance training (Fiatarone *et al*, 1990). Nevertheless, the dose–response relationship between increases in muscle synthetic rates and exercise intensity for older people is unknown. Thus, a comparison of the effects of exercise between young and old across a full spectrum of exercise intensity on MPS and associated changes in signalling activity is unavailable.

On the basis of a limited pilot study at exercise intensities of 60–90% (Bowtell *et al*, 2003), we hypothesized that the dose–response relationship between exercise intensity and increases in MPS would be hyperbolic with linear increases at intensities up to 60% of 1 RM and no further increase above this

value, when all motor units would probably be activated and recruited to generate force. At intensities above 60% 1RM, further power to lift weights would be generated via increasing activity per fiber not the number of active fibers. On the basis of that work, we also hypothesized that there would be a latent period of ~ 1 h before any rise in MPS occurred. We have previously shown (Cuthbertson *et al*, 2005) that in older men (~ 70 years) there is a decreased sensitivity and capacity of increases of MPS (and associated anabolic signalling) across a wide range of essential amino acid availability, a phenomenon we named anabolic resistance. We therefore hypothesized that decreased sensitivity and capacity to increase myofibrillar protein synthesis would occur in older men in response to resistance exercise too.

3.3 SPECIFIC METHODS

The study was approved by the University of Nottingham Ethics Committee and complied with the Declaration of Helsinki. Written informed consent was obtained from the subjects after explaining the study procedure and associated risks.

3.3.1 Study Design

Twenty-five young (24 ± 6 years) and twenty-five older (70 ± 5 years) men were recruited for the study. They were recreationally active, physically independent and healthy overall, with no sign of insulin resistance (fasting blood glucose, 4.5 ± 1.0 versus 5.1 ± 0.9 mM, in young and old). Body mass indices were identical in the two groups (23 ± 4 versus 24 ± 2 kg m²) as were lean body masses (64 ± 17 versus 57 ± 14 kg) and right (11.9 ± 2.7 versus 10.7 ± 4 kg) and left (10.5 ± 2.5 versus 9.3 ± 2.3 kg) lean leg masses (all young

versus older). The only major difference was the 1 repetition maximum (1 RM) weight lifted by unilateral leg extension which was significantly reduced in the older men (75 ± 14 kg *versus* 41 ± 11 kg, $P < 0.05$).

3.3.2 Study Protocol

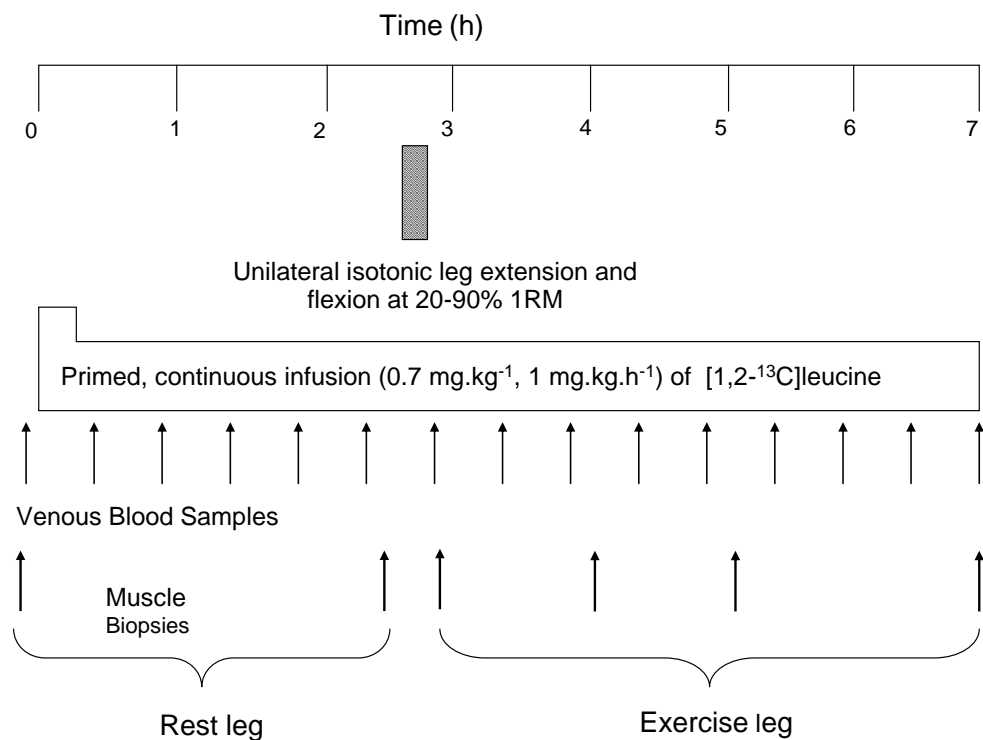


Figure 3.1 Protocol for the measurement of the relationship of alteration in myofibrillar protein synthesis and muscle anabolic signalling molecule phosphorylation and resistance exercise intensity. Each subject was studied over the period shown in the fasted state with 5 young and 5 older subjects carrying out exercise with their dominant leg randomly assigned to 20-90% 1 RM.

The participants were studied after an overnight fast after normal daily activity. On the morning of the study (~09.00 h), they had an 18-g cannulae inserted into the antecubital vein of each arm for tracer infusion and venous blood sampling. Blood samples and muscle biopsies were taken according to the protocol (Fig. 3.1). A primed, continuous (0.7 mg kg^{-1} , $1 \text{ mg kg}^{-1} \text{ h}^{-1}$) infusion of [1,2- $^{13}\text{C}_2$]leucine (99 Atoms%, Cambridge Isotopes, Cambridge, MA, USA) was started immediately after the first biopsy and maintained for ~7 h. After 2.5 h of infusion, the subjects exercised with their dominant legs at intensities, randomly assigned, from 20% to 90% of 1 RM, with five subjects per group per intensity. The seated subjects performed unilateral leg extensions and flexions (1–2 s each) with 2 min rest between sets. The schedule of contractions was designed to equalize, as closely as possible, the volume of exercise, i.e. the force \times time-under-tension product (often described as ‘work’). Thus, at an exercise intensity of 20% of 1 RM, the subjects completed 3 sets \times 27 repetitions (reps); at 40%, 3 sets \times 14 reps; at 60%, 3 sets \times 9 reps; at 75%, 3 sets \times 8 reps and those at 90%, 6 sets \times 3 reps. Total work output (i.e. % 1 RM \times number of repetitions \times number of sets) was 1620–1800 units at different exercise intensities, and total time-under-tension was obtained by multiplying by 4 s.

3.3.3 Measurement of Myofibrillar Protein Synthesis And Cell Signalling Phosphorylation

The fractional synthesis rate (FSR) of myofibrillar protein and the extent of phosphorylation of p70s6K1 on Thr389, 4EBP1 on Thr37/46 and the elongation factor eEF2 on Thr56 were determined as described in chapter 2.

3.4 RESULTS

The only distinguishing features between both groups were age and a 51% smaller 1 RM leg extension force in the older men. Thus, the absolute total force–time integral was less in older men even though the relative work done was the same.

The basal rates of myofibrillar protein synthesis were not different in the two groups of subjects (0.039 ± 0.002 versus $0.043 \pm 0.003\% \text{ h}^{-1}$, young versus older, respectively ($P > 0.05$)). In both groups there was a dose-related effect of resistance exercise on myofibrillar protein synthesis at 1–2 h post-exercise

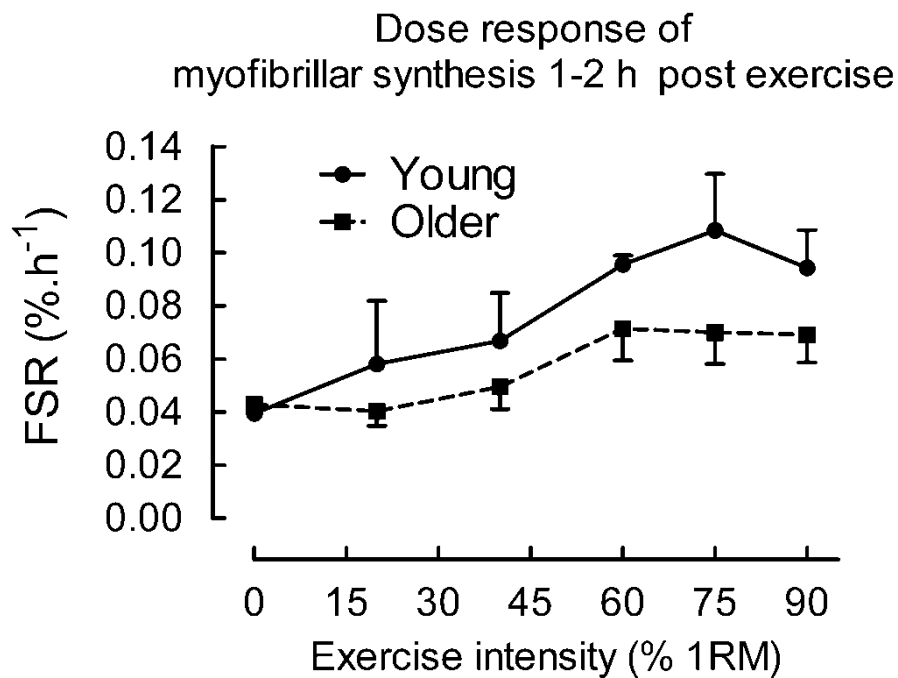


Figure 3.2 Dose response relationship of myofibrillar protein synthesis (FSR, fractional synthetic rate, $\% \cdot \text{h}^{-1}$) measured at 1-2 h post exercise for 5 young men and 5 older men at each intensity. The responses of the young men overall were greater than those of the older men ($P < 0.04$). The responses between 60-90% of 1RM in young and old were indistinguishable from each other but those in the young were together significantly higher than in the older men ($P < 0.01$) for 15 subjects in each group.

which was sigmoidal (Fig. 3.2); thus, there were small increases after exercise at 20 and 40% of 1 RM but a bigger rise to the values at 60, 75 and 90% of 1 RM, that were effectively at a maximal plateau (i.e. with no significant differences between them). There was a significant difference between the overall responses of MPS to exercise in the young and older subjects, with the area under the curves (rate of muscle protein synthesis \times time, i.e. % of total protein synthesized) being $30 \pm 6\%$ higher ($P < 0.04$) in the younger men.

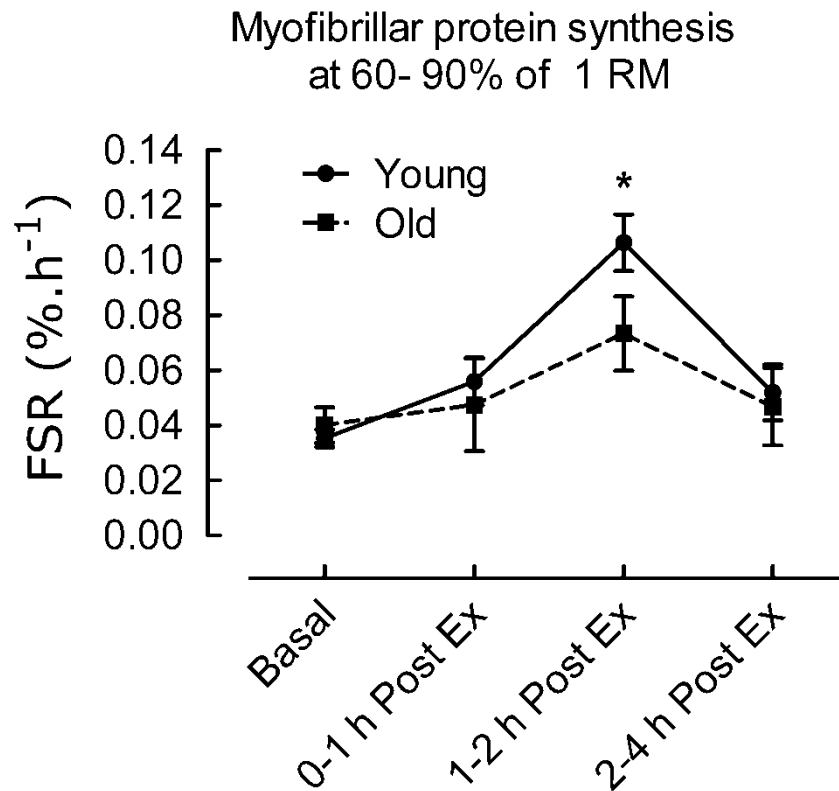


Figure 3.3 Time course of the averaged responses to exercise at 60-90% 1RM of myofibrillar protein synthesis (FSR, fractional synthetic rate, %·h⁻¹) at 60-90% 1 RM for 15 subjects in each group of young and older subjects. * = $P < 0.05$. NOTE: Protein synthesis is measured over 2.5 h in the basal pre-exercise state and then over the periods shown post-exercise.

No values of synthetic rates are presented for the exercise period as the exercise period was variable, and in most cases too short to obtain reliable results. The shapes of the time courses of the changes in protein synthesis thereafter were similar in both groups of subjects, with a lag over the first hour after exercise, a rise whose extent depended on intensity and the group studied, to a peak at 1–2 h and a fall thereafter to near basal values.

The values at 60–90% in each group were not different from each other, and so we averaged the data at these intensities (Fig. 3.3). The values in the young and older men were only different at 1–2 h.

Inspection of the data for the extent of protein phosphorylation of p70s6K and 4EBP1 revealed a much greater degree of variation around mean values than that observed for myofibrillar protein synthesis and it was therefore difficult to discern more than a broad positive relationship between the size of the changes and exercise intensity (data not shown).

Nevertheless, as for myofibrillar protein synthesis, there were no significant differences between the phosphorylation responses of p70s6K and 4EBP1 at 60, 75 and 90% of 1 RM for young and old subjects, and combining these data produced more coherent images of the time courses and differences between the two groups of subjects (Fig. 3.4).

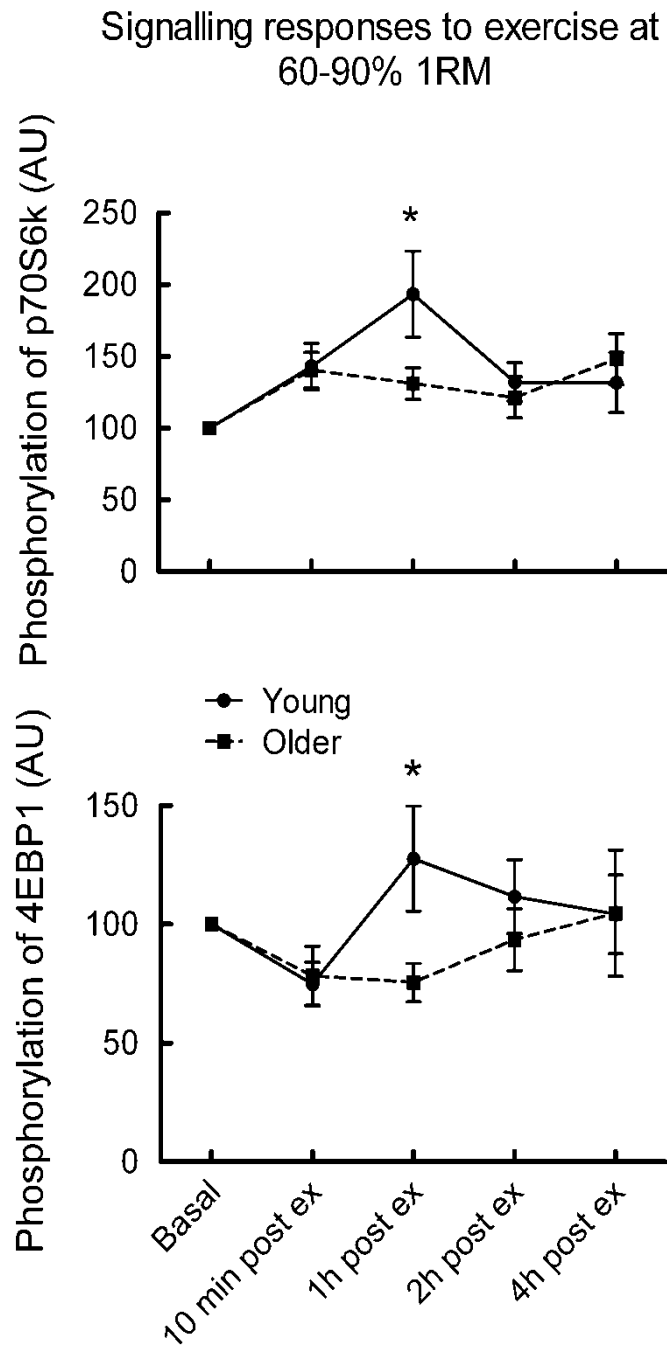


Figure 3.4 Time courses of the responses of phosphorylation of p70s6K and 4EBP1 (arbitrary units as % basal for each subject) averaged for intensities of 60-90 % 1RM (n = 15 in each group) * = P<0.05.

When the extent of phosphorylation of p70s6K at 1 h was related to the extent of MPS at 1–2 h there was a positive correlation for the young but not the old (Fig. 3.5).

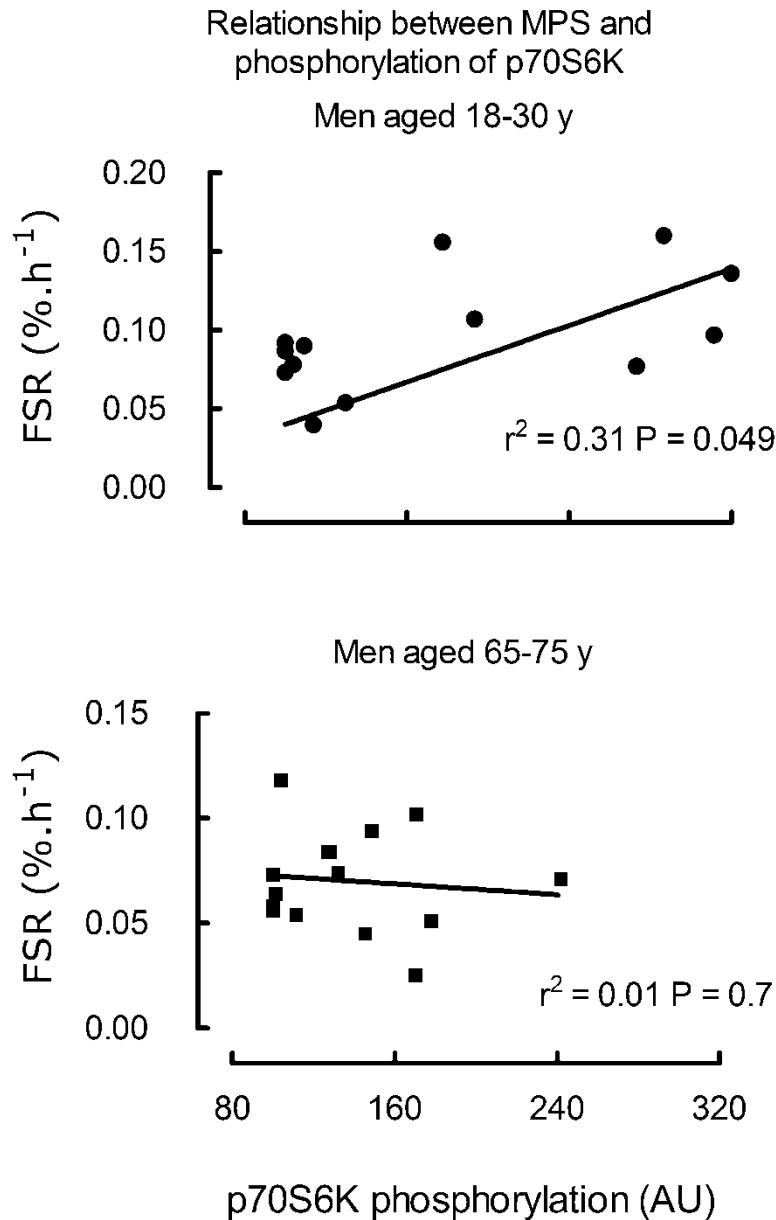


Figure 3.5 Relationship between myofibrillar synthetic rate and extent of phosphorylation of p70s6K averaged for responses at 60-90% in young subjects (above) and older subjects (below). There was a significant relationship ($P=0.049$) between degree of phosphorylation (arbitrary units) and protein synthetic rate (FSR $\%.h^{-1}$) only in the young. NB some points overlaid.

For eEF2, phosphorylation showed a statistically non-significant fall of about 20% immediately after exercise then a rebound to about 120% of basal values by 1 h after exercise with no significant differences between the two age groups (data not shown).

3.5 DISCUSSION

The present results provide new information concerning the responses of myofibrillar protein synthesis and muscle anabolic signalling in young and older men to exercise at a range of resistance exercise intensities, all in the post-absorptive state. In particular, rather than the hyperbolic relationship we postulated, they show a sigmoidal dose–response relationship of myofibrillar protein synthesis to exercise intensity, with little increase from 20 to 40% 1 RM, then a bigger rise at 60% of 1 RM, with no significant further increase up to 90% 1 RM. Older men showed a smaller response than the young subjects.

We previously showed in a pilot study that isometric exercise at 60, 75 and 90% of 1 RM increased myofibrillar protein synthesis at 90–150 min post-exercise to the same extent (Bowtell *et al*, 2003); the present results confirm that above 60% of 1 RM of isotonic exercise, the stimulatory effect is maximized. We ensured that the force \times time integral (i.e. total external work) was equalized so far as possible so the results suggest that the total energy expenditure was constant and not a factor affecting the responses.

Might a change in muscle fibre type composition explain the age-related differences?

It is known that as contraction intensity increases an increasing proportion of type 2 fibres are recruited. Although in the basal or amino acid-stimulated

conditions there is little difference in rates of protein synthesis in human muscle of markedly different fibre type compositions (Mittendorfer *et al*, 2005), it is possible that at high contraction intensities, type 2 fibres would show a greater response than type 1 fibres. Indeed, in type 2 fibres, phosphorylation of sarcoplasmic p70S6K1 occurs to a greater extent (~25–30% more) than in type 1 fibres after resistance exercise at 75% of 1 RM (Koopman *et al*, 2006). This difference was suggested to be due to greater recruitment of type 2 fibres than type 1 fibres, which is feasible. Cross-sectional studies comparing individuals aged 60–70 years to those in their twenties (Larsson, 1983) suggested a slight increase in the proportion of type 1 fibres with age. However, other later cross-sectional studies did not confirm this (see (Porter *et al*, 1995) for review). Furthermore, when fibre type proportions of muscles from the same individuals at 65 years and then at 75 years were assessed, there was a decrease from ~40–60% of type 1 content (Frontera *et al*, 2000). Thus, evidence for a selective loss of type 2 fibres with age is poor and likely provides no explanation for our results.

A loss of total muscle mass would not explain the results given that relative intensity was equalized between groups; in any case we found little difference in the values of muscle mass in the young and the older men (although we may have overestimated this in the older subjects due to the inability of dual-energy X-ray absorptiometry to detect interfascicular fat and oedema in muscle). However, there was a marked difference in strength between the groups which is most commonly explained as being due to differences in tendon properties and efficiency of excitation–contraction coupling in older subjects (Narici &

Maganaris, 2006). Nevertheless, these variations should not affect muscle protein synthesis per se.

Despite the existence of a blunted response of the exercise-stimulated rate of myofibrillar protein synthesis in the elderly, there were no differences in the shape of its post-exercise time course, or that of anabolic signalling, between the two groups. Therefore, there was no indication of any lag in the responses of the older subjects, as has been reported in a comparison of the post-exercise changes in young and old subjects fed after exercise (Drummond *et al*, 2008). However, it is noteworthy that we observed a fall in myofibrillar protein synthesis between 2 and 4 h after the peak at 1–2 h, which we believe has not been reported before in a full paper. The reason for this fall is puzzling but our exercise stimulus (presumably volume rather than intensity) might not have been sufficient to cause a long-lasting effect; after exercise of a greater volume (8 sets of 8 repetitions at 80% 1 RM) in the post-absorptive state by young subjects, the stimulatory effects lasted for up to 24 h (Phillips *et al*, 1997). The effects of volume of work and total time under tension remain to be investigated. Alternatively the fall might have been due to the lack of amino acid availability as occurs after feeding when the increase in MPS can be sustained for at least 24 h (Cuthbertson *et al*, 2006).

The precise molecular mechanisms by which resistance exercise stimulates myofibrillar protein synthesis remain to be determined but it is highly likely that enhanced phosphorylation of mammalian target of rapamycin (mTOR) and its downstream effectors, 4E binding protein 1 (4EBP1) and p70 ribosomal S6 kinase (p70s6K) are involved (Cuthbertson *et al*, 2005; Deldicque *et al*, 2008; Spiering *et al*, 2008; Terzis *et al*, 2008; Wilkinson *et al*, 2008). Accordingly,

we observed quantitatively similar increases in phosphorylation of both p70s6K and 4EBP1, which were maximal for exercise at 60–90% 1 RM at 1 h post-exercise, i.e. just before the period of maximal increase in myofibrillar protein synthesis, and which were blunted in the older participants. Furthermore, we found for the first time in human muscle a positive correlation between extent of phosphorylation of p70s6K and MPS, albeit only in the young subjects. The extent of p70s6K phosphorylation reportedly predicts the extent of accretion of muscle in rats (Baar & Esser, 1999) and in weight lifters (Terzis *et al*, 2008), but no direct correlation between p70s6K phosphorylation and increases in muscle synthesis have been reported and certainly not in human muscle. This strengthens the support for a major role for p70s6K in stimulating MPS after exercise, and short-term changes in both predict the longer term changes. The lack of such a correlation in the older subjects is consonant with a blunted response of MPS to exercise, and reports that muscle hypertrophy after resistance exercise training is less in older men (Kosek *et al*, 2006). Nevertheless, the fact that both myofibrillar protein synthesis and p70S6 phosphorylation showed identical changes at 60–90% of 1 RM suggests that muscle adaptation may occur with exercise at less than the high intensities commonly assumed to be solely efficacious (Spiering *et al*, 2008).

The changes in 4EBP1 phosphorylation were more complex than those for p70s6K, in particular showing a marked fall in the biopsy taken immediately after exercise, which presumably mostly reflected the state of the molecule during exercise, as thereafter the change was reversed. This fall in 4EBP1 phosphorylation has been observed before (Dreyer *et al*, 2006; Koopman *et al*,

2006) and is likely to be associated with a fall in human muscle protein synthesis during exercise (Dreyer *et al*, 2006; Fujita *et al*, 2008). Although, like that of p70s6K, 4EBP1 phosphorylation showed a peak at 1 h post-exercise (although with much greater variability), and with blunted responses in the older subjects, no significant correlations with myofibrillar protein synthesis could be observed in either group.

We were unable to detect any effects of age or exercise intensity on the extent of phosphorylation of the elongation factor eEF2, which suggests that modulation of elongation at least by phosphorylation of eEF2 at Thr56 in the post-exercise period is of little biological relevance.

In summary, we have shown that acute bouts of resistance exercise at different intensities stimulate myofibrillar protein synthesis and anabolic signalling in a dose-dependent manner in both young and old men in the post-absorptive state. The stimulatory effect of exercise peaks at 1–2 h post-exercise is suppressed, but not delayed in older men. Although the extent of p70s6 kinase phosphorylation predicts the stimulation of myofibrillar protein synthesis in young men, older men appear not to match changes in anabolic signalling and myofibrillar protein synthesis, possibly explaining the deficiency in the muscle protein anabolic response.

**CHAPTER 4: EFFECT OF VOLUME OF RESISTANCE EXERCISE
ON MYOFIBRILLAR PROTEIN SYNTHESIS AND CELL ANABOLIC
SIGNALLING IN POSTABSORPTIVE YOUNG AND OLD MEN**

4.1 SUMMARY

We tested the hypothesis that increasing the volume of resistance exercise would counter the blunted synthetic response in muscle of post-absorptive older men so that the response would resemble those of young subjects. Twelve young (24 ± 6 y, body mass index (BMI) 22 ± 3 kg.m⁻²) and twelve older (70 ± 5 y, BMI 23 ± 4 kg.m⁻²) men were randomized, in two groups of six each, to perform unilateral leg extension exercise at one of two different exercise intensities (40% or 75% of their 1 RM). With a three months interval between studies, each subject performed, first 3 and then 6 sets of 14 repetitions at 40% 1RM or 3 then 6 sets of 8 repetitions at 75% 1RM. Biopsies were taken from the m. vastus lateralis of the exercised leg (1% lignocaine anaesthesia) before, immediately after, and 1, 2 and 4 h post- exercise. Myofibrillar protein synthesis (MPS) was measured by incorporation of [1, 2-¹³C₂]leucine; the phosphorylation of p70 ribosomal S6 kinase (p70S6K) Thr389 and eukaryotic initiation factor 4E binding protein 1 (4EBP1) Thr37/46 were measured using western analysis. In young men, doubling exercise volume at 40% and 75% 1RM produced no additional MPS responses (AUC FSR (% protein/4 h); means \pm SEM; Young 3 vs. 6 sets; at 40% 1RM 0.068 ± 0.02 vs. 0.058 ± 0.02 ; at 75% 0.088 ± 0.03 vs. 0.10 ± 0.03 ; $P>0.05$); however, in older men, it resulted in enhanced MPS (AUC FSR; Old 3 vs. 6 sets; at 40% 1RM 0.03 ± 0.01 vs. 0.10 ± 0.02 ; at 75% 0.064 ± 0.01 vs. 0.098 ± 0.02 ; $P<0.05$) and p70S6K responses (AUC; Old 3 vs. 6 sets; p70S6K at 40% 1RM 0.12 ± 0.05 vs. 0.45 ± 0.18 ; at 75% 1RM 0.20 ± 0.03 vs. 0.39 ± 0.07) at both intensities ($P<0.05$), so that responses were similar to those in young men. We conclude that doubling the volume of resistance exercise enhances the stimulatory effect of exercise on anabolic cell signaling and MPS in muscle of older, but not younger men.

4.2 INTRODUCTION

Most previous work on the response of muscle protein synthesis to resistance exercise of high intensity with a large number of sets/repetitions, e.g. 6 -20 sets with 8-12 repetitions at 70 – 80% 1RM, shows that it is stimulated in both fed and fasted conditions (Biolo *et al*, 1995; Chesley *et al*, 1992; Kim *et al*, 2005; Louis *et al*, 2003; Phillips *et al*, 1997; Sheffield-Moore *et al*, 2005; Tipton *et al*, 2001; Kumar *et al*, 2009; Sheffield-Moore *et al*, 2005; Tipton *et al*, 2001).

However, the relationships between intensity and volume of exercise for the stimulation of muscle protein synthesis is still not clear because in previous studies the effect of volume was not explicitly examined, and many results are the aggregate effects of feeding and exercise together. Furthermore, most earlier work has examined the responses of the synthesis of mixed muscle proteins (Biolo *et al*, 1995; Chesley *et al*, 1992; Phillips *et al*, 1997; Sheffield-Moore *et al*, 2005; Tipton *et al*, 2001) whereas it is now becoming clear that myofibrillar protein responses to exercise are usually relatively greater and sustained for longer than those of sarcoplasmic fractions (Kumar *et al*, 2009; Moore *et al*, 2009; Wilkinson *et al*, 2008).

We have recently shown that an acute bout of resistance exercise at higher intensities (60 – 90% 1RM) increases MPS to a greater extent than that at lower intensities (20 – 40% 1RM) but with a larger response in the younger than the older men in the post-absorptive state (Kumar *et al*, 2009). In that study we kept the volume of exercise and total external work output at different exercise intensities (i.e. % 1RM × number of repetitions × number of

sets) constant, so we remain ignorant of how the volume of exercise influences MPS in either young or old muscle in the post absorptive state.

An acute bout of resistance exercise has been shown to activate the mammalian target of rapamycin (mTOR) and its downstream effectors, p70S6 kinase (p70S6K) and eukaryotic initiation factor 4E binding protein 1 (4EBP1), (Baar & Esser, 1999; Cuthbertson *et al*, 2006; Deldicque *et al*, 2005; Deldicque *et al*, 2008; Drummond *et al*, 2009). Furthermore, the acute increase in the phosphorylation of p70S6K after a bout of resistance exercise seems to be closely related to the increase in MPS and degree of eventual hypertrophy in both rat and human muscle (Baar & Esser, 1999; Kumar *et al*, 2009; Terzis *et al*, 2008). Moreover, provision of rapamycin, a potent mTOR inhibitor, abolishes increases in synthesis in human muscle after resistance exercise, suggesting that mTOR-S6 kinase pathway is closely involved in stimulating MPS (Drummond *et al*, 2009). However, it remains unclear whether downstream signalling proteins such as p70S6K and 4EBP1 are differentially activated in response to resistance exercise of different volumes.

Expanding on our previous work (Kumar *et al*, 2009), our aim was to investigate the effect of two different volumes of exercise, 3 vs. 6 sets of unilateral leg extension, at two different exercise intensities, a lower intensity (40% 1RM) and a higher intensity (75% 1RM), on MPS in healthy young and old men. We purposely decided to study subjects in the post-absorptive state to distinguish the exercise- mediated responses from those coupled to increased nutrient availability (Kumar *et al*, 2009). We hypothesized that (i) in muscle of young men, at a given intensity, there are modest thresholds for responses of

MPS and anabolic signalling beyond which any additional volume of work would not induce further increases in them; and (ii) increasing the volume of exercise would increase the amplitude of the MPS and anabolic signalling responses in lesser sensitive muscle of the older men, thus ameliorating the anabolic blunting observed previously (Chapter 3).

4.3 SPECIFIC METHODS

The study was approved by the University of Nottingham Ethics Committee and complied with the Declaration of Helsinki. Written informed consent was obtained from the subjects after explaining the study procedure and associated risks. All subjects were recreationally active and overall healthy.

Table 4.1 Subjects' characteristics (mean±SEM)

	Young men (n=12)	Older men (n=12)
Age (y)	24±6	70±5*
Weight (kg)	72±11	72±16
Height (m)	1.80±0.07	1.74±0.09
BMI (kg.m ²)	22±3	23±4
Lean Mass (kg)	60±11	52±7
Fat mass (kg)	15±7	19±9
% Body fat	20±10	24.5±8.5
1 repetition maximum (N)	750±225	392±196*
Blood glucose (mM)	4.3±1.0	5.0±0.8

* Significant difference between groups P<0.05

4.3.1 Study Design

Subjects were randomly assigned to perform, at 3 month intervals, first 3 and then 6 sets of an isotonic, full cycle unilateral leg extension and flexion exercise at one of two different exercise intensities (either 40% or 75% of 1 RM (6 per group and per intensity)). The protocols differed only in volume of work (see below). All subjects were studied after an overnight fast. Blood samples and muscle biopsies were taken according to the protocol (Figure 4.1).

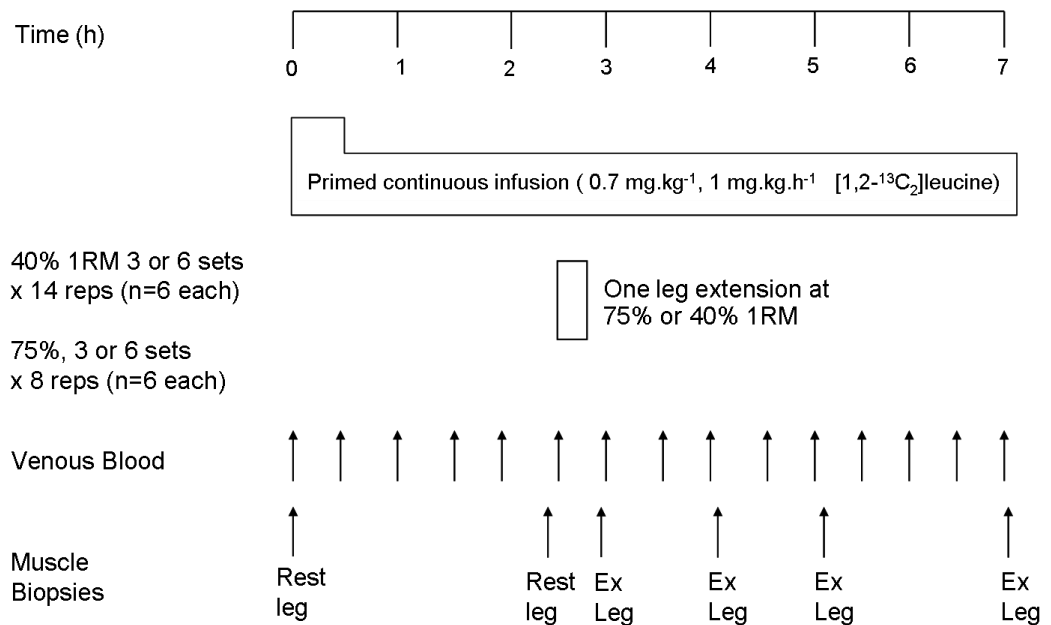


Figure 4.1 Study protocol for the measurement of myofibrillar protein synthesis and muscle anabolic signalling molecule phosphorylation to full cycle unilateral leg extension exercise (3 vs. 6 sets) at 40% or 75% 1RM in post-absorptive young and older men

After taking biopsies at rest at 0 and 2.5 h in the post-absorptive pre-exercise state, the subjects performed full cycle unilateral leg extensions at a moderate contraction velocity (1-2 s concentric, 1-2 s eccentric), with three min rest between sets. Subjects who performed unilateral leg extension at 40% 1-RM subjects completed 3 sets \times 14 repetitions or 6 sets \times 14 repetitions, and those who performed at 75% 1-RM completed 3 sets \times 8 repetitions or 6 sets \times 8 repetitions. After the study, cannulae were removed; the subjects were fed and monitored for half an hour before being escorted home.

4.3.2 Measurement Of Myofibrillar Protein Synthesis And Cell Signalling Phosphorylation

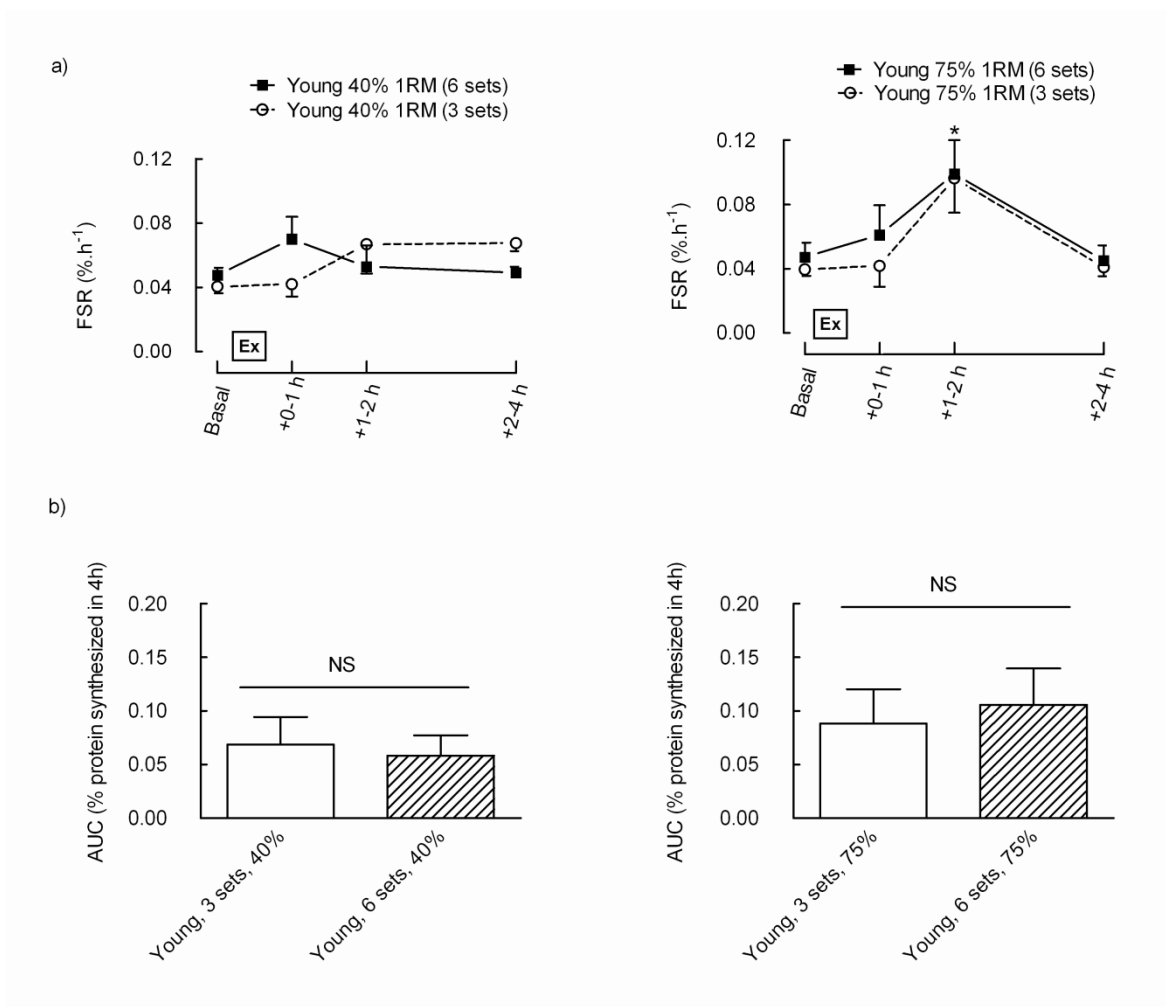
The fractional synthesis rate (FSR) of myofibrillar protein and the extent of phosphorylation of p70s6K1 on Thr389, 4EBP1 on Thr37/46 and the elongation factor eEF2 on Thr56 were determined as described in chapter 2.

4.4 RESULTS

The participants in this study were all healthy, recreationally active individuals with no differences between the groups except age and a diminished 1 RM leg extension force ($P < 0.05$) in the older men (Table 4.1).

As previously reported (Kumar *et al*, 2009) the muscle of the young subjects showed a greater response to exercise at 75% 1RM intensity than after exercise at 40% 1RM (AUC MPS (% protein/4 h); means \pm SEM; young 75% vs. 40%; 0.10 \pm 0.03 vs. 0.058 \pm 0.02; $P < 0.05$) (Figure 4.2a). However increasing the volume of exercise did not supply any additional effect, irrespective of

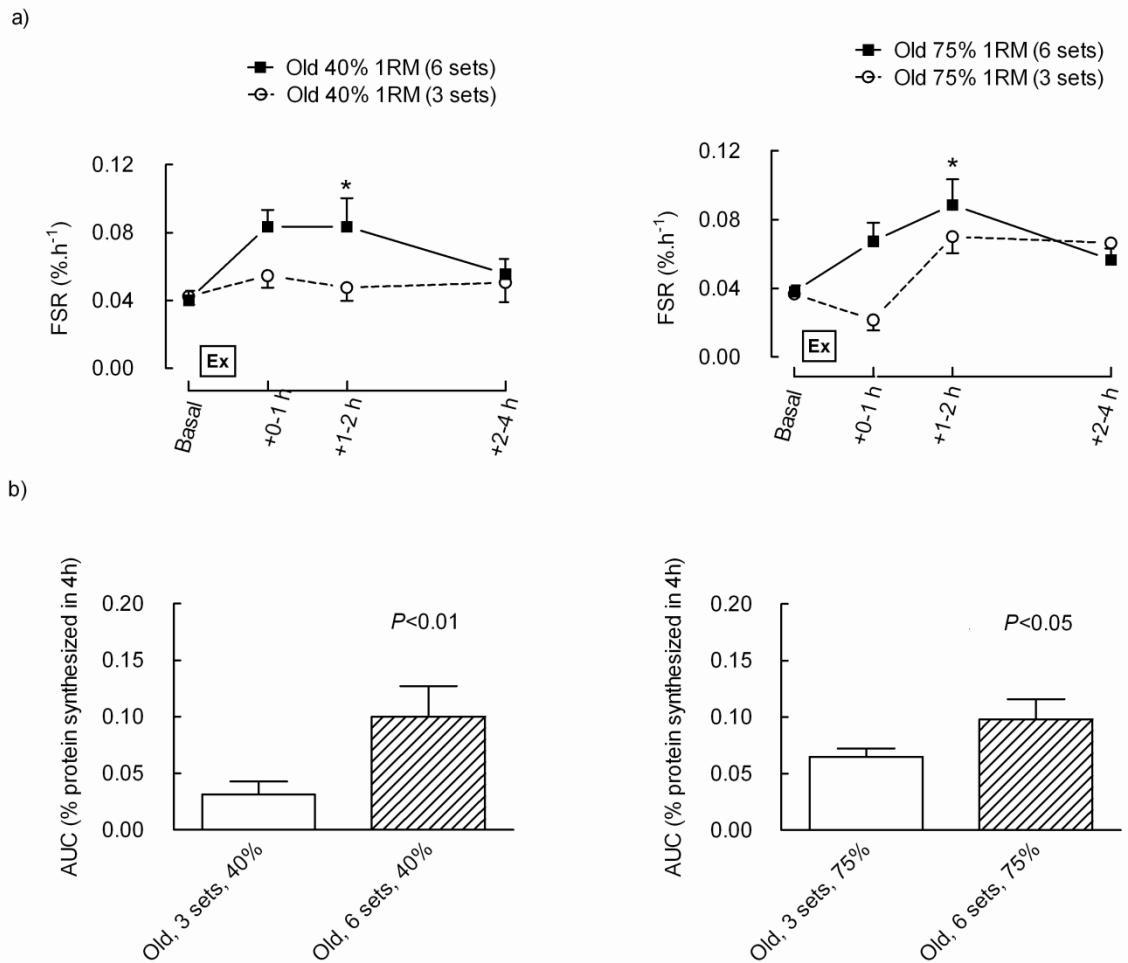
intensity (AUC; young 3 vs. 6 sets; at 40% 1RM 0.068 ± 0.02 vs. 0.058 ± 0.02 ; at 75% 0.088 ± 0.03 vs. 0.10 ± 0.03 ; $P > 0.05$). (Figure 4.2b)



Figures 4.2a and 4.2b

a. Time course of the responses of myofibrillar protein synthesis (FSR, fractional synthetic rate, %·h⁻¹) to 3 or 6 sets of unilateral leg resistance exercise at 40 (left) or 75% (right) 1RM, $n = 6$ young subjects in each group; Values are means \pm SEM; * $P < 0.05$ vs. 40% 1RM and basal FSR at 1-2 h post-exercise (two-way Anova).

b. Areas under the curves (AUC) i.e. % protein synthesized in 4h after 3 or 6 sets of unilateral leg extension exercise at 40% and 75% 1RM in the young subjects; $n = 6$ per group; Values are means \pm SEM. No significant difference exists at different exercise volumes (both $P > 0.05$, 3 sets vs. 6 sets (Student's t test)).



Figures 4.3a and 4.3b

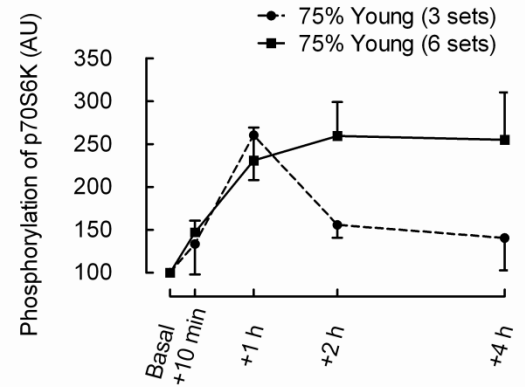
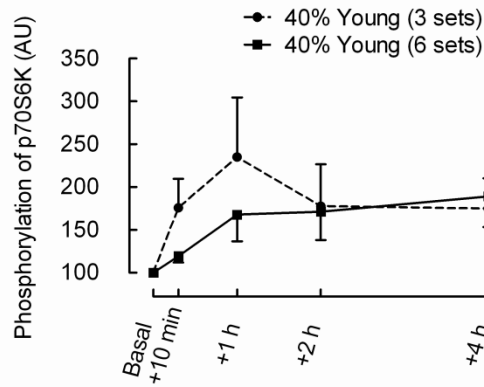
a. Time course of the responses of myofibrillar protein synthesis (FSR, fractional synthetic rate, $\% \cdot h^{-1}$) to 3 or 6 sets of unilateral leg resistance exercise at 40 (left) or 75% (right) 1RM, $n = 6$ older subjects in each group; Values are means \pm SEM; * $P < 0.05$ vs. basal FSR and after 3 sets of exercise (two-way Anova).

b. The areas under the curves i.e. % protein synthesized in 4h after 3 or 6 sets of unilateral leg extension exercise at 40% and 75% 1RM in the older subjects; $n = 6$ per group; Values are means \pm SEM. Both $P < 0.05$, 3 sets vs. 6 sets (Student's t test).

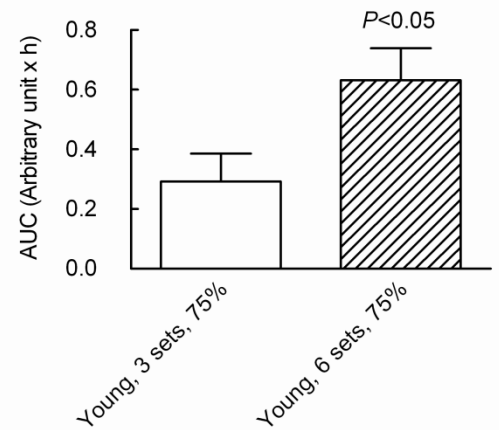
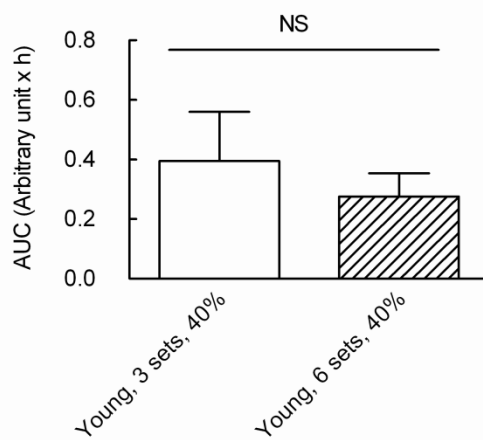
For the older subjects, there were significant differences in the overall responses of post-exercise MPS to resistance exercise at different volumes (Figure 4.3). Increasing the volume of exercise at both 40% and 75% 1RM from 3 to 6 sets significantly enhanced the MPS responses in the older subjects (area under the curves i.e. rate of myofibrillar protein synthesis \times time or % of total myofibrillar protein synthesized in the period; $P < 0.05$ vs. values from 6 sets) (AUC; old 3 vs. 6 sets; at 40% 1RM 0.03 ± 0.01 vs. 0.10 ± 0.02 ; at 75% 0.064 ± 0.01 vs. 0.098 ± 0.02 ; $P < 0.05$). When the time course of post-exercise responses at the greater exercise volume for each workload were compared, there was a faster increase in MPS over the first hour after exercise with this being sustained at 1-2 h post exercise before returning towards near basal values at 2-4 h.

In the young group, there was no significant difference between the phosphorylation responses of p70S6K to resistance exercise at different volumes at 40% 1RM; however increasing exercise volume from 3 sets to 6 sets at 75% 1RM resulted in a sustained elevation of phosphorylated p70S6K in young men (AUC; young 3 vs. 6 sets; at 40% 1RM 0.39 ± 0.16 vs. 0.30 ± 0.07 ; at 75% 1M 0.30 ± 0.09 vs. 0.63 ± 0.10 ($P < 0.05$) (Figures 4.4a and 4.4b).

a)



b)

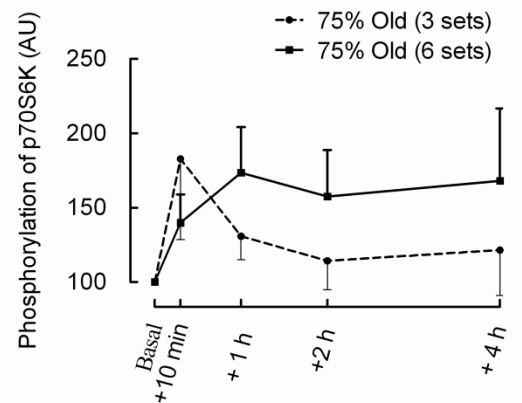
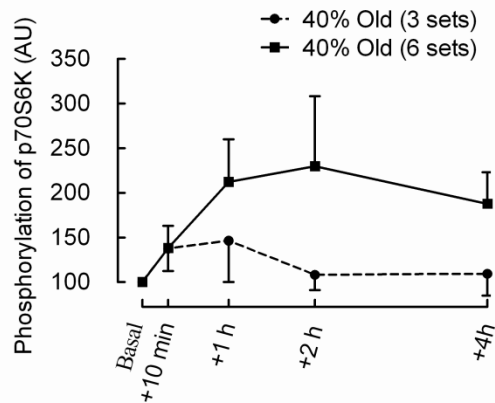


Figures 4.4a and 4.4b

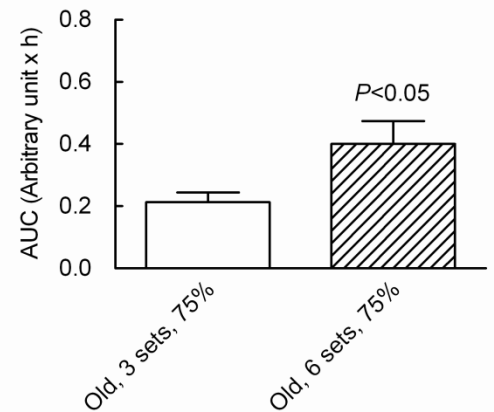
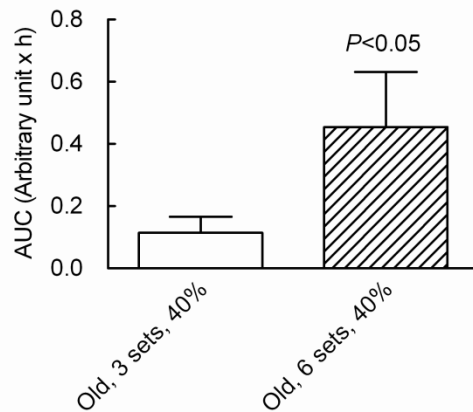
a. Time course of the responses of phosphorylation of p70S6K (arbitrary units (AU) as percentage basal values for each subject) to 3 or 6 sets of unilateral leg resistance exercise at 40 (left) or 75% (right) 1RM, $n = 6$ young subjects in each group.

b. The areas under the curves in 4 h after 3 or 6 sets of unilateral leg extension exercise at 40% and 75% 1RM in the young subjects; $n = 6$ per group; Values are means \pm SEM. The AUC was greater with 6 than 3 sets at 75% 1RM, $P < 0.05$ vs. 3 sets at 75% of 1RM.

a)



b)



Figures 4.5a and 4.5b

a. Time course of the responses of phosphorylation of p70S6K (arbitrary units (AU) as percentage basal values for each subject) to 3 or 6 sets of unilateral leg resistance exercise at 40 (left) or 75% (right) 1RM, $n = 6$ older subjects in each group.

b. The areas under the curves in 4h after 3 or 6 sets of unilateral leg extension exercise at 40% and 75% 1RM in the older subjects; $n = 6$ per group; Values are means \pm SEM. Both $P < 0.05$, 3 sets vs. 6 sets (Student's t test).

The extent of post-exercise p70S6K phosphorylation was significantly higher after 6 sets of exercise in comparison to that after 3 sets of exercise at both intensities in older men (AUC; old 3 vs. 6 sets; at 40% 1RM 0.12 ± 0.05 vs. 0.45 ± 0.18 ; at 75% 1RM 0.20 ± 0.03 vs. 0.39 ± 0.07) ($P<0.05$). The concentration of phosphorylated p70S6K stayed elevated throughout the 4 h post-exercise period after 6 sets, whereas it fell to near basal values at 2 h after 3 sets at both 40% and 75% 1RM ($P<0.05$) (Figures 4.5a and 4.5b).

For 4EBP1, phosphorylation showed a significant fall of about 40% immediately after exercise then rebounded, to basal values by 1-2 h after exercise with no significant differences between the two age groups at different exercise volumes (data not shown).

4.5 DISCUSSION

We report here novel information concerning the responses of MPS in the young and the older men to different volume of resistance exercise at two different intensities in the post-absorptive state. Increasing the volume of exercise from 3 to 6 sets at a given intensity enhanced the post-exercise myofibrillar synthetic responses in the older men whereas it had no additional effect in the young men. The other major finding of the present study was that the phosphorylation of p70S6K was increased after resistance exercise to an extent depending on the exercise volume at higher intensity in the young group, and at both lower and higher intensities in the older group. The existence of a potential explanatory link between the exercise volume and extent of anabolic signalling responses in post-absorptive young and old men is new information not previously available.

In the older men, the myofibrillar synthetic responses are enhanced by doubling the volume of exercise, even at the lower intensity, thereby achieving similar responses to those in young men. It has been previously demonstrated that “anabolic blunting” is a pervasive feature of ageing muscle, which is revealed in a reduced sensitivity to the anabolic effects of feeding (Cuthbertson *et al*, 2005; Guillet *et al*, 2004; Volpi *et al*, 2000; Wilkes *et al*, 2008) as well as exercise (Kumar *et al*, 2009). These lower synthetic responses of older muscle to exercise are associated with its inability to adequately activate mTOR signalling compared with that of young muscle after exercise (Kumar *et al*, 2009). We would therefore expect that the less sensitive muscle of older men would require a greater anabolic stimulus, (i.e. more muscular contractions) to activate the protein synthetic machinery sufficiently to achieve synthetic responses comparable to those seen in younger men. This was indeed what we observed.

According to Henneman's size principle, during low intensity muscular contraction, slow twitch fibres with small motor units are primarily recruited, whereas increasing muscular force gradually recruits increasing numbers of type II fibres (Henneman *et al*, 1965). However, several workers have shown that in hypoxic or fatiguing conditions early recruitment of type II fibres can occur with the effect of maintaining the muscular force during low-intensity resistance exercise (Moritani *et al*, 1992; Takarada *et al*, 2000). In comparison to young muscle, older muscle has decreased exercise capacity (i.e. reduced fatigue resistance - for reviews see (Doherty, 2003; Vandervoort, 2002) and a higher number of repetitive muscular contractions even at low intensity (40% 1RM) causes increased fatigue, an associated larger metabolic stress (Takarada

et al, 2000; Tanimoto & Ishii, 2006) and likely the additional recruitment of type II fibres. These observations may explain the stimulation of MPS in the older group at a lower intensity but with no effect in young muscle, in which fewer type II muscle fibres are likely recruited by increasing the exercise volume at 40% 1RM (Kraemer *et al*, 1993; Spiering *et al*, 2008).

Surprisingly, MPS was not enhanced in young muscle after exercise at a higher volume at 75% 1RM despite increased p70S6K phosphorylation. Therefore, it may be that young muscle has a lower threshold for protein synthetic responses to exercise (Kumar *et al*, 2009). Nevertheless increased exercise-stimulated MPS cannot be sustained, presumably due to insufficient amino acid availability in the postabsorptive state.

In conclusion, we have found that in young men doubling the volume of exercise at 40% and 75% 1RM had no additional effect on the response of MPS in the post absorptive state; however, in older men, it resulted in faster response with greater amplitude at both intensities. The results suggest that there is increased latency of response to exercise in muscle of older men and increasing the volume of exercise enhances the stimulatory effect of resistance exercise on MPS in them, even at relatively low intensities i.e. 40% of 1RM.

Although, it still remains unclear how long term resistance exercise of lower intensities with higher volume would affect muscle synthetic responses and muscle mass in the elderly, the findings of this work have implications for the exercise recommendations for elderly people, and the frail elderly in particular, for whom muscle maintenance is of crucial importance and who may not be

capable of undertaking high intensity exercise, (>80% 1RM): they would clearly benefit from doing moderate intensity exercise with a higher volume of exercise in order to maintain their muscle mass.

**CHAPTER 5: EFFECT OF POST RESISTANCE EXERCISE LEUCINE
ENRICHED PROTEIN SUPPLEMENTATION ON MYOFIBRILLAR
PROTEIN SYNTHESIS AND CELL ANABOLIC SIGNALLING IN YOUNG
AND ELDERLY MEN**

5.1 SUMMARY

The muscle anabolic responses to postabsorptive resistance exercise (RE) are less in older (O) than younger (Y) men. We hypothesized that amelioration of this would occur by supplementing a protein drink (SlimFast Optima, SFO, 10 g protein and 24 g carbohydrate) with leucine (Leu), taken immediately after RE. Three groups (n=9) of healthy young (24±6 y, BMI 23±2 kg.m⁻²) and older men (70±5 y, BMI 25±2 kg.m⁻²) were randomized to (i) RE alone, (ii) RE followed by 325 ml SFO with 4.2 g of leucine, (iii) RE+SFO with 4.2 g of alanine (Ala), as control. Subjects performed 6×8 repetitions full-cycle unilateral leg RE at 75% of 1-RM. Also, a 4th group of older men (70±5 y, BMI 25±2 kg.m⁻² n=9) ingested SFO+4.2 g leucine at rest. Muscle biopsies were taken before, immediately after, and 1, 2 and 4 h after RE or SFO. MPS was measured by incorporation of [1, 2-¹³C₂]leucine and the phosphorylation of p70S6K by Western analysis. In young men, SFO+leucine after resistance exercise stimulated ($P<0.05$) MPS more than SFO+alanine (AUC MPS (% protein/4 h) Y 0.15±0.016 vs. 0.125±0.013) and more than exercise alone (AUC: Y 0.15±0.016 vs. 0.09±0.03); in old men, SFO+leucine after resistance exercise stimulated MPS ($P<0.05$) more than SFO+alanine (AUC: O 0.14±0.01 vs. 0.11±0.01) and more than exercise alone (AUC: O 0.14±0.01 vs. 0.09±0.001), and more than SFO+leucine at rest (AUC: O 0.14±0.01 vs. 0.09±0.01); in older men, postexercise SFO+leucine caused responses of MPS after resistance exercise as great as those in the young men (Y 0.15±0.016 vs. O 0.14±0.01). In older but not younger men, SFO+Leu enhanced post-RE p70S6K phosphorylation above that after RE alone (AUC P70s6K (% increase/4 h): O, 0.50±0.05 vs. 0.26±0.11; Y, 0.60±0.14 vs. 0.56±0.40). Combining RE with SFO+leucine rejuvenates the time-averaged responses of MPS in the older men, mimicking responses in young men.

5.2 INTRODUCTION

Ingestion of protein (Rasmussen *et al*, 2002; Smith *et al*, 1998; Rennie *et al*, 2002; Svanberg *et al*, 1996; Volpi *et al*, 2003) or performance of resistance exercise (Chesley *et al*, 1992; Phillips *et al*, 1997) stimulate muscle protein synthesis and anabolic signaling (activation of m-TOR signaling pathway) responses in both young and elderly muscle. However, the muscle synthetic responses to acute resistance exercise in the fasted state (Kumar *et al*, 2009) or after feeding (Guillet *et al*, 2004; Katsanos *et al*, 2005; Volpi *et al*, 2000; Cuthbertson *et al*, 2005; Katsanos *et al*, 2006) are blunted in elderly muscle compared to that in younger muscle. Furthermore, it has recently been shown that the effect of insulin (15 μ IU/ml) on muscle protein breakdown as well as on Akt-PKB activation is blunted in older muscle (Wilkes *et al*, 2009). Since basal muscle protein turnover in the post-absorptive condition in healthy old people is found to be similar to rates seen in young muscle, these blunted muscle synthetic responses of elderly muscle seem to be key factors in the aetiology of sarcopenia (Cuthbertson *et al*, 2005; Volpi *et al*, 2001). Therefore stimulation of these blunted synthetic responses in elderly would be a useful intervention to prevent or slow the progression of sarcopenia.

Post-exercise ingestion of nutrients (EAA+CHO) has been clearly shown to elevate MPS above that measured following resistance exercise alone in both young and elderly (Borsheim *et al*, 2004; Koopman *et al*, 2005; Miller *et al*, 2003; Rasmussen *et al*, 2000; Biolo *et al*, 1995; Tipton *et al*, 1999) and this effect is most likely due to amino acids alone (Bennet *et al*, 1989; Smith *et al*, 1992) and particularly leucine (Smith *et al*, 1992) without much influence of insulin (Bohe *et al*, 2003; Cuthbertson *et al*, 2005). Furthermore, recent studies have shown that the attenuated muscle protein synthetic and anabolic signalling responses to food intake at rest in the

elderly can be compensated by increasing the leucine concentration of a meal (Rieu *et al*, 2006; Katsanos *et al*, 2006). Taken together, it seems that the ingestion of leucine enriched EAA supplementation following an acute bout of resistance exercise can significantly enhance the MPS and may provide an effective strategy to combat sarcopenia in the elderly.

Therefore, the aim was to assess the impact of leucine enriched protein ingestion after an acute bout of resistance exercise on myofibrillar protein synthesis and anabolic signalling, particularly activation of the mTOR signalling pathway, in post-absorptive young and older subjects. We hypothesised that the age-related blunting of muscle synthetic responses to feeding and exercise in elderly could be overcome by combining both leucine enriched protein supplementation and resistance exercise of optimal intensity and volume (based on data from chapter 3 and 4).

5.3 SPECIFIC METHODS

The study was approved by the University of Nottingham Ethics Committee and complied with the Declaration of Helsinki. Written informed consent was obtained from the subjects after explaining the study procedure and associated risks. It was unlikely that anyone would agree to participate in all three exercise studies (explained below) required because of the 6 biopsies required in each study, so we recruited separate groups of participants through newspaper and web advertisements. Three, groups of 9 healthy, young and older men were recruited for the exercise \pm nutritional intervention studies plus an extra group of 9 older men of the same characteristics for the studies involving ingestion of protein drink and leucine capsules without preceding exercise (Subject characteristics, see Table 5.1). All subjects were recreationally active, physically independent and healthy overall.

Table 5.1 Subjects' characteristics (mean \pm SEM)

	Young men (n =27)	Older men (n=36)
Age (y)	24 \pm 6	70 \pm 5*
Weight (kg)	75 \pm 10	76 \pm 10
Height (m)	1.79 \pm 0.05	1.74 \pm 0.05
BMI (kg.m ²)	23 \pm 2	25 \pm 2
Lean Mass (kg)	59 \pm 7	54 \pm 4
Fat mass (kg)	13 \pm 5	19 \pm 8
% Body fat	17 \pm 6	25 \pm 9
1 RM (N)	683 \pm 171	392 \pm 111*
Blood glucose (mM)	4.6 \pm 0.5	5.0 \pm 0.4

* Significant difference between groups $P < 0.05$

5.3.1 Study Design

3 groups each (n=9) of young and elderly subjects were randomly assigned to study protocols involving (i) resistance exercise (RE) alone; (ii) RE + 325 ml SlimFast Optima (SFO) with 4.2 g of Leucine (Leu) (RT+SFO+Leu); (iii) RE+SFO with 4.2 g of alanine (Ala) (RE+SFO+Ala) as control. All subjects performed 6 sets of 8 repetitions of an isotonic, full cycle unilateral leg extension and flexion exercise at 75% of 1 RM (exercise regimen based on data from chapter 3 and 4). A further 9 older subjects were studied at rest, who took SlimFast Optima (SFO) with 4.2 g of Leucine (Rest+SFO+Leu) without preceding resistance exercise.

All subjects were studied after an overnight fast. They were asked to refrain from any heavy exercise for 72 h before the study day. Blood samples and muscle biopsies were taken according to the protocol (Figure 5.1).

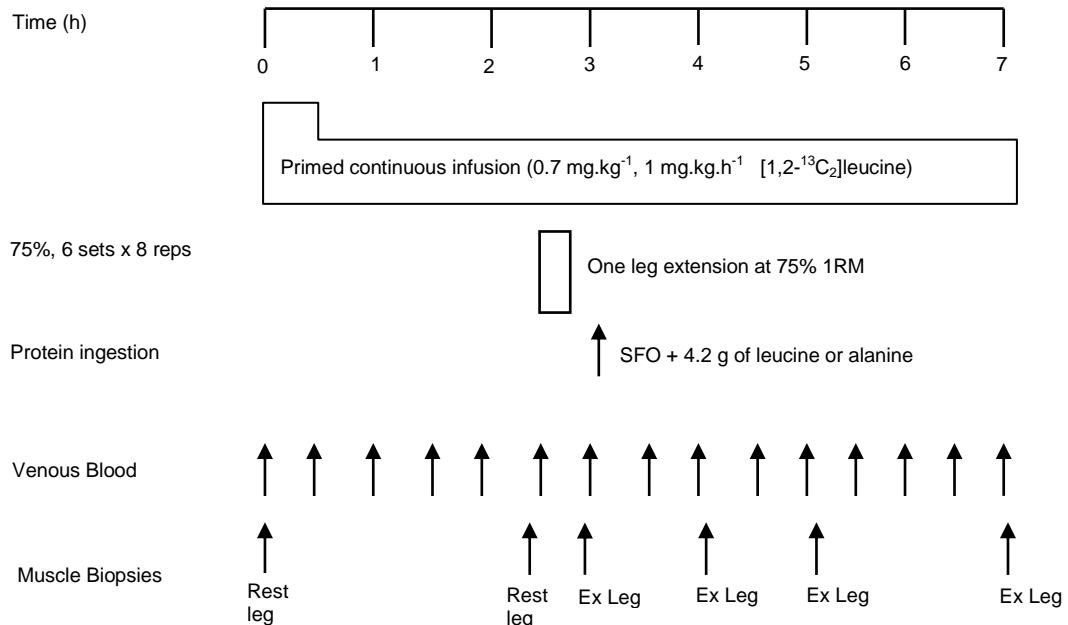


Figure 5.1 Study protocol for the measurement of myofibrillar protein synthesis and muscle anabolic signalling phosphorylation to full-cycle unilateral leg extension exercise at 75% 1RM followed by the ingestion of Slim-Fast Optima (SFO) with 4.2 g leucine or alanine in post-absorptive young and older men ($n=9$). NB 9 older men were studied at rest consuming SFO and 4.2g leucine without preceding exercise.

A primed, continuous infusion (0.7 mg.kg^{-1} , 1 mg.kg.h^{-1}) of $[1, 2-^{13}\text{C}_2]$ leucine tracer (99 Atoms %, Cambridge Isotopes Limited, Cambridge, MA, USA) was started (at 0 h) after the first biopsy and maintained until the end of the study. After taking

biopsies at rest at 0 and 2.5 h in the post-absorptive pre-exercise state, the subjects performed full-cycle unilateral leg extensions at a moderate contraction velocity (1-2 s concentric, 1-2 s eccentric), with three min rest between sets. After the exercise, each subject took first 4.2 g of alanine or leucine capsules and then SFO at 30 min intervals (on the basis of feeding optimization studies described below). Subjects in the rest group first took 4.2 g of leucine capsules and then SFO at 30 mins interval following their 2nd muscle biopsy. Muscle biopsies were taken from the m. vastus lateralis under sterile conditions using the conchotome biopsy technique with 1% lignocaine as local anaesthetic.

After the study, cannulae were removed; the subjects were fed and monitored for half an hour before being escorted home.

5.3.2 Composition Of The EAA + CHO Drink And Optimization Of Feeding Timing

Each subject in the study received a full can (325 ml) of SlimFast Optima (10 g protein + 24 g CHO; protein and EAA composition: 8g casein, 2g whey, 0.05g soy, 1.05 g leucine, 0.36 g alanine, 0.64 g isoleucine and 0.73 g valine) and 4.2 g of leucine or alanine (as control for the leucine) capsules. Preliminary studies were undertaken to: (i) determine the time- course of the rise in blood amino acids after consumption of a can of SlimFast Optima, and particularly the timing of the peak amino acid concentration in blood; (ii) determination of the time-course of the rise of leucine concentration in the blood after consuming gelatine capsules containing 4.2 g of leucine; (iii) adjusting the timing of ingestion of the leucine capsule in relation to the SlimFast Optima, and ensuring that the peak amino acid concentration coincided, thereby determining the post-exercise feeding schedule.

These studies were performed on three young volunteers (n=3; Age 24±6 y; BMI 24±4), who took part in all three studies. In each case an 18 G cannula was inserted into an antecubital vein of the post-absorptive volunteer at ~0900 and a basal blood sample was taken. Then subjects ingested either, (i) a full can (325ml) of SlimFast Optima; or (ii) 4.2 g of leucine alone; or (3) 4.2 g leucine capsules followed by SlimFast Optima at 30 min interval (estimated from the difference in peak amino acid concentrations from study (1) and (2) (figure 5.2) to confirm coincident appearance in the blood. Blood was drawn over 2.5 h at 20 min intervals into Li Heparin tubes, plasma separated immediately and analyzed for amino acids using an ion-exchange amino acid analyser (Biochrom) (see chapter 2). The results are shown below (figure 5.2).

5.3.3 Measurement Of Myofibrillar Protein Synthesis And Cell Signalling Phosphorylation

The fractional synthesis rate (FSR) of myofibrillar protein and the extent of phosphorylation of p70s6K1 on Thr389, 4EBP1 on Thr37/46, Akt on Ser473 and the elongation factor eEF2 on Thr56 were determined as described in chapter 2.

5.4 STUDY RESULTS

The participants in this study were all healthy, recreationally active individuals, well matched with no differences between groups except for age and a diminished 1 RM leg extension force ($P < 0.05$) in the older men (Table 5.1).

5.4.1 Optimization Of Feeding Timing Results

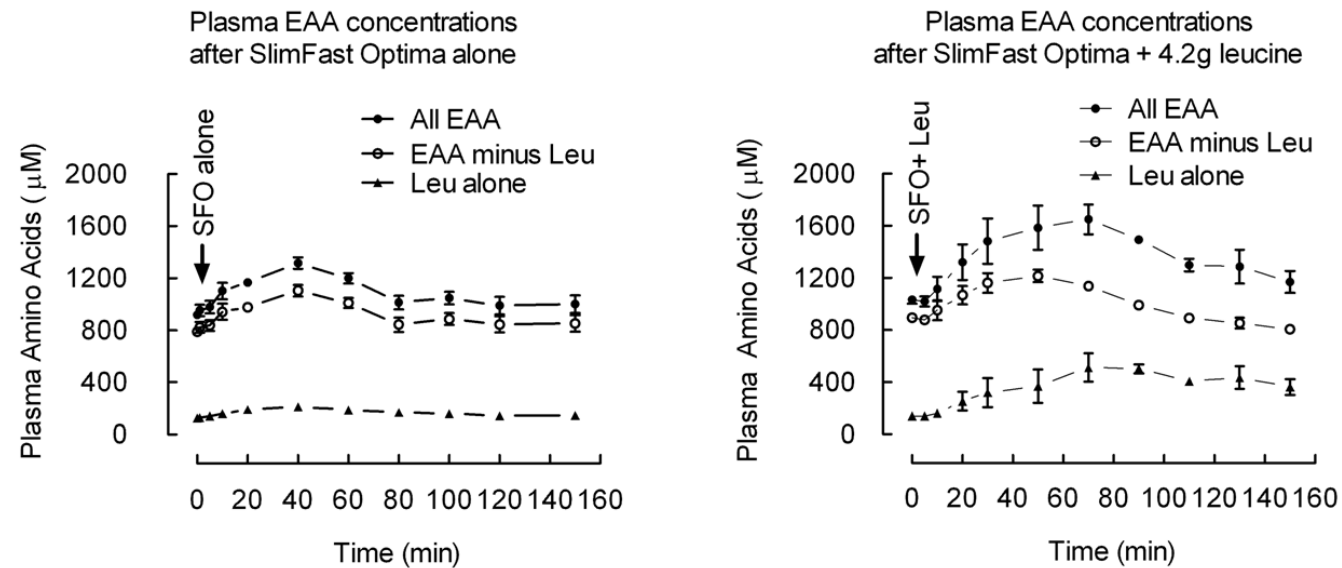


Figure 5.2 Concentrations of essential amino acids (total or with leucine subtracted) or leucine in plasma after drinking 325 ml of SlimFast Optima without (left) or with (right) 4.2 g of leucine taken in a gelatine capsule 30 min before the SlimFast Optima. Values are means \pm SEM for n = 3. In some cases the error bars are within the symbols. The results clearly show a perfect superimposition of the peak of essential amino acid concentrations and a boosting of the total essential amino acid concentrations when leucine was given.

5.4.2 Post RE Plasma EAA After Slimfast Optima Ingestion With 4.2 g Of Leucine In Young And Older Men

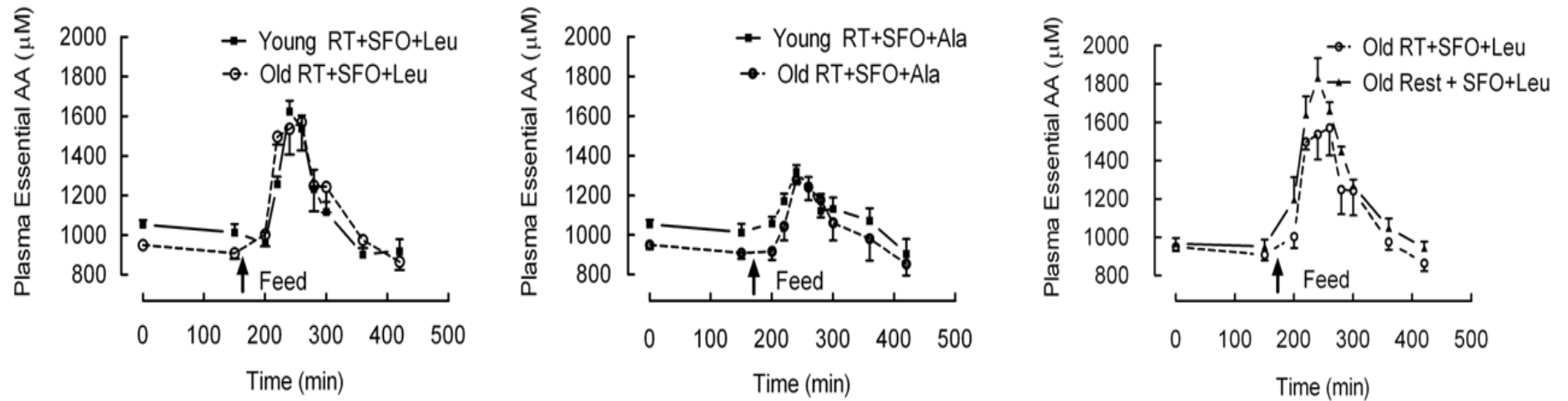
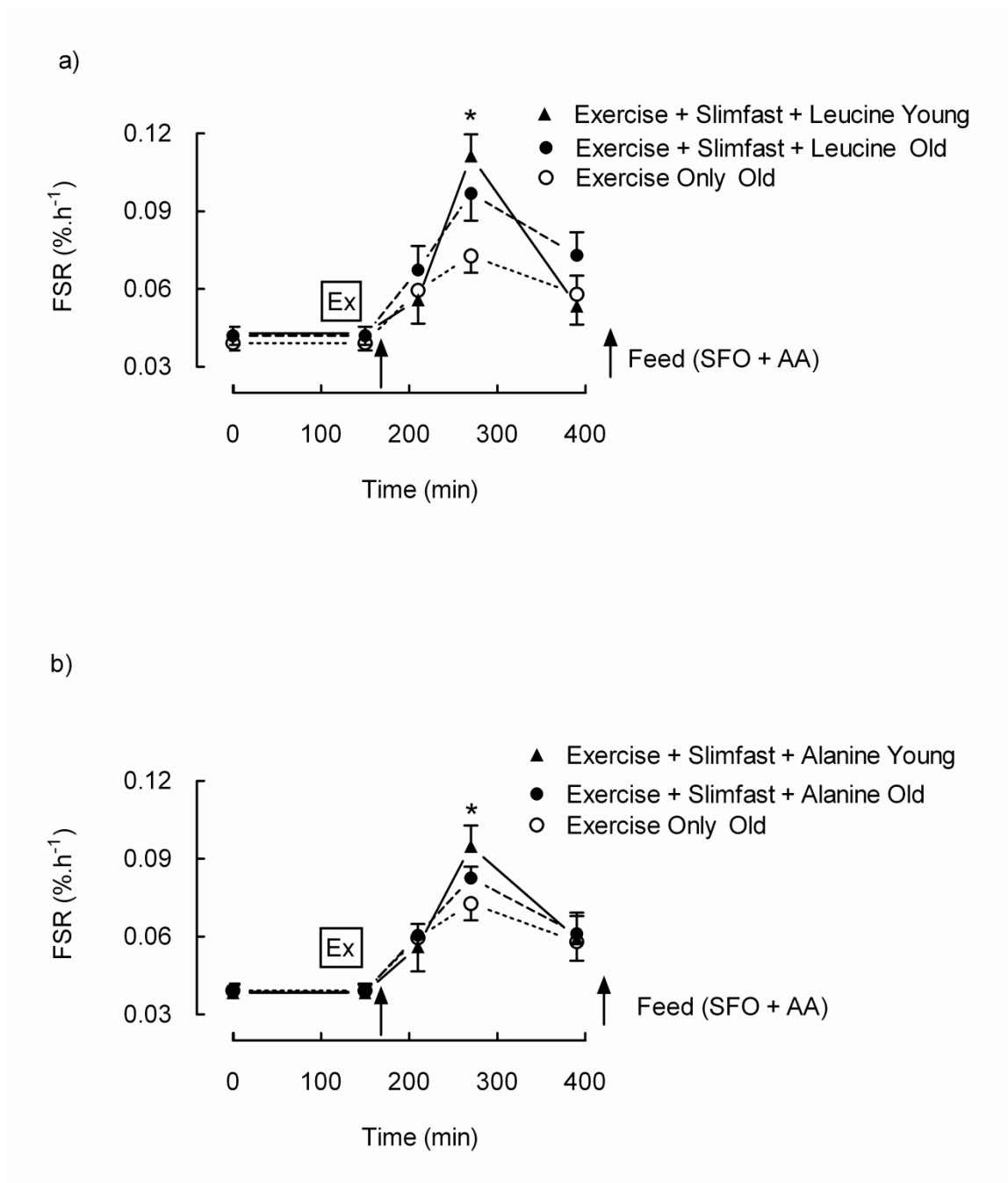


Figure 5.3 Plasma essential amino acid concentrations after 6×8 repetitions unilateral leg extension exercise at 75% 1RM in older and young men (RT) after SlimFast Optima supplemented with leucine (left) (RT+SFO+Leu), alanine (middle) (RT+SFO+Ala) or in older men at rest (Rest+SFO+Leu) and after exercise (RT+SFO+Leu) with SlimFast Optima supplemented with leucine (right). The results clearly show higher plasma essential amino acid concentration after SFO with 4.2 g leucine than after alanine supplementation following resistance exercise and the time course of this rise was similar in both young and older group. The plasma essential amino acid concentration in older men at rest (Rest+SFO+Leu) was slightly higher but statistically not significantly different ($P>0.05$) than those observed after exercise (RT+SFO+Leu) with SlimFast Optima supplemented with leucine.

5.4.3 Myofibrillar Protein Synthesis (MPS)

**Figure 5.4 a and b**

Time course of the responses of myofibrillar protein synthesis (FSR, fractional synthetic rate, %·h⁻¹) to 6 sets of unilateral leg resistance exercise at 75% 1RM in older men with or without SlimFast Optima plus leucine (top) or alanine (bottom) and in young men after resistance exercise with SlimFast Optima + leucine (top) or alanine (bottom). n = 9 young and old subjects in each group; Values are means ± SEM. Responses in young and older exercise only group were not significantly different from each other (therefore, young exercise only data not displayed on the figure for the sake of clarity). * *P* < 0.05 from basal FSR.

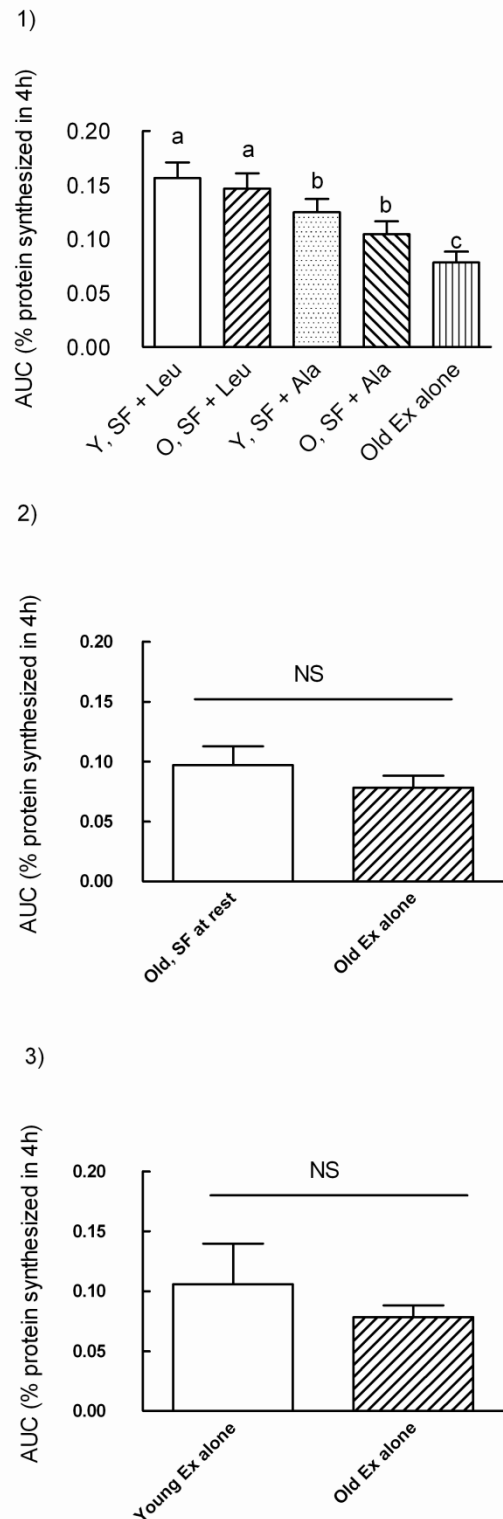


Figure 5.5

1) Areas under the curves (AUC) i.e. % protein synthesized in 4h after 6 sets of 8 unilateral leg extension exercise at 75% 1RM in older men with or without SlimFast Optima plus 4.2 g

leucine or alanine and in young men after resistance exercise with SlimFast Optima + 4.2 g leucine or alanine. n = 9 young and old subjects in each group; Values are means \pm SEM; a, b, c significant difference in response between the groups $P < 0.05$ (Student's t test).

2) AUC i.e. % protein synthesized in 4h after ingestion of SlimFast Optima + 4.2 g leucine with or without 6 sets of 8 unilateral leg extension exercise at 75% 1RM in older men. n = 9; Values are means \pm SEM; NS, no significant difference in responses between the groups $P > 0.05$ (Student's t test).

3) AUC i.e. % protein synthesized in 4h in young and old after 6 sets of 8 unilateral leg extension exercise at 75% 1RM alone; n = 9 young and old subjects in each group; Values are means \pm SEM; NS, no significant difference in response between the groups $P > 0.05$ (Student's t test).

On examination of the responses of MPS: 1) in young men, RE+SFO+Leu stimulated ($P<0.05$) MPS more than RE+SFO+Ala (AUC MPS (% protein/4 h) Y 0.15 ± 0.016 vs. 0.12 ± 0.013) and more than exercise alone (AUC: Y 0.09 ± 0.03); 2) in old men, RE+SFO+leucine stimulated MPS ($P<0.05$) more than RE+SFO+Ala (AUC: O 0.14 ± 0.01 vs. 0.11 ± 0.01) and more than exercise alone (O 0.09 ± 0.001), and more than Rest+SFO+leu (AUC: 0.09 ± 0.012); 3) in older men, RE+SFO+leucine caused responses of MPS as great as those in the young men (Y 0.15 ± 0.016 vs. O 0.14 ± 0.01) ($P>0.05$); 4) in older men, MPS following RE+SFO+Leu did not returned to baseline at 4 h as seen in other groups therefore the net positive balance (effect of feeding over ex alone) was probably even greater as it lasted beyond the 4 h; 5) the MPS responses in old Rest+SFO+Leu group were not statistically significantly different to those seen in exercise only group (AUC: 0.09 ± 0.012 vs. 0.09 ± 0.001 ; $P>0.05$).

5.4.4 Muscle Cell Anabolic Signalling

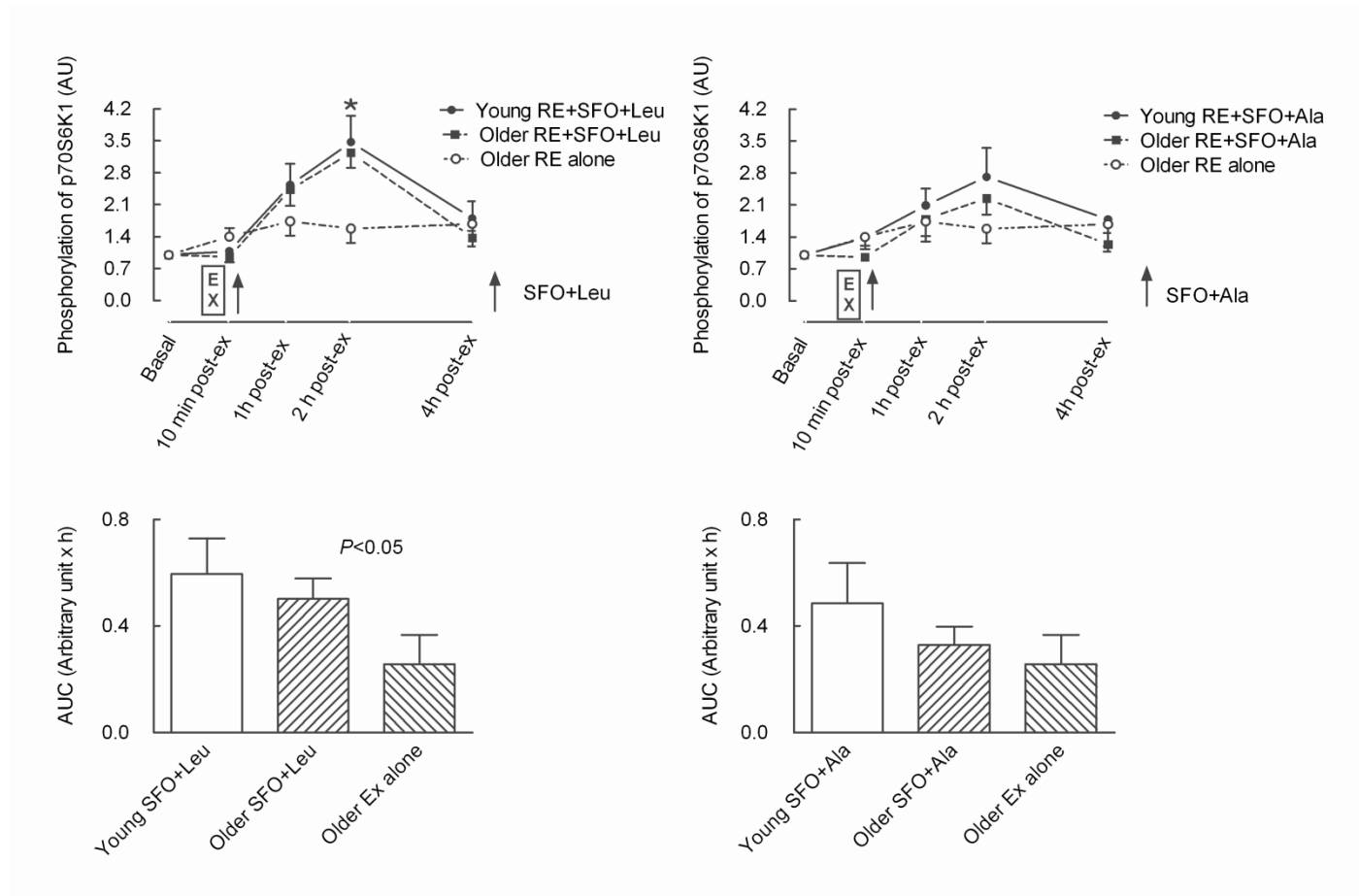


Figure 5.5 Time course and AUC of p70S6K1 phosphorylation in 4 h to resistance exercise at 6×8 repetitions at 75% 1RM in older with or without SlimFast Optima plus 4.2 g leucine or alanine and in young men after resistance exercise with SlimFast Optima + 4.2 g leucine or alanine. $n = 9$; * $P < 0.05$ in older RE+SFO+Leu group.

We measured the degree of phosphorylation of a large variety of signalling molecules (eEF2^{Thr56}, p70S6K, 4EBP1 and Akt-PKB; data not shown) but the clearest results came from the measurement of p70S6kinase signalling protein. This showed clearly that SlimFast Optima supplemented with leucine rejuvenated the responses of the older men to be significantly enhanced ($P < 0.05$), so that they were not different from those of the young (AUC P70s6K (% increase/4 h): O, 0.50 ± 0.05 vs. Y, 0.60 ± 0.14), but markedly better than those seen after resistance exercise alone (AUC P70s6K (% increase/4 h): O, 0.50 ± 0.05 vs. 0.26 ± 0.11 ; Y, 0.60 ± 0.14 vs. 0.56 ± 0.20). Supplementing SFO with alanine had no significantly enhanced effect on p70S6K phosphorylation in elderly from that after RE alone (0.32 ± 0.06 vs. 0.26 ± 0.11).

5.5 DISCUSSION

This study has provided new physiological and nutritional information, that it is possible, through exercise and leucine ingestion, to rejuvenate the acute MPS and muscle cell anabolic signalling responses in older men to a level at which they are no different from those in younger men. Specifically, ingestion of 325 ml of CHO + PRO drink (10 g protein) containing 5.2 g of leucine in total, immediately after an acute bout of RE at 75% 1RM markedly enhanced acute MPS and p70S6K1 responses of the older men, raising their rates to values similar to those of the young, when compared to those with SFO + Ala containing 1 g leucine.

Several studies have highlighted the importance of combining RE and amino acid supplementation to maximize the MPS response and shown that consuming essential amino acids (Rasmussen *et al*, 2000; Moore *et al*, 2009b; Tipton *et al*, 1999; Biolo *et*

al, 1997) after resistance exercise augments the contractile induced stimulation of MPS. Furthermore, Dreyer et al showed that leucine-enriched EAA+CHO ingestion following an acute bout of resistance exercise enhanced both mTOR signaling and mixed muscle protein synthesis in human beings when compared to those following exercise without nutrition (Dreyer *et al*, 2008). However, only young men were studied in the study and the effect of additional free leucine supplementation on postexercise MPS response was not specifically examined. To our knowledge, this is the first study reporting a comparison of the time course of acute changes in MPS and p70S6K responses after RE and the provision of the amino acids, in particular the branched-chain amino acid, leucine in both young and old men. In addition, the results also show the ‘on and off’ nature of muscle protein synthetic machinery to anabolic stimuli and that we can measure these acute changes over very short periods.

Previously we looked at the effect of intensity of leg extension exercise at constant exercise volume and showed that an acute bout of resistance exercise at higher intensities (60 – 90% 1RM) increases MPS in the post-absorptive state over 4-h post-exercise to a greater extent than that at lower intensities (20 – 40% 1RM) but with a larger response in young than older men (Kumar *et al*, 2009). Then we examined the effect of volume of exercise at constant exercise intensity and found that increasing the volume of exercise from 3 to 6 sets at 75% 1RM enhanced the post-exercise myofibrillar synthetic responses in older men whereas it had no additional effect on them in young men in postabsorptive state (Kumar et al, chapter 4). Taken together, we purposely decided to use exercise protocol using 6 sets of leg extension exercise

at 75% 1RM to maximally stimulate muscle synthetic responses to exercise in both young and elderly subjects.

We purposely decided to give about 20 g of protein (SFO + capsules) to our subjects in both leucine and alanine groups after the resistance exercise as Moore et al (Moore *et al*, 2009a) has recently shown in six healthy young men that ingestion of 20 g intact protein (or about 8.6 g EAAs) was sufficient to maximally stimulate MPS after resistance exercise and excess dietary protein consumed after exercise stimulated irreversible oxidation with no further increase in protein synthesis.

Recently, Katsanos et al showed that ingestion of 6.7 g of an EAA mixture containing 41% leucine (1.7 g over a 3.5 h period) was more effective in stimulating MPS rates when compared to responses with EAA mixture containing only 26% leucine in elderly and producing similar synthetic responses to those seen in young muscle (Katsanos *et al*, 2006). Also Rieu et al showed that co-ingestion of 3 g leucine with protein, carbohydrate and fat over a 5 h period further improved MPS in elderly men (Rieu *et al*, 2006). This ability of leucine to independently stimulate MPS in elderly indicates that the leucine in high proportion should maximally stimulate the postexercise MPS in elderly, that's what indeed we observe in our present study.

The present data is in contrast with recently published study by Koopman et al (Koopman *et al*, 2008), who showed that co ingestion of leucine with carbohydrate and protein (4.7 g leucine vs.17.6 g leucine over a 6h period) following physical activity did not further elevate MPS in elderly men. Whole body protein balance was

2.8% greater ($p < 0.05$) in higher leucine group. The apparent discrepancy could possibly be explained by the fact that in the present study, MPS responses were measured at regular intervals (at 1, 2 and 4 h) after exercise + nutritional intervention, where MPS rates showed a faster rise and peaked over the 1-2 h post exercise before showing a downwards trend at 2-4 h. However, in the Koopman study (Koopman *et al*, 2008) MPS was measured only at 6h post exercise, thereby missing this peak of MPS rise, thereby giving almost identical synthetic MPS responses.

Regarding signalling proteins, it has been shown that the leucine supplementation in resting conditions as well as following resistance exercise enhance MPS via activating insulin-dependent and as well as insulin-independent mTOR pathway signalling proteins (Anthony *et al*, 2002; Karlsson *et al*, 2004; Kimball & Jefferson, 2001; Norton & Layman, 2006; Proud, 2004). Recently, Atherton *et al* showed that leucine exclusively, amongst all other essential amino acids, stimulates anabolic signalling in skeletal muscle cells through the distinctive stimulation of mTOR, 4EBP1 and p70s6K1 phosphorylation (Atherton *et al*, 2010). Accordingly, we observed quantitatively similar increases in phosphorylation p70s6K, which were maximal at 2 h post-exercise + nutritional supplementation in all groups, however it was significantly enhanced ($P < 0.05$) in the old RE+SFO+Leu group, so that it was not different from those of the young, and markedly better than those seen after resistance exercise alone. This enhanced response of p70S6K in the old RE+SFO+Leu group could explain their maximal increase in MPS.

Despite the existence of a blunted response of the exercise-stimulated rate of myofibrillar protein synthesis in the elderly in postabsorptive state, in present study we observed enhanced MPS response in the old RE+SFO+Leu group, almost identical to those seen in the young. Interestingly MPS was still elevated at 4 h after the exercise in this group which is different to their responses at exercise alone and feeding alone, thus highlighting the potential of combining RE with leucine enriched protein supplementation to counteract sarcopenia.

In conclusion, the results show that it is possible to rejuvenate the MPS and muscle cell anabolic signalling responses in older men to become identical to those in younger men after exercise by giving leucine enriched protein drink immediately after exercise.

CHAPTER 6: SUMMARY AND GENERAL DISCUSSION

6.1 Overall summary of results

6.1.1 Effect of exercise intensity on MPS

The results from this study showed a sigmoidal dose-response relationship of myofibrillar protein synthesis to exercise intensity, with little increase from 20-40% 1RM, and a bigger (2- fold at 1-2 h post exercise) rise at 60 % of 1 RM with no significant further increase up to 90% 1RM in both postabsorptive healthy young and old men. Older men compared to young subjects demonstrated a blunted rise in MPS after exercise at different work intensities, suggesting a general pattern of reduced protein synthesis in response to exercise in the elderly. This blunted response to exercise may be important in the progression of age-related sarcopenia. The molecular mechanism of this ‘anabolic blunting’ in older people involves reduced mRNA translation, as evidenced by lower activation of mTOR signalling pathway proteins associated with mRNA translation initiation, specifically p70S6K and 4EBP1.

6.1.2 Effect of resistance exercise volume on MPS

Increasing exercise volume from 3 to 6 sets at 40% and 75% 1RM produced no additional MPS response in young men; however, in older men, MPS was enhanced as was p70S6K phosphorylation status at both intensities, demonstrating that the muscle of older men requires a greater volume of exercise to activate the protein synthetic machinery sufficiently to obtain synthetic responses comparable to those seen in younger men. On the basis of these results, the “double volume” protocol i.e. 6 sets at 75% of 1RM was chosen to test objective 3.

6.1.3 Effect of feeding a leucine enriched protein – carbohydrate mixture (SFO) after resistance exercise on MPS

On examination of the responses of MPS and anabolic signalling the results reveal that (i): in young men, SFO+leucine after exercise stimulated MPS and signalling more than SFO+alanine and more than exercise alone; (ii): in older men, SFO+leucine after exercise stimulated MPS and signalling more than SFO+alanine and more than exercise alone, and more than SFO+leucine at rest, but most striking, (iii): in older men, SFO+leucine elicited responses of MPS and anabolic signalling after resistance exercise as great as those in the young men. In other words, ingestion of SFO+leucine immediately post exercise rejuvenated the post exercise responses of the older men to mimic those seen in the younger men.

6.2 General Discussion

The findings of the work provide evidence that the human skeletal muscle protein synthetic response to exercise in the post absorptive state as well as with feeding are, as hypothesized, specific to intensity, the volume of exercise in the post absorptive state and to the leucine content of a protein meal, and that this response is altered with ageing.

6.2.1 How robust are the findings

The sample size for stage 1 and 2 was determined on the basis of data from previous muscle metabolism studies of healthy young men aged 20-30 and healthy men aged 65-75 (Louis *et al*, 2003; Cuthbertson *et al*, 2006). For repeated measures of muscle protein labelling in the same sample, taken through the entire extraction and analysis process, the coefficient of variation (CV) is ~3.8%; CV of leucine metabolites in

blood is ~5%. The population CV is ~10% for young men and ~12% for the elderly men. A power calculation to discover the smallest number of subjects needed (taking a population variance of 12%), studied using laboratory techniques with a combined CV of 15%, to detect (with 80% confidence at the 5% significance level) a cross-sectional difference (between two groups) or a one-way difference of 20% (on a paired basis) suggested we needed 6 subjects per group. Subjects recruited to study 1 were allocated to respective trial conditions using a fractional replication approach. This method balanced the combination of pair trials (each subject completed 2 trials) to prevent trial order bias (i.e. one subject completed exercise at 20% and 60% intensity, then next subject at 60% and 20% intensity) and subject intensity bias (i.e. one subject completed a high and low intensity, rather than two high or two low).

The final calculation of sample size ($n = 9$ per group and treatment), and the statistical methods and tools employed to analyze the data in study 3 were largely determined following analysis of results data from the first two studies, which provided the biological data necessary to calculate the sample size needed to enable the detection of differences of 20% in MPS and signalling responses after exercise and feeding with 80% confidence at the 5% significance level.

6.2.2 Measurement of 1RM

The 1 repetition maximum of all subjects was confirmed by asking the subjects to perform a 1RM at the previously determined weight following an interval of 30 minutes during the screening visit (or 2-3 days after the screening visit). For correct determination of the 1RM, the variation of scores between the two assessments had

to be less than 5% (Wilkinson *et al*, 2008). In the present work, no more than three attempts were required for all subjects to accurately determine the 1RM.

6.2.3 *Exercise protocol*

In contrast to most previous work on the response of muscle protein synthesis to resistance exercise, in which the exercise protocol involved a greater number of sets e.g. 6 -20 sets at 70 – 80% 1RM, in fasted conditions (Phillips *et al* 1997; Biolo *et al* 1995), I used an exercise schedule in which subjects performed only three sets of exercise at various intensities (Chapter 3). So the question arises whether the training stimulus in both young and older groups was strong enough to stimulate MPS effectively? The schedule of contractions at different intensities was designed to equalize, as closely as possible, the volume of exercise, i.e. the force \times time-under-tension product (often described as ‘work’). Thus, at an exercise intensity of 20% of 1 RM, the subjects completed 3 sets \times 27 reps; at 75%, 3 sets \times 8 reps and e.g. those at 90%, 6 sets \times 3 reps. Initial pilot studies using various exercise protocols revealed that the young healthy untrained volunteers were unable to perform adequately exercise at 90% 1RM than above mentioned exercise schedule or more than 6 sets optimally (i.e., same range of leg extension and flexion 2 s each) at any given intensity. Therefore, in order to keep the total work output (i.e. % 1 RM \times number of repetitions \times number of sets \times time-under-tension) similar at different exercise intensities and to extend this work to investigate the effect of doubling the volume of exercise on muscle synthetic responses, I chose to use 3 and then 6 sets (Chapter 4) of exercise so that even older subjects could complete the exercise protocol optimally. Although the exercise design did not reflect typical training behaviour, it

was not designed to do this, but rather to discover the acute effect of age and exercise intensity and volume on muscle synthetic responses.

6.2.4 *Muscle biopsies*

Each acute study involved the subject fasting overnight and throughout the duration of the study and also involved six serial muscle biopsies. Hypothetically prolonged fasting (Essen *et al*, 1992) and serial muscle biopsies from the same area of vastus lateralis of quadriceps muscle, possibly producing local inflammation (Lang *et al*, 2007), could decrease muscle protein synthesis. Therefore, each muscle biopsy from the quadriceps muscle in a subject was taken 3 cm apart in a rhomboid pattern rather than parallel to each other. Furthermore, it has been shown that extended fasting up to 18 h including five muscle biopsies from the same muscle did not affect basal mixed MPS in human subjects (Volpi *et al* 2008).

6.2.5 *Stable isotope [1,2-¹³C₂]leucine tracer to measure MPS*

The method using [1,2-¹³C₂]leucine is considered the method of reference (Matthews *et al*, 1980) for studying protein and amino acid metabolism in human subjects and has been subsequently validated (Rennie *et al*, 1982; Halliday *et al*, 1988; Nair *et al*, 1988; McNurlan *et al*, 1994).

There has been a lot of discussion in the literature on which amino acid pool best reflects of the precursor pool for protein synthesis during continuous-infusion studies (Garlick *et al*, 1991; Rooyackers & Nair, 1997; Watt *et al*, 1991). It is unquestionably the intracellular amino acyl-tRNA pool, but it is technically difficult to measure the enrichment in that pool. The plasma venous α -KIC enrichment is an acceptable alternative in the case of the [1,2-¹³C₂]leucine tracer (KIC, the

transamination product of leucine, is formed intramuscularly and closely reflects the intracellular leucine labelling which can also be used as the surrogate precursor, but can only be measured when muscle is sampled, whereas the KIC labelling can be determined easily throughout the study) and its use has been validated in human muscle (Watt *et al*, 1991). The ratio of venous KIC: leucine (~0.85) remains constant in the postabsorptive state and α -KIC is underestimated by ~15% in fasted state. However, in the fed state this discrepancy (venous KIC: leucine (~ 1) becomes less as all the pools become closer due to the fact that feeding inhibits breakdown and therefore the dilution is less (Vazquez *et al*, 1986).

Despite all of its associated problems, this technique has become the most widely adopted method has been successfully applied in our laboratory to investigate human muscle protein turnover (Rennie *et al* 1982; Louis *et al* 2003).

6.2.5 *Postprandial vs. postabsorptive state*

Resistance exercise does not lead to a positive AA balance when measurements are made in the postabsorptive state (Biolo *et al*, 1995). However, I deliberately chose to separately investigate the effects of exercise from the effects of feeding as there is confusion in the literature concerning the magnitude and time course of the effects of exercise alone and those due to exercise and feeding. Thereby resolving the question of how much exercise alone contributes to the anabolic effect. The results clearly demonstrate that feeding and resistance exercise act synergistically to increase MPS and lead to greater short term muscle synthetic responses than that achieved by food or exercise alone in older men.

Previously, it has been shown that the stimulatory effect of exercise in both the postabsorptive and fed states lasted for up to 24 or 48 h in the young (Chesley *et al*, 1992; Phillips *et al*, 1997). Interestingly, we observed a return to basal rates in myofibrillar protein synthesis after 4 h following the peak at 1–2 h in young group in both fasted as well as fed exercise studies. The only study documenting similar short term time course of muscle synthetic responses to exercise and feeding (Moore *et al* 2009), showed that in the rested leg myofibrillar protein synthesis returned to basal values at 5 h after consumption of 25 g of whey protein in healthy young men. The reason for the return to basal rates is puzzling, however one can speculate that the MPS response might be biphasic (falling at 4-5 h and then rising again) i.e., a latent “readiness” of synthetic apparatus to be reawakened for at least 24 h post exercise and up to 72 h (AA might be used up initially to synthesize new proteins from the intracellular AA pool after an anabolic stimulus and then second wave of AAs is provided from plasma or recycling of AA from MPB). Furthermore, timing of the biopsies may play an important role with respect to determining the timing of peak rates and duration of the responses. Although the time course, but not the magnitude, of muscle protein synthesis rate over 4 h post exercise period were almost identical in the young groups in all studies; whether this trend persists beyond 4 h post exercise is unclear.

6.2.6 Signalling responses

Alterations in the activity of several signalling molecules (eEF2, Akt-PKB and mTOR) involved in translation initiation were unable to explain the differential protein synthetic responses between young and old men. Furthermore, there was a mismatch between the change in the phosphorylation status of signalling proteins

which activate the translational machinery and the rise in MPS in both exercise + feeding studies in both older and young groups. Indeed, recent work has revealed that alteration in signalling protein phosphorylation can be almost completely unconnected from protein synthesis with a stimulus such as insulin, exercise or feeding (Greenhaff *et al*, 2008; Drummond *et al*, 2009; Glover *et al*, 2008). It is possible that due to the timing of muscle samples correlative changes in the phosphorylation status of the target phosphoproteins measured were missed, or perhaps there are other phosphorylation sites (that were not quantified) or very likely there exist other signalling proteins such as mammalian vacuole protein sorting 34 (mVsp34) are involved in regulating the exercise \pm feeding-induced responses (Mackenzie *et al*, 2009).

6.3 Implications in practical terms

There are obvious implications of this work in respect of exercise and dietary protein recommendations for healthy elderly people.

The fact that with training intensities above 60% 1 RM, the muscle synthetic response to training does not increase further is good news for elderly, particularly for frail elderly who may have difficulty in coordinating strength training with high workloads that may present a risk of injury. The data indicate that they can safely train at more moderate loads (40% 1 RM) performing 6 sets of 14 repetitions and optimally stimulate MPS by ingesting a leucine supplemented protein-carbohydrate drink (containing 5g of leucine and a modest 10 g of protein). Further investigation of the effect of chronic exercise training (e.g. for 20 weeks) using lower exercise intensities and higher volume to provide evidence about the optimal resistance

exercise regime to obtain maximal beneficial effects i.e. muscle mass and strength gain (or reduced rates of muscle loss) to help counteract the debilitating nature of sarcopenia is warranted. Furthermore, the optimisation of training regimes may also have beneficial effects in counteracting the muscle wasting that accompanies a number of acute and chronic disease conditions i.e. cancer and cardiac cachexia, sarcopenic obesity, diabetes and cirrhosis to name but a few.

6.4 Future directions

6.4.1 Effects of exercise on muscle synthetic responses

As the volume of exercise at different intensities, i.e. the force \times time-under-tension, was kept constant in the study, we remain ignorant of differential responses of human muscle protein synthesis to workload until failure at lower and higher intensities.

Using indirect measures of chronic muscle protein synthetic responses such as muscle strength, previous work has shown a positive relationship between the effect of chronic resistance training of different intensities (20%, 50% and 80% 1RM) and muscle strength increases in the older adults (de Vos *et al*, 2005); however, it still remains unclear how long term resistance exercise of different intensities or volume would affect the muscle protein synthetic responses, cell anabolic signalling and gain of muscle mass in the elderly.

6.4.2 *Effect of leucine enriched feeding coupled with optimal resistance exercise on muscle synthetic responses*

It also remains unclear how long term leucine supplementation of feeding coupled with optimal resistance training would affect chronic muscle synthetic responses and cell anabolic signalling in healthy elderly. Provision of low energy value protein drinks supplemented with leucine may prove to be an important method of maximizing the response to exercise and consequently maintaining muscle mass and function as we age.

6.4.3 *Muscle protein breakdown*

Although the alterations in rate of MPB are often adaptive responses to alterations in the rate of MPS, and change acutely in response to a bout of resistance exercise (i.e. depressed during exercise and elevated after resistance exercise) nonetheless a study involving measurement of both arms of muscle protein turnover in response to resistance exercise of varying intensities or duration with or without leucine supplemented protein feeding would be important in determining the net muscle protein balance.

6.4.4 *Exercise responses in women*

Women constitute 50% of general population; however research related to measurement of muscle protein turnover in women is sparse. Recent findings have suggested the sex related differences in muscle anabolic responses to exercise and feeding (as discussed in chapter 1.10). Furthermore, the rate of sarcopenia is accelerated in postmenopausal women suggesting there might be a relationship between deficiencies of ovarian hormones i.e. oestrogen and the rate of loss of

muscle mass. Conducting similar studies in postmenopausal women or patients post bilateral oophorectomies would help uncover potentially important gender differences.

6.6.5. *Molecular targets*

It is well recognized that the mTOR signaling pathway is very important in controlling skeletal MPS however the signalling pathways are extremely complex with many overlapping and redundant aspects. Therefore further studies are required to fill the gaps in understanding the critical molecular steps involved in the acute exercise-induced regulation of muscle protein synthesis or training induced muscle hypertrophy. Much more work is required to uncover the precise role of signalling proteins such as ERK, hVps34, TSC complex, Rheb, REDD1 and REDD2 that regulate muscle synthesis and breakdown and ultimately control the adaptive response, i.e. muscle hypertrophy or increased mitochondrial biogenesis.

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Conference presentations

- I. Anabolic resistance of muscle maintenance in the elderly: an effect extending to the effects of exercise and insulin action; poster presentation; British Society for Research on Ageing meeting, Brighton, UK; 15/07/08 BSRA 17, P47 (2008).
- II. Differential responses to exercise at 40% and 75% of 1 repetition maximum of myofibrillar protein synthesis and anabolic signaling in muscle of postabsorptive young and old men; oral presentation; meeting of The Physiology Society, Cambridge, UK; 17/07/08 Proc Phys Soc C53,P37.(2008) <http://www.physoc.org/custom2/publications/proceedings/archive/article.asp?ID=Proc%20Physiol%20Soc%2011C53>
- III. Time course and dose responses of myofibrillar protein synthesis and intracellular signaling to resistance exercise between 20 and 90% 1 repetition maximum in young and elderly in the postabsorptive state; poster presentation; American Physiology Society meeting, Hilton Head, South Carolina, USA ; 27/09/08 APS 28.18 P64. (2008)
- IV. Effect of doubling the volume of resistance exercise on myofibrillar protein synthesis (MPS) in postabsorptive young and old men; oral presentation; The Physiology Society meeting, Dublin, Ireland; 08/07/09 University College Dublin Proc Physiol Soc 15, C38 (2009) <http://www.physoc.org/custom2/publications/proceedings/archive/article.asp?ID=Proc%20Physiol%20Soc%2015C38>
- V. Leucine supplementation overcomes age-related anabolic blunting of myofibrillar protein synthesis and p70S6K phosphorylation after resistance exercise in skeletal muscle of older men; oral presentation: Joint Meeting of the Scandinavian and German Physiological Societies in Copenhagen; 27/03/10 – 30/03/10
- VI. Strategies for overcoming the anabolic resistance of muscle protein synthesis (MPS) and muscle protein breakdown (MPB) to essential amino acids (EAA), insulin and exercise in the elderly; selected for an oral presentation: The 2010 IANA (International Academy on Nutrition and Aging) Albuquerque, Hyatt Regency Tamaya, Santa Ana Pueblo, New Mexico, USA; July 26-27, 2010

Prizes and awards received

1. The University of Nottingham Endowed Postgraduate Prize 2009/10
2. The University of Nottingham McCallaun Research Prize 2010

Appendix

1 General Chemicals

Urease, o-phenylenediamine, Dowex resin (50W X-8, H⁺ form, 100-2 mesh) and the N-methyl-tert-butylsilyltrifluoroacetamide (MTBSTFA) for GC-MS derivatization were obtained from Sigma-Aldrich, Poole, Dorset, UK.

Universal indicator and pyridine for GC-MS derivatization were obtained from Sigma Poole, Dorset. The GC-MS (EC-1) capillary column was obtained from Alltech (Carnforth, Lancashire, UK) and the GC-C-IRMS, (DB1701) capillary column, was obtained from J & W Columns (Agilent Technologies, South Queensferry, West Lothian, UK).

2 Preparation of Urease

Urease was prepared fresh as required. Approximately 10 mg urease was washed free of contaminating amino acids in 2 ml of 70% ethanol and centrifuged at 6000 x g for 1 min at room temperature. The supernatant was discarded and the urease re-suspended in distilled deionised water ready for use.

3 Preparation of o-phenylenediamine (OPD)

0.15% of o-phenylenediamine (OPD) (Sigma) in 4N HCL was prepared fresh as required by dissolving 20 mg of OPD in 12 ml of 4N HCL. The bottle was then wrapped in foil to prevent photolysis and stored at 4 degree prior to use.

4 Preparation of Dowex resin for cation-exchange chromatography

The resin underwent extensive pre-washing to remove contaminating amino acids. Firstly it was washed with an excess of 2M NH₄OH to elute the amino acids, washed with DDW until neutral, then it was washed with 1M HCl to prime ready for use. Prior to use it was stored in ddW as slurry.

School of Biomedical Sciences
University of Nottingham
Derby City General Hospital



Title: Effects of resistance exercise (intensity and duration) and nutritional supplementation on muscle protein synthesis and cell anabolic signalling in old and young men

Name of Investigators:

Professor M. J. Rennie

Dr K. Smith

Dr V Kumar

Dr V P Rao

Dr E. A. Wilkes

Healthy Volunteer's Consent Form

Please read this form and sign it once the above named or their designated representative, has explained fully the aims and procedures of the study to you

- I voluntarily agree to take part in this study.
- I confirm that I have been given a full explanation by the above named and that I have read and understand the information sheet given to me which is attached.
- I have been given the opportunity to ask questions and discuss the study with one of the above investigators or their deputies on all aspects of the study and have understood the advice and information given as a result.
- I agree to the above investigators contacting my general practitioner to make known my participation in the study where relevant.
- I agree to comply with the reasonable instructions of the supervising investigator and will notify him immediately of any unexpected unusual symptoms or deterioration of health.
- I authorise the investigators to disclose the results of my participation in the study but not my name.
- I understand that information about me recorded during the study will be kept in a secure database. If data is transferred to others it will be made anonymous. Data will be kept for 7 years after the results of this study have been published.
- I authorise the investigators to disclose to me any abnormal test results.
- I understand that I can ask for further instructions or explanations at any time.
- I understand that I am free to withdraw from the study at any time, without having to give a reason for withdrawing.

- I confirm that I have disclosed relevant medical information before the study.
- I shall receive an inconvenience allowance of £ 100 per acute study. If I withdraw from the study for medical reasons not associated with the study a payment will be made to me proportional to the length of the period of participation, but if I withdraw for any other reason, the payment to be made, if any, shall be at the discretion of the supervising investigator.
- I have not been a subject in any other research study in the last three months which involved: taking a drug; being paid a disturbance allowance; having an invasive procedure (e.g. venepuncture >50ml, endoscopy) or exposure to ionising radiation.
- I confirm that I have not been exposed to more than 5 mSv of ionising radiation in the last 12 months.

Name: **Date of Birth:**

Address:

Telephone number:

Signature: **Date:**

I confirm that I have fully explained the purpose of the study and what is involved to:

.....

I have given the above named a copy of this form together with the information sheet.

Investigators Signature: **Name:**

Study Volunteer Number: