The Specificity of Training Adaptation in Skeletal Muscle

A thesis submitted in fulfilment of the requirements for the Doctorate of Philosophy

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Abstracts

1. **Camera D.M**, Edge J, Short M.J, Hawley J.A, Coffey V.G. Time Course of Akt signalling events following resistance and aerobic exercise in human skeletal muscle. Oral presentation given at: Australian and New Zealand Association for the Advancement of Science Conference, April 18 2010, Melbourne, Australia

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4. **Camera D.M**, West D.W, Burd N.A, Phillips S.M, Garnham A.P, Hawley J.A, Coffey V.G. Interaction of muscle glycogen availability and nutrition on cell signalling and myofibrillar protein synthesis following resistance exercise. Oral presentation given at: European College of Sports Science Conference July 6 – 9 2011, Liverpool, England

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research programme; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged, and ethics procedures and guidelines have been followed.

Signed:

Donny M. Camera

Date: _____

Acknowledgements

"It's not so much the journey that's important; as is the way that we treat those we encounter and those around us, along the way"

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Abbreviations

ACC	acetyl-CoA carboxylase
AMP	adenosine monophosphate
AMPK	5’ adenosine monophosphate kinase
AS160	AKT substrate of 160 kDa
ATP	adenosine triphosphate
BCAA	branch chain amino acid
CaN	calcineurin
CaMK	calmodulin kinase
Ca^{2+}	calcium
cDNA	complementary DNA
CHO	carbohydrate
CK	casein kinase
c-miRNA	circulating microRNA
Con	concentric
COX	cytochrome C oxidase
CP	creatine phosphate
CREB	cAMP response element binding protein
CSA	cross sectional area
Ct	threshold cycle
CYC	cycle

DNA	deoxyribose nucleic acid
DTT	dithiothreitol
Dvl	dishevelled
dw	dry weight
EAA	essential amino acid
Ecc	eccentric
EDTA	ethylene-diamine-tetra-acetic acid
eEF	eukaryotic elongation factor
eIF	eukaryotic initiation factor
ERK	extra-cellular regulating factor
ERR	estrogen-related receptor alpha
ES	effect size
FABPpm	fatty acid binding protein plasma membrane
FAD	flavin adenine dinucleotide
FAK	focal adhesion kinase
FAT/CD36	fatty acid translocase/Cluster of Differentiation 36
Foxj3	forkhead/winged helix transcription factor 3
FoxO	Forkhead box O
FSR	fractional synthetic rate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GβL	G beta L protein

GC-IRMS	gas chromatography isotope ratio mass spectrometry
GCMS	gas chromatography mass spectrometry
Glut4	glucose transporter 4
GS	glycogen synthase
GRB	growth factor receptor bound protein
GSK	glycogen synthase kinase
HDAC	histone deacteylase
HCl	hydrochloric acid
HFB	heptafluorobutyric
HPLC	high-performance liquid chromatography
HSP	heat shock protein
hVps34	human vacuolar protein sorting 34
IC	intracellular
IGF	insulin-like growth factor
IGFR	insulin-like growth factor receptor
IκB	inhibitor of NFκB
IKK	IκB kinase
IL	interleukin
IRS	insulin receptor substrate
JAK	janus kinase
JNK	c-Jun N-terminal kinase

KE	knee extension
kJ	kilojoule
LAT1	L-type amino acid transporter 1
LE	leg extension
LKB1	liver kinase B1
LOW	low muscle glycogen concentration
LP	leg press
MAFBx	muscle atrophy F box protein
MAPK	mitogen activated protein kinase
MEF	myocyte enhancer factor
MEK	mitogen activated kinase
MGF	mechanical growth factor
miRNA	microRNA
MITO	mitochondrial
mLST8	mammalian lethal with Sec13 protein 8
MPB	muscle protein breakdown
MPS	muscle protein synthesis
MRF	myogenic regulatory factor
mRNA	messenger ribose nucleic acid
mSIN1	mammalian stress-activated protein kinase interacting protein 1
mTOR	mammalian target of rapamycin

MuRF	muscle ring finger protein
MYO	myofibrillar
myomiRs	muscle-specific microRNA
MyoG	myogenin transcription factor
MyoD	myogenic differentiation transcription factor
NAD/NADH	nicotinamide adenine dinucleotide/hydroxide
NFAT	nuclear factor of activated T-cells
NFκB	nuclear factor kappa enhancer binding protein
NORM	normal muscle glycogen concentration
NRF	nuclear respiratory factor
NUT	nutrient
PA	phosphatidic acid
PDH	pyruvate dehydrogenase
PDK1	3’ phosphoinositide-dependent protein kinase 1
PDK4	pyruvate dehydrogenase kinase 4
PGC-1α	peroxisome proliferator activated receptor gamma co-activator-1 alpha
PI3K	phosphatidylinositol-3-OH kinase
PKB	protein kinase B
PLA	placebo
PLD	phospholipase D
PMSF	phenylmethanesulfonylfluoride

PPAR	peroxisome proliferator activated receptor
PRAS40	proline-rich AKT substrate 40
PRO	protein
Proctor1	protein observed with Rictor1
p70 S6K	ribosomal protein p70 S6 kinase
Rag	ragulator
RAPTOR	regulatory associated protein of mTOR
REDD	regulated in development and DNA damage responses
REX	resistance exercise
Rheb	Ras homologue enriched in brain
Rictor	rapamycin-insensitive companion of mTOR
RM	repetition maximum
RPM	revolutions per minute
ROS	reactive oxygen species
rpS6	ribosomal protein S6
RSK	ribosomal S6 kinase
RT-PCR	real time-polymerase chain reaction
S	squat
SDS	sodium dodecyl sulphate
SE	standard error
SEM	standard error mean

SHP	Sarcoma oncogene homology 2-domain-contain protein tyrosine phosphatase
SIRT	sirtuin
SLC7	solute carrier family 7
SNAT	sodium-coupled Neutral Amino Acid Transporter
SOS	son of sevenless
SRF	serum response factor
STARS	striated muscle activator of Rho signalling
S6K	ribosomal protein S6 kinase
TBS	tris-buffered saline
TBST	tris-buffered saline with tween
TCA	tricarboxylic acid
TFAM	mitochondrial transcription factor A
TNFα	tumor necrosis factor alpha
tRNA	transfer ribonucleic acid
TSC	tuberous sclerosis complex
UPS	ubiquitin proteasome system
VEGF	vascular endothelial growth factor
VO_{2peak}	maximal oxygen uptake
YY1	yin yang 1
yr	year
W	watts

W_{max}	maximal power
Wnt	winglesstype MMTV integration site family
1RM	one repetition maximum
4E-BP1	eukaryotic initiation factor 4E-binding protein
5’TOP	5′-terminal oligopyrimidine tracts

Abstract

It is generally accepted that skeletal muscle adaptation to repeated bouts of contractile activity are specific to the mode, intensity, and duration of the exercise stimulus, but the molecular mechanisms regulating this specificity of adaptation remain poorly defined. The interaction of nutrient and substrate availability is also critical in determining skeletal muscle adaptation and has the capacity to modulate the specificity of training response. Accordingly, the primary aims of the studies undertaken for this thesis were: 1) to determine the molecular adaptations regulating the specificity of training adaptation following resistance and endurance exercise, and 2) to demonstrate how substrate (glycogen) and nutrient (protein/carbohydrate) availability impact adaptation responses following 'concurrent' training.

The first study (Chapter Two) determined the time course of Akt-mediated and AMPK signalling during the acute post-exercise recovery period following resistance and endurance exercise in human skeletal muscle. Sixteen male subjects were randomly assigned to either a cycling (n = 8, 60 min, 70% $V_{O_{2peak}}$) or resistance (n = 8, 8 × 5 leg extensions, 80% one-repetition maximum, 3-min recovery) exercise group. Muscle biopsies were obtained from the *vastus lateralis* at rest, immediately after exercise, and at 15, 30, and 60 min of recovery. Muscle glycogen concentration decreased with both exercise modes but glycogen utilisation was greater following cycling compared to resistance exercise ($P < 0.05$). Post-exercise increases in $Akt^{Thr308/Ser473}$ and $mTOR^{Ser2448}$ phosphorylation peaked at 30 – 60 min and were comparable between cycling and resistance exercise ($P < 0.05$). Similar patterns in $p70S6K^{Thr389}$ and $4E\text{-}BP1^{Thr37/46}$ phosphorylation were also observed. In contrast, $AMPK^{Thr172}$, glycogen $synthase^{Ser641}$ and AS160 phosphorylation were only elevated after cycling ($P < 0.05$). The results show a similar time course for Akt-mTOR-S6K phosphorylation during the initial 60-min between resistance and endurance exercise. However, selective increases in phosphorylation responses for proteins promoting glucose uptake (AS160) and glycogen synthase only occurred with endurance exercise. These findings indicate that endurance and resistance exercise initiate comparable translational signalling responses during the acute post-exercise recovery period, but pathways regulating glucose metabolism are selectively activated following endurance exercise.

The second study described in Chapter Three determined the effect of muscle glycogen concentration and post-exercise nutrient ingestion on anabolic signalling and rates of myofibrillar protein synthesis after resistance exercise. Sixteen young, healthy men matched for age, body mass, peak oxygen uptake ($V_{O_{2peak}}$) and strength (1RM) were randomly assigned to either a nutrient or placebo group. After 48 h diet and exercise control, subjects undertook a glycogen depletion protocol consisting of one-leg cycling to fatigue (LOW), whereas the other leg rested (NORM). The next morning following an overnight fast, a primed, constant infusion of L-[*ring*-$^{13}C_6$] phenylalanine was commenced and subjects completed 8 sets of 5 unilateral leg press repetitions at 80% 1RM. Immediately after resistance exercise and 2 h later, subjects consumed a 500 ml bolus of a protein/carbohydrate (20 g whey + 40 g maltodextrin) or placebo beverage. Muscle biopsies from the *vastus lateralis* of both legs were taken at rest and 1 and 4 h after resistance exercise. Muscle glycogen concentration was higher in NORM than LOW at all time points in both nutrient and placebo groups ($P < 0.05$). Post-exercise Akt-p70S6K-rpS6 phosphorylation increased in both groups with no differences between legs ($P < 0.05$). mTOR^{Ser2448} phosphorylation in placebo increased 1 h after exercise in NORM ($P < 0.05$), whereas mTOR increased ~ 4-fold in LOW ($P < 0.01$) and ~ 11 fold in NORM with nutrients ($P < 0.01$; different between legs $P < 0.05$). Post-exercise rates of myofibrillar protein synthesis were not different between NORM and LOW in the nutrient group (0.070 ± 0.022 vs. 0.068 ± 0.018 %/h^{-1}) or with placebo ingestion (0.045 ± 0.021 vs. 0.049 ± 0.017 %/h^{-1}). These results show that commencing high-intensity resistance exercise with low muscle glycogen availability does not compromise the anabolic signal and subsequent rates of myofibrillar protein synthesis during the early (4 h) post-exercise recovery period. Ingestion of a protein/CHO beverage enhanced the anabolic response to resistance exercise but failed to induce differences between the normal and low glycogen legs.

The final study (Chapter Four) elucidated the effect of protein supplementation on anabolic signalling and rates of myofibrillar and mitochondrial protein synthesis after a single bout of consecutive resistance and endurance exercise. Using a randomised cross-over design, 8 healthy males were assigned to experimental trials consisting of resistance exercise (8 × 5 leg extensions, 80% 1-RM) followed by cycling (30 min at ~70% $V_{O_{2peak}}$) with either post-exercise protein (25 g whey protein; PRO) or placebo (PLA) ingestion. Muscle biopsies were obtained at rest, 1 and 4 h post-exercise. Akt^{Ser473} and mTOR^{Ser2448} phosphorylation increased 1 h after exercise with protein

ingestion (175 - 400%, $P < 0.01$) and was different from placebo (150 - 300%, $P < 0.001$). MuRF1 and Atrogin-1 mRNA were elevated post-exercise but were higher with PLA compared to PRO at 1 h (50 - 315%, $P<0.05$), while PGC-1α mRNA increased 4 h post-exercise (620 - 730%, $P < 0.001$) with no difference between treatments. Post-exercise rates of myofibrillar protein synthesis increased above rest in both trials (75 - 145%, $P < 0.05$) but were higher with PRO (67%, $P < 0.05$) while mitochondrial protein synthesis did not change from baseline with either exercise (placebo) or protein ingestion. These results indicate a concurrent training session promotes anabolic adaptation responses and increases in metabolic/oxidative mRNA expression in skeletal muscle. Moreover, protein ingestion after combined resistance and endurance exercise enhances myofibrillar protein synthesis and attenuates markers of muscle catabolism. Therefore, protein ingestion with concurrent training promotes the anabolic response and may promote/protect muscle mass and reduce the potential interference effect of endurance exercise on hypertrophy.

In summary, the results from the studies undertaken for this thesis provide novel information regarding the effects of exercise mode, glycogen availability, and nutrient supplementation on the molecular mechanisms involved in the specificity of training adaptation. Specifically, resistance and endurance exercise are equally capable of mediating translational signalling responses during the acute recovery period. In addition, low glycogen availability does not appear to attenuate anabolic adaptations to resistance exercise but protein ingestion is integral to the stimulation of myofibrillar protein synthesis with concurrent training. The increase in myofibrillar protein synthesis with protein ingestion following consecutive resistance and endurance exercise may ameliorate potential interference of endurance exercise on muscle hypertrophy with chronic concurrent training.

Chapter One

Literature Review

1.1 Introduction

Skeletal muscle comprises ~40 - 50% of body mass (Wackerhage and Rennie 2006) and contains ~ 60% of all proteins in the human body (Matthews 1999). Mature skeletal muscle is a highly malleable tissue with the capacity to alter its phenotype in response to repeated bouts of contractile activity (i.e. exercise) and altered nutrient availability (Coffey and Hawley 2007a, Nader 2006, Holloszy 1967, Baar 2006, Goldberg 1968, Hawley et al. 2011). The mechanisms eliciting these changes are likely initiated by metabolic and/or mechanical factors that disrupt cellular homeostasis, with contraction kick-starting an adaptation response that continues during the post-exercise recovery period. The process of exercise-induced adaptation in muscle is the result of a cascade of molecular events that selectively enhance the expression of genes and increases the type and amount of proteins specific to the contractile stimulus (Figure 1.1).

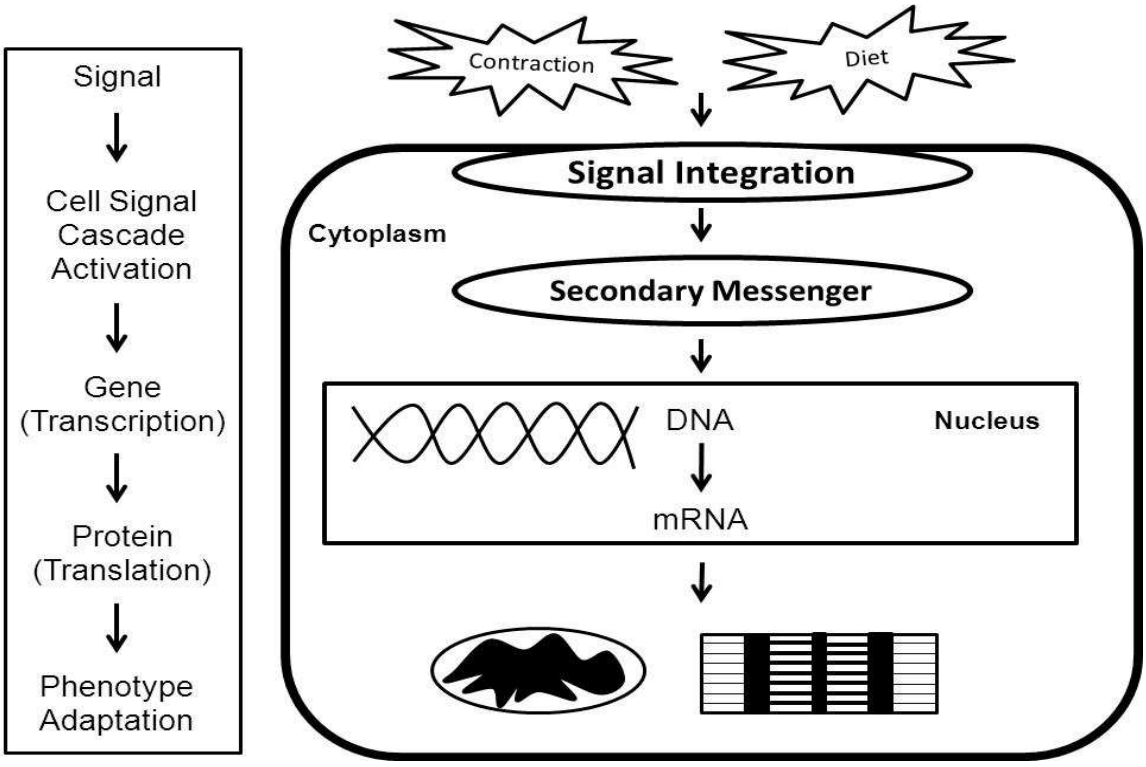

Figure 1.1 Simplified overview of the process of adaptation which generates alterations in skeletal muscle phenotype in response to exercise and nutrient stimuli.

The seminal work of Holloszy (Holloszy 1967) and the introduction of the needle biopsy technique (Bergstrom 1975, Bergstrom 1962) during the 1960's and 70's provided the first insight into the biochemical adaptations to exercise in skeletal muscle. Holloszy (Holloszy 1967) first demonstrated that regular bouts of prolonged endurance exercise significantly increased mitochondrial enzyme activity and content in rodent skeletal muscle, and the augmented capacity to generate adenosine tri-phosphate (ATP) via oxidative metabolism. These findings were later complemented by work in human skeletal muscle showing enhanced oxidative potential (Henriksson and Reitman 1977) and increased size of type I 'slow' twitch fibres (Gollnick et al. 1973) following endurance training, and together provide a molecular basis for the endurance training-induced increase in aerobic capacity and endurance performance. These pioneering studies coincided with the first evidence of contraction-induced increases in skeletal muscle mass (i.e. hypertrophy). Using radioactive-labelled amino acids in order to measure their incorporation into tissue, Goldberg showed that the chronic mechanical loading of skeletal muscles resulted in an increase in muscle mass (Goldberg 1968), and later demonstrated that sustained skeletal muscle unloading results in a decrease in mass (i.e. atrophy) (Goldberg et al. 1975). Together, these findings provided the initial evidence for muscle plasticity. However, only in the past two decades has the molecular machinery and associated signalling proteins mediating these adaptations begun to be elucidated. To this extent, changes in gene expression that promote increases in specific proteins are being characterised as the molecular underpinning for alterations in muscle phenotype to contractile stimuli.

The intrinsic ability of skeletal muscle cells to modulate turnover of key structural and functional proteins (e.g. mitochondrial, myofibrillar) is controlled by a complex network of highly regulated molecular processes. Important rate limiting steps in the adaptation response include, but are not limited to, regulation of transcription and translation, and subsequent synthesis of proteins (Coffey and Hawley 2007b). The nature and specificity of this response is determined by the characteristics of the contractile stimulus, such as mode, frequency, intensity and duration/volume. Chronic structural and metabolic adaptations in skeletal muscle have been shown to result from the cumulative effects of repeated exercise bouts (Perry et al. 2010, Wilkinson et al. 2008, Widegren, Ryder and Zierath 2001). The time-course of the molecular adaptation responses in skeletal muscle to exercise training ranges from immediately post-exercise to weeks (Figure 1.2). Numerous studies investigating mRNA responses in human

skeletal muscle to a single bout of exercise have shown anabolic, catabolic and metabolic genes are acutely and transiently activated (Coffey et al. 2006, Harber et al. 2009, Vissing, Andersen and Schjerling 2004). Gene expression induced early in the post-exercise period mainly correspond to the group of 'immediate early genes' involved in transcriptional cell regulation (e.g.: c-fos, fosB, c-jun, junB) (Hoppeler and Flück 2002, Puntschart et al. 1998, Booth and Neufer 2005). This response is then complemented by an increase in the abundance of many other exercise-induced genes ~3 - 12 h post-exercise, before returning to resting levels within 24 h (Pilegaard et al. 2000, Bickel et al. 2005, Yang et al. 2005, Psilander, Damsgaard and Pilegaard 2003).

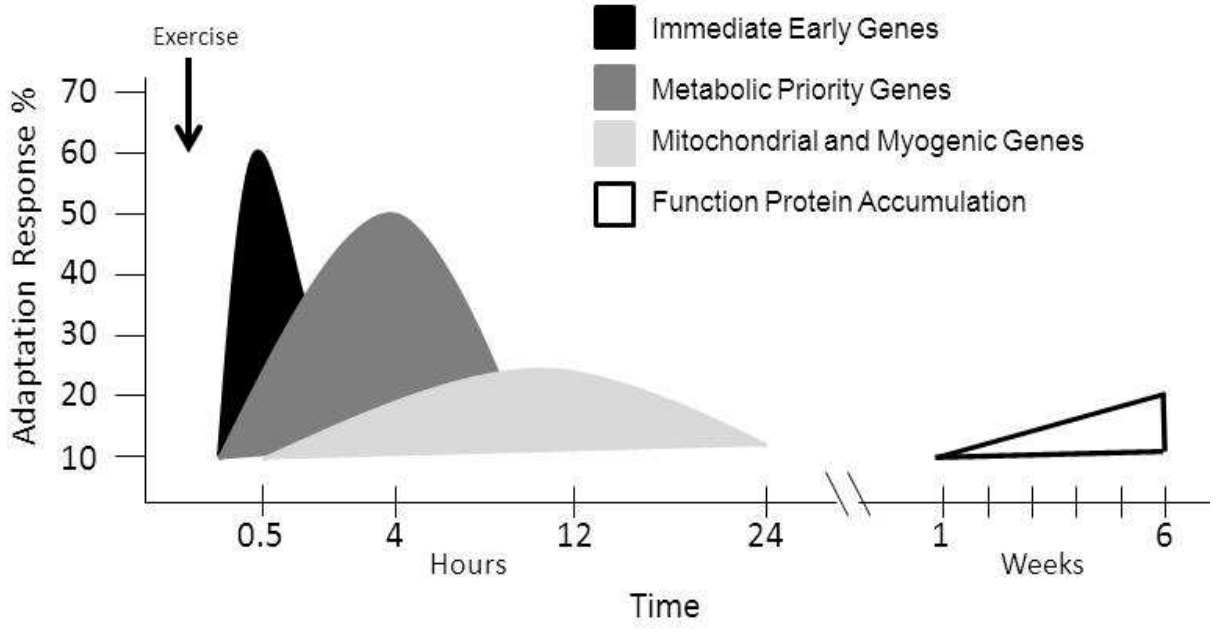

Figure 1.2 Representation of the approximate time-course of exercise-induced gene expression in skeletal muscle that can lead to training adaptation. Adapted from (Neufer and Booth 2005).

The increase in expression of genes following an acute exercise bout is an important factor in the chronic adaptation to an exercise stimulus, whether resistance- or endurance-based exercise, that is primarily dependent upon the magnitude and specificity of the many mRNA's up- or down-regulated with each transient response (Coffey and Hawley 2007b, Zambon et al. 2003). The

acute response is also reflected at the level of protein synthesis with exercise-induced increases in signalling cascades that mediate mRNA translation initiation and elongation, and subsequent synthesis of nascent proteins (Kimball and Jefferson 2010). Accordingly, repeated bouts of similar contractile activity induce early gene responses and protein synthesis in skeletal muscle that ultimately result in an altered phenotype. The specificity of training dictates that diverse contractile stimuli results in divergent adaptation responses and determines the training-induced phenotype (Coffey and Hawley 2007b). Endurance training increases the size and number of mitochondria, mitochondrial enzyme activity (Holloszy 1967, Gollnick et al. 1973, Henriksson and Reitman 1977) and capacity to oxidise lipids (Hawley 2002, Henriksson 1977). Moreover, endurance training (6 - 8 weeks) in previously untrained participants has been shown to increase mitochondrial volume and capillary density by ~ 30% (Hoppeler et al. 1985). In contrast, resistance training promotes an increase in muscle size and strength through neural adaptations (Sale 1988), increases in myofibrillar volume (i.e.: contractile protein) (MacDougall et al. 1979, Phillips et al. 1999), and increased cross sectional area of type II fibres (Adams et al. 1993, Zierath and Hawley 2004, D'Antona et al. 2006, Fry et al. 2003). Importantly, the specific molecular profile generated following an acute exercise bout and chronic changes in protein at the cellular level with divergent exercise modes has yet to be clearly established.

A critical factor in the adaptation response promoting the exercise-induced muscle phenotype to each respective training mode is nutrient/substrate availability (Hawley et al. 2011). The extent to which substrate levels are altered before and during exercise and in the subsequent post-exercise recovery period is an important factor in modifying subsequent training adaptations. For example, the capacity for carbohydrate ingestion to replenish muscle glycogen stores during recovery to enhance subsequent performance has long been established (Hawley, Tipton and Millard-Stafford 2006, Bergström et al. 1967, Ivy et al. 1988a). Likewise, the ability of protein or amino acid ingestion to stimulate muscle protein synthesis, reduce protein breakdown, and promote net muscle protein accretion is also well recognised (Rennie et al. 1982, Phillips, Hartman and Wilkinson 2005). These nutrient-related responses can be mediated by the same molecular targets activated by exercise/muscle contraction. In particular, the phosphatidylinositol 3-kinase- (PI3K-) mammalian (more recently termed “mechanistic”) Target Of Rapamycin (mTOR) signalling pathway and cell energy sensor AMP-activated protein kinase (AMPK) are

convergence points for nutrient (i.e.: insulin, amino acids) and exercise stimuli (Hardie, Ross and Hawley 2012, Hawley et al. 2006).

To date, the majority of research examining the specificity of training adaptation has focused on the distinct phenotypes generated in response to chronic endurance or resistance exercise, and the exercise-nutrient interactions that influence these training-induced adaptations. However, an important training modality that has received far less attention is the simultaneous incorporation of resistance and endurance exercise in a training regimen, referred to as concurrent training. Early work dating back to the 1980's has proposed an 'interference' paradigm in which resistance (i.e.: strength) and aerobic (i.e.: endurance) exercise bouts performed in close proximity appear to inhibit strength development when compared with strength training alone (Hickson 1980). The paucity of studies investigating the molecular response to concurrent training have failed to provide clarity regarding the specificity of training adaptation (Coffey et al. 2009a, Coffey et al. 2009b, Donges et al. 2012, Lundberg et al. 2012, Wang et al. 2011). Furthermore, few studies have investigated the effects of nutrient availability/supplementation within a concurrent training paradigm and its potential to modulate a specificity of training adaptation response with divergent contractile activity.

This chapter will provide a review of the putative primary and secondary messengers involved in regulating the adaptation response to single mode (resistance and endurance exercise) and concurrent training. The focus will include the IGF-signalling cascade and proteins regulating translation initiation due to their purported role in the specificity of adaptation to divergent forms of exercise. Additionally, the capacity for nutrition (protein and carbohydrate) and muscle glycogen availability to promote/inhibit the exercise-induced adaptation response in skeletal muscle will also be reviewed.

1.2 Skeletal Muscle Mechanotransduction

The process of converting a mechanical force (e.g.: muscle contraction) into an internal cellular response is termed mechanotransduction (Wu et al. 2011) while mechanisms responsible for coupling mechanical stimuli with intracellular biochemical events can be referred to as mechanoreception (Hornberger and Esser 2004). Mechanoreception involves the up-regulation of

primary and secondary messengers that initiate a cascade of events resulting in the activation and/or repression of specific pathways that ultimately alter the morphological, biochemical, and physiological characteristics of the muscle (Hornberger and Esser 2004). How these signalling events are deciphered and integrated is not completely understood. Moreover, the complexity of the mechanisms that enable skeletal muscle cells to respond to contraction is complicated by the numerous primary messengers that act as intermediaries for mechanoreception. Such primary messengers can include i) calcium flux, ii) ATP turnover, iii) cellular stress, iv) stretch, v) redox potential and vi) reactive oxygen species (Coffey and Hawley 2007b, Sanders Williams and Neufer 1996, Flück 2004, Sakamoto and Goodyear 2002). These putative messengers are unlikely to act in isolation, but rather as a complex, multifaceted signal. This section will focus on several important exercise-induced primary messengers.

Calcium

Neural activation of skeletal muscle releases acetylcholine from the neuromuscular junction causing the depolarization of the plasma membrane and subsequent generation of an action potential and calcium (Ca^{2+}) release from the sarcoplasmic reticulum. Skeletal muscle Ca^{2+} release is an 'all-or-none' principle where the duration of a Ca^{2+} burst will determine the extent of Ca^{2+} signalling (Lamb 2002). As such, the greater the firing rates of action potentials that reach the muscle, the greater the Ca^{2+} release (Baar 2009, Chin 2010). This Ca^{2+} release is essential for facilitating the interaction between myosin and actin during muscle contraction. The frequency, intensity and duration of the contraction stimulus determine the 'spike' and duration of the Ca^{2+} transients (Westerblad and Allen 1991). There is evidence linking the transient alterations in cellular Ca^{2+} concentration to the activation of calcium-dependent protein kinases and phosphatases integral to the adaptive response to exercise (Chin 2004, Ojuka 2004, Berchtold, Brinkmeier and Müntener 2000). In this regard, changes in intracellular Ca^{2+} levels appear to be central to modulating the different contractile properties and subsets of genes expressed between slow type I oxidative and fast type II glycolytic muscle fibres (Chin 2010).

Oscillations in intracellular Ca^{2+} concentration are initially transduced by the protein calmodulin (CaM) which is responsible for regulating the activities of several calmodulin binding protein

kinases and phosphatases. The CaM family of serine/threonine protein kinases (CaMK) is composed of various isoforms that differ in their functions. In response to increases in intracellular Ca^{2+} levels and subsequent Ca^{2+} binding, the CaMKII and CaMKIV isoforms are activated (Pelosi and Donella-Deana 2000). These isoforms in turn translocate from the cytoplasm to the nucleus and can activate specific transcription factors that appear to mediate muscle plasticity and hypertrophy (Hook and Means 2001, McKinsey et al. 2000, Olson and Williams 2000). Further, both the CaMKII and CaMKIV isoforms are implicated in the putative regulation of mitochondrial biogenesis (Wu et al. 2002). These putative responses are likely to involve the interaction of secondary messengers such as nuclear factor of activated T-cells (NFAT), histone deacetylase (HDAC) and myocyte enhancer factor 2 (MEF2) (Liu et al. 2005).

Increases in intracellular Ca^{2+} are also proposed to activate the serine/threonine protein phosphatase calcineurin. Activated calcineurin has been shown to induce hypertrophy of skeletal muscle cells in culture, while inhibition of calcineurin activity blocks the hypertrophic response to insulin-like growth factor (IGF)-1 (Musaro et al. 1999, Semsarian et al. 1999). Calcineurin also plays a major role in mediating neural activity-dependent muscle fibre specification and remodelling (Al-Shanti and Stewart 2009, Klee, Crouch and Krinks 1979). In addition, calcineurin has been identified in the regulation of glucose transport. This role was first demonstrated when GLUT4 gene expression was elevated in transgenic mice overexpressing an active form of calcineurin (Ryder et al. 2003). Recent evidence has also implicated CaMKII in the regulation of GLUT4 (Ojuka, Goyaram and Smith 2012).

The multiplicity of physiological processes and responses to contraction mediated by Ca^{2+} flux makes determining a role in the specificity of training adaptation difficult. However, if prolonged moderate exercise can increase Ca^{2+} uptake from the sarcoplasmic reticulum by increasing the number of active Ca^{2+}-ATPase pumps (Schertzer et al. 2004) and high intensity exercise generates a decrease in Ca^{2+} uptake and release (Matsunaga et al. 2002), differences in Ca^{2+} flux may initiate diverse signalling responses to promote specificity of training adaptation.

Redox Potential

The oxidation of glucose, fatty acids and amino acids to produce acetyl CoA, via the Krebs (or Tricarboxylic Acid) Cycle, generates ATP and the ubiquitous high-energy electron carriers nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (Adams et al. 2002, Bickel et al. 2005). NAD and FAD can exist in reduced (NADH and $FADH_2$) and/or oxidised (NAD^+ and FAD^+) forms depending on whether they are donating or accepting electrons from other molecules, respectively (Silverthorn 2004). This tendency for NADH and $FADH_2$ to gain and lose electrons constitutes a redox potential. During exercise or muscle contractions where there is an impending need to generate energy, the cytosolic NAD^+/NADH ratio is subject to dynamic fluctuations imposed by the rate of NAD^+ reduction to NADH and the reciprocal rate of NADH oxidization to NAD^+ (Robergs, Ghiasvand and Parker 2004). For many years fluctuations in the NADH/NAD^+ ratio were thought to simply reflect the balance of redox state but are now considered potential intracellular signals affecting skeletal muscle gene expression (Hawley and Zierath 2004, Powers, Talbert and Adhihetty 2011). An example is the NAD^+-dependent changes in the protein and enzymatic activity of the deacetylase sirtuin (SIRT) (Rodgers et al. 2005b, Lagouge et al. 2006) which has been implicated as a key regulator of exercise-mediated mitochondrial adaptations in skeletal muscle (Dali-Youcef et al. 2007, Gurd 2011, Haigis and Sinclair 2010) and may also down-regulate translation and muscle protein synthesis through negatively regulating mTOR signalling via the tuberous sclerosis protein (TSC)1/2 complex (Ghosh, McBurney and Robbins 2010).

Maintenance of the redox potential produces volatile reactive oxygen species (ROS) and increased activity of metabolic pathways by muscle contraction represents a stimulus capable of generating elevated levels of ROS. The first study to report an increase in the production of ROS in human skeletal muscle following exercise appeared in the late 1970's (Dillard et al. 1978). While multiple anti-oxidant systems exist in skeletal muscle (Stofan et al. 2000, Arbogast and Reid 2004), it has been suggested oxidative stress can limit exercise capacity and may also modulate exercise-induced adaptation (Carrero et al. 2000). Previous investigations in human skeletal muscle *in vivo* have shown prolonged endurance exercise to be a stimulus more likely to promote oxidative stress and ROS activity than heavy resistance training, possibly due to its fatiguing nature (Medved et al. 2004, Matuszczak et al. 2005). For example, there is evidence linking ROS production to the maintenance and stability of contraction-induced peroxisome

proliferator-activated receptor gamma co-activator (PGC)-1α expression in skeletal muscle cells (Irrcher, Ljubicic and Hood 2009). The presence of oxygen radicals has also been shown to increase susceptibility of myofibrillar proteins to proteolysis (Smuder et al. 2010). Therefore, oxidative stress and ROS activity may potentially impact increases in myofibrillar synthesis in response to resistance exercise. Whether redox state and reactive oxygen species promotes or inhibits exercise-induced adaptation is unclear and more work is required to ascertain the nature of redox-controlled signalling pathways that may regulate the molecular machinery governing adaptation to exercise in skeletal muscle.

Mechanical Stretch

Contractile activity places the sarcolemmal, cytoskeletal and extracellular matrix of skeletal muscle cells under mechanical tension (Sanders Williams and Neufer 1996, Goldberg et al. 1975). This contraction-induced tension is thought to be a primary regulator of several diverse cellular processes including, but not limited to, cell growth, differentiation, gene expression and protein synthesis (Alenghat and Ingber 2002).

Skeletal muscle mechanoreceptors that initiate signal transduction include disruption of the phospholipid bilayer and activation of surface adhesion receptors (Hornberger and Esser 2004). Additionally, the tension generated by mechanical stretch within the lipid bilayer may release components such as IGF that can activate cell signalling pathways (Hamill and Martinac 2001). The mechanotransduction process also involves the dystrophin–glycoprotein complex and focal adhesion proteins that physically couple the extracellular matrix and cytoskeleton (Rando 2001, Hornberger and Esser 2004). Focal adhesion proteins possess transmembrane integrin receptor proteins and may act as a mechanoreceptor to transmit mechanical information between the outside and inside of the cell. Integrins and dystrophin–glycoprotein complexes have been shown to interact with various signalling proteins (Rando 2001) and focal adhesion kinase (FAK) signalling has been shown to increase within minutes following deformation of integrin proteins by mechanical forces (Ingber 2006, Huijing 1999). Thus, integrins and dystrophin–glycoprotein complexes may serve as integration sites where mechanical information is converted into biochemical signals to ultimately regulate muscle protein synthesis.

Another potential mechanism by which contraction-mediated mechanical stretch may initiate cell signalling is through phosphatidic acid (PA) (Hornberger 2011). Several studies have demonstrated mechanical stimuli capable of inducing hypertrophy can initiate increases in PA concentration (Hornberger et al. 2006a, O'Neil et al. 2009). While several enzymes are implicated in the regulation of PA concentration, phospholipase D (PLD) has received the most attention as it catalyses the hydrolysis of the phosphodiester bond of phosphatidylcholine to generate PA and choline (Sun et al. 2008). Hornberger and co-workers established that inhibiting PLD activity blocked both the mechanical activation of anabolic signalling and increased PA concentrations in skeletal muscle (Hornberger et al. 2006a). Furthermore, activation of anabolic signalling proteins following eccentric contractions was blocked by inhibitors preventing PLD-catalysed PA formation (Hornberger et al. 2007, O'Neil et al. 2009). These results provide evidence that the mechanically induced increases in PA concentrations contribute to activation of cell signalling pathways. However, further work is required to elucidate other potential regulators of PA activity and whether increases in PA are specific to certain modes of exercise. Taken together, it appears mechanotransduction in skeletal muscle has an intrinsic capacity to delineate between distinct models of mechanical stress. When extrapolated to an exercise setting where the nature of the contractile stimulus varies (e.g. eccentric vs. concentric contraction, or both), this specificity implies the signalling events initiated by mechanical stress and tension are likely to contribute to specific exercise-induced adaptations.

Phosphorylation Potential

Energy-requiring processes, most notably muscle contraction, are initiated by hydrolysis of ATP (Polekhina et al. 2003). If the demand for ATP exceeds availability, increases in the free ADP: ATP and AMP: ATP ratios result. Increases in the AMP:ATP ratio are approximately 5-fold higher compared to increases in ADP:ATP, making AMP a focal point for monitoring cellular energy status (Hardie and Hawley 2001).

Exercise is characterised by large increases in muscle energy turnover that is dependent on the type, intensity and duration of the stimulus (Richter and Ruderman 2009). Most exercise protocols used to investigate cellular energy status in human skeletal muscle have involved

endurance-based exercise and have shown increases in the free cellular AMP:ATP ratio in response to endurance cycling is intensity-dependent (Chen et al. 2003, Birk and Wojtaszewski 2006). Interestingly, changes in free AMP and subsequent AMP: ATP ratio following exercise appear to be attenuated after short-term training (Chesley, Heigenhauser and Spriet 1996, McConell et al. 2005, Talanian et al. 2010). This response may suggest an adaptation in mitochondrial function by providing tighter control between ATP supply and demand and thus reducing the dependence on intramuscular phosphocreatine and glycogen stores to regenerate ATP anaerobically (Chesley et al. 1996). In contrast, very few studies have examined changes in cell energy status following resistance exercise. This may be due to the original concept that energy requirements for resistance exercise can be met solely through phosphocreatine and intramuscular glycogen (Keul et al. 1978). However, studies in rat skeletal muscle have shown increases in free AMP following electrical stimulation mimicking resistance exercise (Hutber, Hardie and Winder 1997, Sahlin, Gorski and Edstrom 1990). Moreover, significant reductions in ATP and creatine phosphate have been observed following a bout of strenuous whole body resistance training in strength trained athletes (Tesch, Colliander and Kaiser 1986, MacDougall et al. 1999). These results suggest that both endurance and resistance exercise can mediate changes in cell energy status provided there are adequate levels of contractile intensity and duration.

Numerous studies have demonstrated that elevated cellular AMP concentration appears to principally exert its effect via the secondary messenger AMPK (Sakamoto and Goodyear 2002, Hawley and Zierath 2004, Hardie and Sakamoto 2006). AMPK is proposed to function as a ‘fuel gauge’ by sensing the energy status of the cell and has been implicated in the regulation of diverse processes such as glucose uptake, fatty acid oxidation, hypertrophy and gene expression (Aschenbach, Sakamoto and Goodyear 2004, Hardie et al. 2012). How AMPK is regulated in response to divergent contractile stimuli and its role in the adaptation response to exercise will be discussed subsequently.

1.3 Signalling Mechanisms Regulating Muscle Protein Synthesis

The biochemical signals generated during mechanotransduction activates a complex network of intracellular protein kinases and phosphatases. These signalling pathways are regulated at

multiple levels, and depending on the nature of the contractile stimulus, are likely to involve signalling pathway cross-talk to transduce a co-ordinated signal (Figure 1.3). Detailed examination of every canonical signalling pathway characterised in skeletal muscle is beyond the scope of this thesis, and several recent reviews on the different signalling pathways and mechanisms mediated by muscle contraction exist (Berdeaux and Stewart 2012, Bootman 2012, Coffey and Hawley 2007b, Harrison 2012, Kramer and Goodyear 2007, Nusse 2012, Sandri 2008, Tsivitse 2010). This section will focus specifically on the Ca^{2+} Calmodulin-dependent kinase (CaM) / Calcineurin, 5'Adenosine Monophosphate Activated Protein Kinase (AMPK), and the Insulin/ Insulin-like Growth Factor (IGF) signalling pathways and their putative role regulating the adaptation response to resistance and endurance exercise.

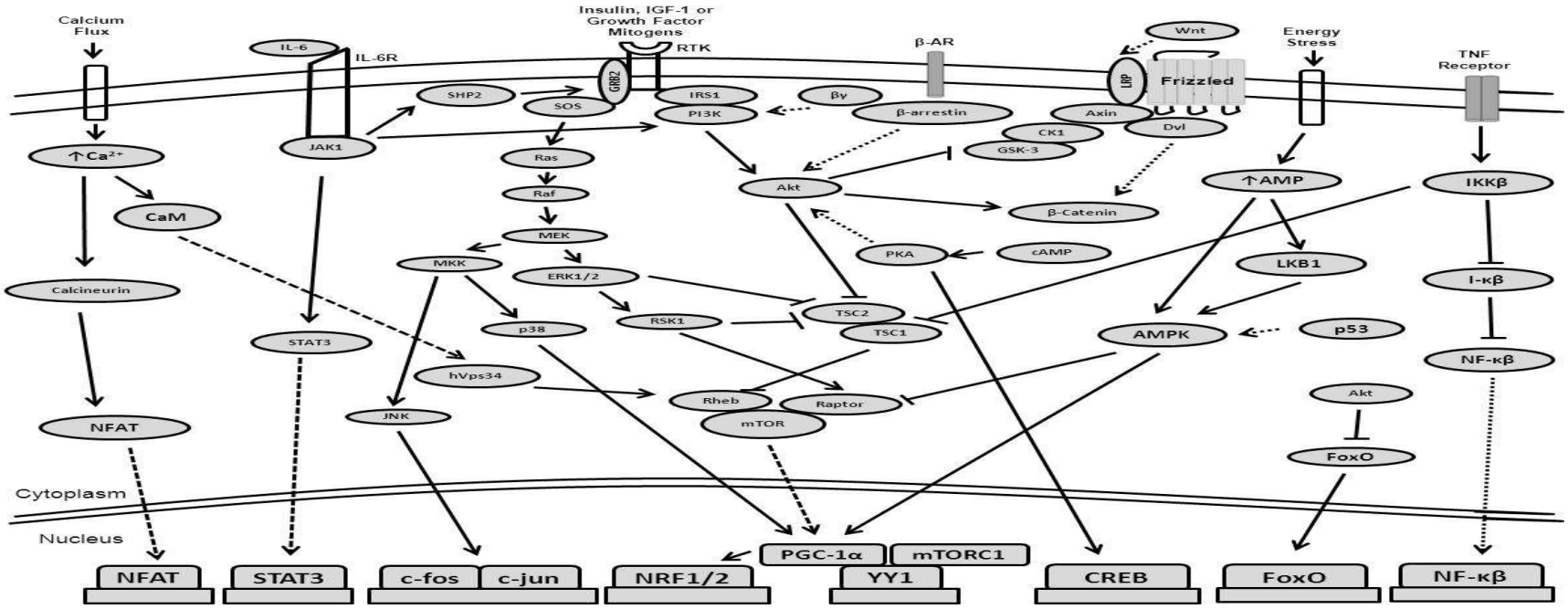

Figure 1.3 Schematic representation illustrating some of the principal signalling pathways that may regulate adaptation responses to muscle contraction. Dotted lines depict pathways yet to be completed defined. Bars denote inhibition and arrows denote activation. AMPK, 5'Adenosine Monophosphate Activated Protein Kinase; CaM, Calmodulin; CK1, Casein Kinase 1; CREB, cAMP response element binding protein; Dvl, Dishevelled; ERK, Extracellular-signal-Regulated kinase; FoxO, Forkhead box subgroup O; GSK-3, Glycogen Synthase Kinase 3;GRB2, Growth factor receptor bound protein 2; hVps34, human vacuolar protein sorting 34; IKK, Inhibitor NF-κB kinase; IL-6, Interleukin 6; IRS1, Insulin receptor substrate 1; JAK1, Janus kinase 1; JNK, c-Jun N-terminal kinase; LKB1, Liver kinase B1;mTORC1, Mechanistic target of rapamycin complex 1; MEK, MAPK-ERK kinase; NFAT, Nuclear factor of activated T-cells; NRF, Nuclear Receptor Factor; NF-κB, Nuclear Factor-KappaB; PGC-1α, peroxisome-proliferation-activated receptor-gamma co-activator-1; PI3K, Phosphoinositide 3-kinase; PKA, Protein Kinase A; RAPTOR, Regulatory associated protein of mTOR; Rheb, Ras homolog enriched in brain; RSK, Ribosomal S6 kinase; SHP2, Sarcoma oncogene homology 2-domain-contain protein tyrosine phosphatase-2; SOS, Son of sevenless; STARS, Striated activator of Rho signalling; STAT3, Signal transducer and activator of transcription 3; TSC, Tuberous sclerosis complex; TNF, Tumour Necrosis Factor; YY1, yin yang 1.

Ca^{2+} Calmodulin-dependent kinase (CaMK) / Calcineurin Signalling

Changes in intracellular Ca^{2+} levels can activate transcriptional pathways responsible for muscle-specific gene expression (Berchtold et al. 2000, Pette and Staron 1997). The CaMKII isoform is the most abundant isoform in human skeletal muscle (Flück et al. 2000, Rose and Hargreaves 2003, Rose, Kiens and Richter 2006). CaMKII is activated through autophosphorylation of its Thr^{286} amino acid side chain (Hook and Means 2001, Hudmon and Schulman 2002) and localises to the cytoplasm where it regulates the transcription of several proteins involved in the diverse functions of muscle hypertrophy and mitochondrial biogenesis (Chin 2004). In response to increases in intracellular Ca^{2+} levels, activated CaMKII translocates to the nucleus where it phosphorylates and deactivates histone deacetylase C 4 (HDAC4) causing it to dissociate from myocyte enhancer factor 2 (MEF2). This dissociation allows MEF2 to activate transcription of specific MEF2-dependent target genes such as MyoD and myogenin (Molkentin et al. 1995) both of which are putative regulators of muscle hypertrophy (McKinsey et al. 2000) (Figure 1.4).

Phosphorylation of CaMKII has been shown to increase ~50 - 70% in human skeletal muscle during 40 min of cycling (Rose and Hargreaves 2003). CaMKII activity was also shown to increase within the first minute of exercise and remained elevated during a 90 min cycling bout (~ 67% $V_{O_{2peak}}$) (Rose et al. 2006). This increase in CaMKII activation appears to be intensity-dependent, with greater levels of phosphorylation and activity observed when cycling intensity increases from ~ 35% to ~ 85% $V_{O_{2peak}}$ (Benziane et al. 2008, Egan et al. 2010, Rose et al. 2006). Increases in CaMKII activity have also been demonstrated in response to acute repeated sprint exercise and short-term (2 - 3 weeks) endurance training (Rose et al. 2007, Benziane et al. 2008).

Few studies have investigated CaMKII responses following resistance exercise in humans despite its potential contribution to the hypertrophy response. Wang and colleagues recently reported no change in CaMKII phosphorylation when endurance and resistance exercise were combined (Wang et al. 2011). Whether other resistance exercise protocols that manipulate different training variables (i.e.: intensity, repetitions, sets, etc) can activate CaMKII post-exercise remains to be established. The role of CaMKIV in skeletal muscle hypertrophy and/or oxidative metabolism remains controversial. Transgenic animals that selectively over-expressed the CaMKIV isoform in skeletal muscle possessed increased mitochondrial volume, expression of mitochondrial enzymes regulating fatty acid metabolism and electron transport, and enhanced recovery from

fatigue (Wu et al. 2002). While such phenotype adaptations suggest a role for CaMKIV in generating an oxidative/ metabolic phenotype in response to exercise, several human and animal studies have failed to detect the CaMKIV protein in skeletal muscle using immunoblotting techniques (Akimoto et al. 2004, Rose et al. 2006), currently precluding any direct role in skeletal muscle adaptation to exercise.

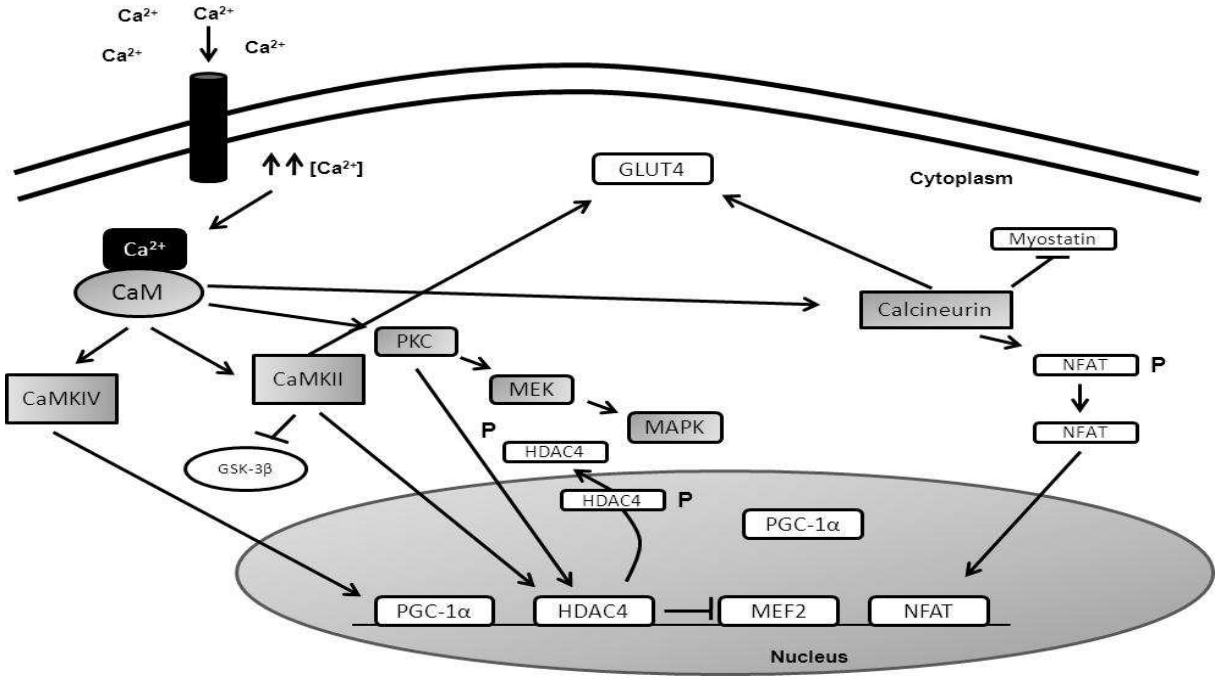

Figure 1.4 Schematic of putative skeletal muscle calcium (Ca^{2+})/calmodulin, mitogen-activated protein kinase (MAPK) and calcineurin signalling pathways that may modulate calcium-induced changes in gene expression following contraction. Bars denote inhibition and arrows denote activation. CaM, Calmodulin; CaMK, calmodulin kinase; GSK-3β, Glycogen Synthase Kinase-β; PKC, Protein Kinase C; MEK, MAPK-ERK kinase; HDAC, histone deacetylase; MEF2, myocyte enhancer factor 2; peroxisome-proliferation-activated receptor-gamma co-activator-1, PGC-1α; NFAT, nuclear factor of activated T-cell.

The protein phosphatase calcineurin is also proposed to act as a Ca^{2+} sensor (Bassel-Duby and Olson 2006, Chin et al. 1998). The calcineurin/NFAT signalling pathway has been shown to regulate muscle hypertrophy through activation of IGF-1 signalling but others have shown no effects on muscle hypertrophy with administration of calcium/ calcineurin inhibitors (Bodine et

al. 2001b). Interestingly, work in skeletal muscle of transgenic mice down-regulating calcineurin activity identified myostatin, a negative regulator of skeletal muscle mass, as a possible downstream target of the calcineurin-NFAT pathway (Michel et al. 2007, Michel, Dunn and Chin 2004, Muthuri, Chin and Michel 2007).

Calcineurin has also been associated with activation of the striated muscle activator of Rho signalling (STARS) protein (Kuwahara et al. 2007). STARS is proposed to stimulate hypertrophy by increasing serum response factor (SRF)-mediated gene transcription (Kuwahara et al. 2005, Wei et al. 1998). Calcineurin's most well characterised role in skeletal muscle is its putative regulation of type II to type I muscle fibre conversion (Chakkalakal et al. 2003, Chin et al. 1998, Wu et al. 2001). Studies of transgenic mice expressing constitutively active calcineurin show an increase in gene expression of oxidative genes and total number of type I muscle fibres (Chin et al. 1998, Naya et al. 2000, Wu et al. 2000, Michel et al. 2004). However, similar findings are yet to be confirmed in human skeletal muscle.

Increases in intracellular Ca^{2+} concentration and subsequent activation of signalling intermediaries such as CaM kinase, calcineurin and protein kinase C are likely to play a role in the adaptation response to exercise. However, much of the research investigating calcium signalling has been limited to cell culture and transgenic animal models. It remains to be established whether divergent contractile activity and substrate/ nutrient availability exerts specificity in calcium-related signalling responses.

The 5'Adenosine Monophosphate Activated Protein Kinase (AMPK) Mediated Signalling

AMP binding to AMPK increases phosphorylation at Thr^{172} of the α-subunit by a conformational change allowing phosphorylation (and activation) by the upstream liver kinase B1 (LKB1) (Hawley et al. 2003, Hardie et al. 2003). Calcium/calmodulin-dependent protein kinase β has also been identified as an upstream activator of AMPK capable of inducing phosphorylation at Thr^{172} in response to rises in cytosolic Ca^{2+} concentration (Hawley et al. 2005, Jensen et al. 2007, Woods et al. 2005). Given the increases in AMP and cytosolic Ca^{2+} associated with muscle contraction (Al-Shanti and Stewart 2009, Chen et al. 2003), it is not surprising that an acute exercise bout increases phosphorylation of AMPK and its enzymatic activity (Chen et al. 2003,

Rasmussen and Winder 1997, Wojtaszewski et al. 2003, Wojtaszewski et al. 2000, Yeo et al. 2010).

Muscle contraction activates AMPK which "switches on" catabolic pathways that generate ATP while "switching off" anabolic pathways and other ATP-consuming processes to restore homeostasis (Corton, Gillespie and Hardie 1994, Kahn et al. 2005) (Figure 1.5). AMPK may enhance ATP production through activating glucose uptake via the glucose transporter type (GLUT) 1 and GLUT4 glucose transporters (Pehmøller et al. 2009) and by stimulating glycolysis (Hardie 2011b). Moreover, AMPK can also stimulate fatty acid uptake by the translocation of fatty acid transporters (Bonen et al. 2007) and fatty acid oxidation through the phosphorylation and inactivation acetyl- coenzyme A carboxylase (Winder et al. 1997).

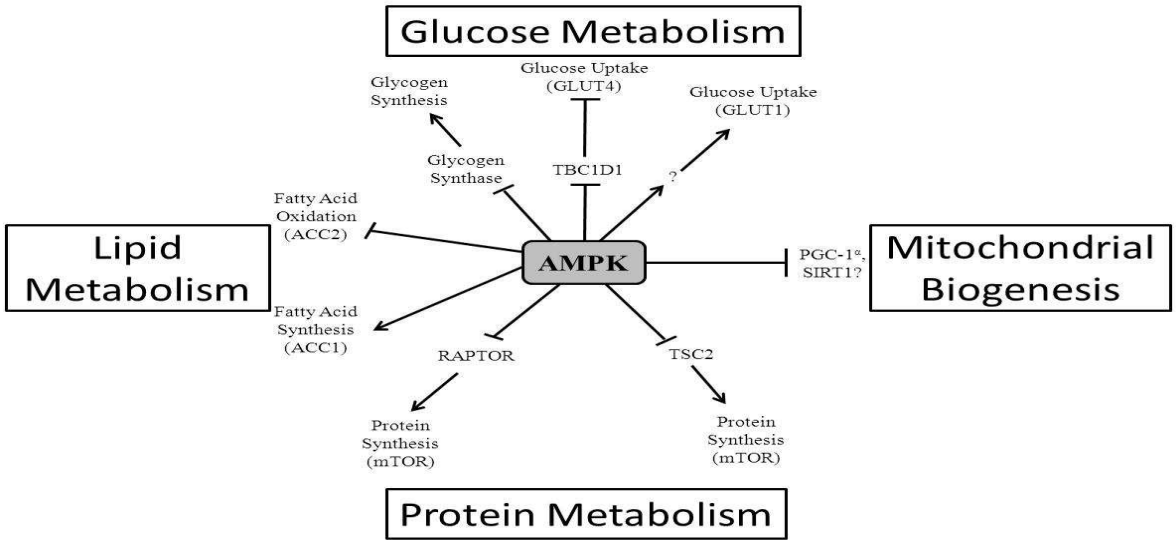

Figure 1.5 Putative AMPK targets in muscle cell metabolism. AMPK may mediate several processes involved in the adaptation response to exercise. Such processes include mitochondrial biogenesis, protein metabolism, glucose metabolism, and lipid metabolism. ACC 1/2, Acetyl-CoA carboxylase 1/2; GLUT 1/4, Glucose transporter type 1/4; mTOR, mammalian target of rapamycim; PGC-1α, Peroxisome proliferator-activated receptor gamma coactivator-1α; RAPTOR, Regulatory associated protein of mTOR; SIRT 1, Sirtuin 1; TSC2, Tuberous sclerosis complex; TBC1D1, TBC1 domain family member 1. Adapted from (Hardie et al. 2012).

AMPK has also been proposed to directly influence muscle metabolism by regulating the expression of a specific set of nuclear gene targets (Leff 2003). AMPK activation has been associated with regulation of transcription factors including nuclear receptor factor-1 (NRF-1), MEF2 (Bergeron et al. 2001, Zheng et al. 2001) and the mitochondrial transcription co-factor PGC-1α (Zong et al. 2002). AMPK may also increase activation of the NAD^+-dependent silent information regulator two number 1 (SIRT1) protein that deacetylates PGC-1a (Cantó et al. 2010) and phosphorylates and inactivates HDAC5, leading to its removal from the nucleus and allowing MEF2 to bind and activate PGC-1α (Hardie 2011a). These findings suggest a prominent role for AMPK in regulating an oxidative phenotype and endurance training-induced adaptation. However, a clear cause and effect between AMPK activity and PGC-1α expression and direct evidence *in vivo* human skeletal muscle for AMPK-mediated increases in mitochondrial biogenesis is lacking.

AMPK is also putatively involved in repressing the mechanistic target-of-rapamycin complex-1 (mTORC1), an important regulator of translation initiation and subsequent muscle protein synthesis (Gwinn et al. 2008, Inoki, Zhu and Guan 2003, Zoncu, Efeyan and Sabatini 2011). AMPK has been shown to attenuate mTOR signalling by phosphorylation of its upstream regulator TSC2 (Inoki et al. 2003). AMPK can also directly phosphorylate and subsequently inhibit mTORC1 at the Thr^{2446} residue (Zoncu et al. 2011). However, protein synthesis increases during the post-exercise recovery period (Moore et al. 2005, Phillips et al. 1999), a response that can occur despite concomitant increases in AMPK activity following exercise (Dreyer et al. 2006). The possibility exists that the concomitant increase in AMPK activity and elevated rates of muscle protein synthesis in the hours after exercise may reflect AMPK's contribution to other metabolic processes such as glucose uptake (Richter et al. 1982 , Richter et al. 1989) or re-glycogen synthesis (Richter et al. 1982), without directly inhibiting protein synthesis. Notably, an AMPK- mediated inhibition of translation initiation or protein synthesis during recovery from exercise *in vivo* human skeletal muscle remains to be established. It is also unknown if this potential inhibition is specific to the mode of exercise and/or other factors such as substrate availability.

AMPK has been implicated in many of the phenotypic changes and chronic adaptations in muscle that occur with endurance training (Frøsig et al. 2004). Most studies have shown an association

between AMPK and an oxidative/metabolic phenotype with chronic endurance-based exercise where there is a greater reliance on oxidative metabolism of glucose and fatty acids. In contrast, AMPK is unlikely to be a critical mediator in resistance-based exercise adaptation due to modest perturbation of ATP concentrations, or accumulation of ADP or AMP. Therefore, it seems plausible that AMPK activation and greater total protein content may mediate part of the specificity of training adaptation observed with chronic endurance but not resistance exercise.

Insulin/ Insulin-like Growth Factor (IGF) Signalling

Insulin/ IGF signalling is an important contraction and nutrient stimulated pathway that increases translation and protein synthesis in skeletal muscle (Bodine et al. 2001b, Coffey and Hawley 2007b, Glass 2005, Rommel et al. 2001, Clemmons 2009). The increase in autocrine/ paracrine expression of IGF-1 in response to skeletal muscle contraction activates the type I IGF receptor (IGF-IR) (Clemmons 2009). The subsequent activation of downstream signalling targets including mTORC1 and p70S6K1 through the insulin/IGF-1- phosphatidylinositol 3-kinase (PI3K)-Akt signalling pathway provided an initial basis for exercise-induced increases in skeletal muscle hypertrophy (Figure 1.6). Indeed, early work by Baar and Esser (1999) established that gains in muscle mass following 6 weeks high-frequency electrical stimulation were associated with mechanical loading-induced phosphorylation of p70S6K (Baar and Esser 1999). This finding was closely followed by work from Bodine and colleagues who demonstrated the activity of p70S6K and subsequent increases in muscle mass were mTORC1-dependent (Bodine et al. 2001b). However, despite evidence implicating IGF-mediated increases in muscle protein synthesis with contraction through its canonical growth factor pathway, recent studies showing IGF-1 independent activation of downstream signalling targets indicates IGF-1 is not essential for increases in anabolic signalling for skeletal muscle growth (Hornberger et al. 2004 , Philp, Hamilton and Baar 2011, Spangenburg et al. 2008, Spiering et al. 2008).

Goodman and colleagues used electroporation in skeletal muscle from mice to demonstrate mTOR activation independent of upstream PI3K-Akt signalling, and that this activation was sufficient to induce skeletal muscle hypertrophy (Goodman et al. 2010). These findings are supported by West and co-workers who manipulated the volume of muscle mass used during an

acute resistance exercise bout to generate a 'high' versus 'low' hormonal milieu. Despite a 10-fold disparity in circulating plasma IGF-1 concentration no differences in acute Akt-p70S6K phosphorylation or muscle protein synthesis were observed during the early (4 h) recovery period (West et al. 2009). Accordingly, the physiological effects, if any, of IGF during recovery from exercise appear inconsequential compared with the direct effect of contraction and/or nutrition in modulating IGF signalling pathway activity for translation initiation and protein synthesis in skeletal muscle.

Akt, also referred to as protein kinase B (PKB), is a critical junction in the insulin/IGF pathway and regulates glucose transport and maintenance of cell size in skeletal muscle. When co-localized to the cell membrane, Akt is phosphorylated and activated by phosphorylation at Thr^{308} by pyruvate dehydrogenase kinase 1 (PDK1) (McManus et al. 2004) and at Ser^{473} by mechanistic target of rapamycin complex 2 (mTORC2) (Sarbassov et al. 2005) for full activation. Three isoforms of Akt exist and are expressed in a tissue specific manner but Akt1 and Akt2 are predominantly expressed in skeletal muscle (Jones et al. 1991, Nader 2005). Akt activation down-regulates pathways controlling muscle protein breakdown via the ubiquitin-proteasome and autophagy/lysosomal systems. The phosphorylation of the nuclear transcription factor FoxO by Akt has been shown to sequester FoxO from the nucleus to the cytosol preventing transcription of a number of atrogenes including the atrophy related E3 ubiquitin ligase atrogin-1 (also known as MAFbx) and muscle-specific ring finger-1 (MuRF-1) (Sandri et al. 2004, Stitt et al. 2004). Moreover, Akt-mediated control of FoxO attenuates the transcription of several autophagy related genes that promote lysosomal degradation (Mammucari et al. 2007, Zhao et al. 2007).

Akt activation can also mediate uptake of glucose into muscle cells through its phosphorylation and inhibition of the downstream target Akt substrate of 160 kDa (AS160). The phosphoylation of AS160 allows GLUT4 to translocate to the cell membrane and promote glucose transport into the muscle cell (Howlett et al. 2008). Akt can also direct glycogen synthesis through phosphorylation (and inhibition) of glycogen synthase kinase 3 (GSK-3) to activate glycogen synthase (Graham et al. 2010).

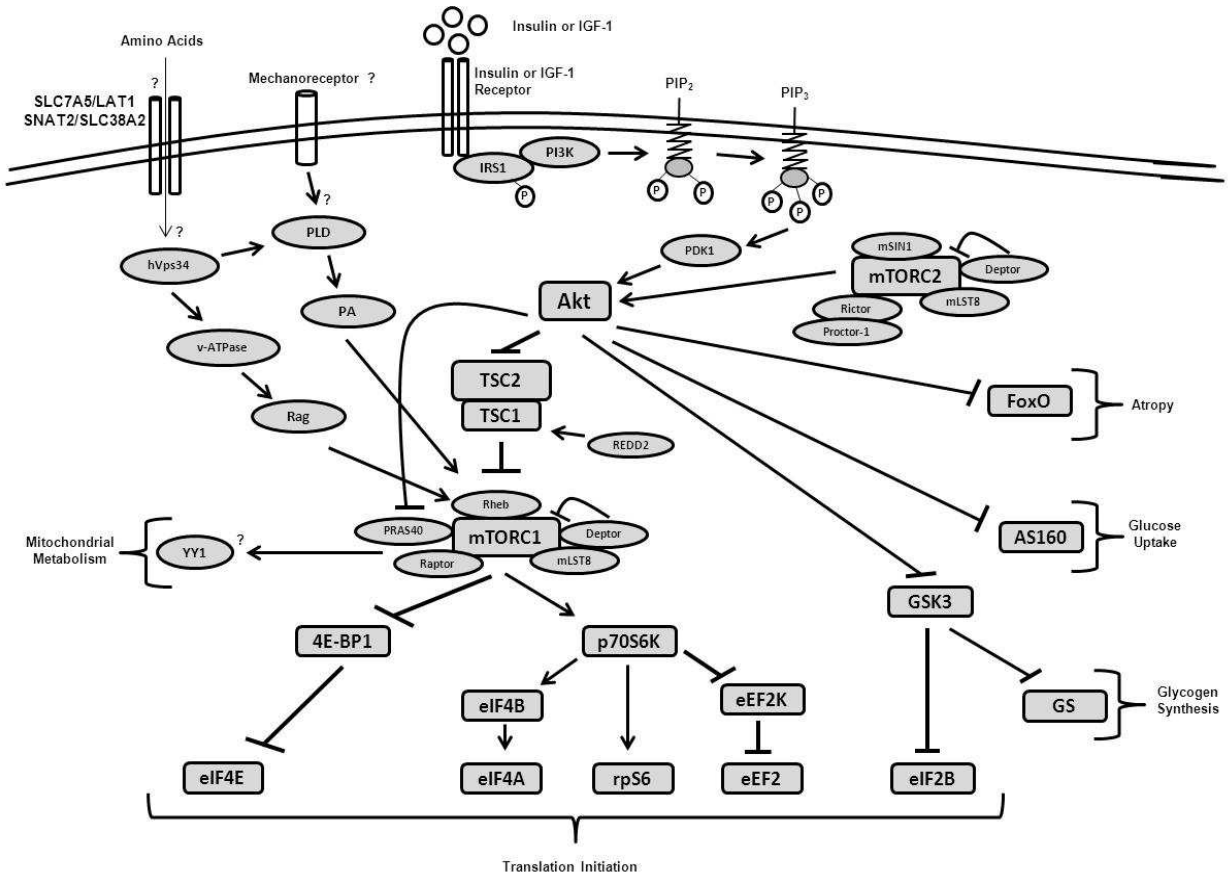

Figure 1.6 Schematic representation of the IGF signalling pathway involved in the muscle adaptation response to exercise. Bars denote inhibition and arrows denote activation. AS160, Akt substrate of 160 kDa; Deptor, DEP-domain-containing mTOR-interacting protein; eEF2, Elongation factor 2; eEF2K, Elongation factor 2 kinase; eIF4A/B/E, Eukaryotic translation initiation factor 4A/B/E; FoxO, Forkhead box subgroup O; GS, Glycogen Synthase; GSK-3, Glycogen Synthase Kinase 3; hVps34, human vacuolar protein sorting 34; IRS1, Insulin receptor substrate 1; LAT1, L-type amino acid transporter; PDK1, Phosphoinositide-dependent kinase-1;PRAS40, proline-rich AKT substrate 40 kDa; Proctor 1, protein observed with Rictor-1; mLST8, Mammalian lethal with Sec13 protein 8; mSIN1, mammalian stress-activated protein kinase interacting protein 1; mTORC1/2, Mechanistic target of rapamycin complex 1/2; PA, Phosphatidic Acid; PLD, Phospholipase D; PI3K, Phosphoinositide 3-kinase; Raptor, Regulatory associated protein of mTOR; Rag, Ragulator; REDD2, regulated in development and DNA damage response 2; Rheb, Ras homolog enriched in brain; Rictor, rapamycin-insensitive companion of mTOR; rps6, ribosomal protein S6; SLC7A5, Solute carrier family 7 member 5; SNAT2, Sodium-coupled Neutral Amino Acid Transporter 2; TSC, Tuberous sclerosis complex; YY1, yin yang 1.

Another downstream target of Akt includes the highly conserved serine/threonine protein kinase mTOR. mTOR has emerged as a major regulator of cell growth and proliferation through integrating signals from growth factors, contraction, nutrients, and changes in energy status to regulate the initiation of protein translation (Hay and Sonenberg 2004). mTOR exists as two multi-protein complexes with each exhibiting different cellular functions (Figure 1.6). Regulation of mTORC1 by Akt occurs either directly by phosphorylation at Ser^{2448} or through the phosphorylation of TSC2 and PRAS40.

mTORC1 is activated by amino acids but the primary/upstream amino acid sensor/s is currently unknown. Recent evidence indicates the Rag family of small GTPases are the primary signalling intermediaries between amino acids and mTORC1 (Kim et al. 2008, Sancak et al. 2008). The Rag GTPases are found in an inactive conformation and amino acids are able to switch the Rag GTPases to their active conformation and are proposed to activate mTORC1 through interaction with the Raptor subunit (Kim et al. 2008, Sancak et al. 2008, Sancak et al. 2007, Saucedo et al. 2003). An additional regulatory mechanism of mTORC1 signalling is the lipid second messenger phosphatidic acid (PA) (Foster 2007). O'Neil and co-workers have demonstrated mechanical stress-induced elevations in PA are able to activate mTOR through a PI3K–Akt-independent mechanism (O'Neil et al. 2009) but these findings are yet to be confirmed *in vivo* human skeletal muscle. Finally, mTORC1 activity may also be modulated by an indirect interaction with the regulated in development and DNA damage response 2 (REDD2) protein. Miyazaki and Esser (2009) recently demonstrated that overexpression of REDD2 reduced activation of mTOR in muscle cells despite mechanical stretch and amino acid stimulation (Miyazaki and Esser 2009).

mTORC1's regulation of muscle protein synthesis is primarily mediated through the phosphorylation of its downstream targets including the ribosomal protein S6 kinase (S6K1) and the eukaryotic initiation factor 4E binding protein 1 (4E-BP1) (Navé et al. 1999). mTORC1's phosphorylation of p70S6K at Thr^{389} can promote activation of the ribosomal protein S6 (rpS6) via phosphorylation at $Ser^{235/236}$ and the eukaryotic elongation factor 2 kinase (eEF2K) at Ser^{366} (Roux et al. 2007, Wang et al. 2001). Activation of rpS6 promotes translation initiation by increasing the affinity of ribosomes for binding 5'terminal oligopyridimine (5'TOP) mRNAs (Ruvinsky et al. 2005) which encode ribosomal proteins and elongation factors (eEF1A and eEF2) (Terada et al. 1994). The phosphorylation of eEF2 kinase removes its inhibition on eEF2,

resulting in enhanced translation elongation (Browne and Proud 2002). mTORC1 can also enhance cap-dependent translation through its phosphorylation of 4E-BP1 at $Thr^{37/46}$ derepressing its inhibition of eIF4E- eIF4G complex formation (eIF4F) for translation initiation (Holz et al. 2005, Parsa and Holland 2004, Wang and Proud 2006).

mTOR may regulate other cellular processes that can mediate adaptation to exercise. For example, mTORC1 activity has been shown to directly correlate with mitochondrial metabolism, and the expression of select mitochondrial genes are controlled by mTORC1 (Cunningham et al. 2007, Schieke et al. 2006). The investigations into mitochondrial biogenesis led to the discovery that the activity of transcription factor yin yang 1 (YY1) was regulated by mTORC1 (Cunningham et al. 2007, Shi et al. 1991). Considering the 'master regulator' of mitochondrial biogenesis, PGC-1α, is known to function as a transcriptional co-activator for YY1 in an mTOR-dependent manner it has been proposed that a decrease in mTOR activity inhibits YY1-PGC-1α function leading to decreased expression of mitochondrial genes (Cunningham et al. 2007).

Numerous studies have reported increases in exercise-induced IGF signalling in human skeletal muscle while others have reported decreases or no change in the phosphorylation status of this cascade with exercise. Table 1.1 provides an overview of literature reporting insulin/IGF pathway signalling responses after an acute bout of resistance or endurance exercise in human skeletal muscle in the fasted state. The variability in responses is likely the result of differences in the type, intensity and duration of the contractile stimulus, subject training status, and timing of muscle biopsies. Consequently, the precise role of the IGF signalling pathway in promoting or inhibiting molecular processes important for exercise-induced adaptation with divergent exercise (i.e. endurance and resistance training) and their contribution to the specificity of training adaptation in skeletal muscle remains unclear.

Nonetheless, exercise-induced activation of cell signalling pathways (i.e: IGF pathway) modulating the phosphorylation of proteins proximal to translation contributes to the overall control of protein synthesis in skeletal muscle. The next section of this review describes the molecular mechanisms regulating muscle protein balance and turnover (i.e.: muscle protein synthesis and breakdown).

Table 1.1: Summary of insulin/IGF pathway signalling responses in the *vastus lateralis* after acute exercise conducted in humans in the fasted state

Reference	**Protocol**	**Akt**	**mTOR**	**TSC2**	**p70S6K**	**rpS6**	**4E-BP1**
		Ser^{473}	Ser^{2448}	Thr^{1462}	Thr^{389}	$Ser^{235/6}$	$Thr^{37/46}$
Resistance Exercise							
Apro 2010	LP: 4 x 10 @ 80% 1RM + 4 x 15 @ 65% 1RM	↔	↑	—	↔	↑	—
Borgenvik 2012	LP: 4 X 10 @ 80% 1RM + 4 x 15 @ 65% 1RM	—	↑	—	↑	—	—
Breen 2011a	LP: 8 X 10 @ 75% 1RM + LE: 8 X 10 @ 75% 1RM	↑	—	—	↔	—	—
Créer 2005	LE: 3 x 10 @ 70% 1RM	↑	↔	—	—	—	—
Deldicque 2008	LE: 10 x 10 @ 80% 1RM	↓	—	—	↔	—	↓
Dreyer 2006	LE: 10 x 10 @ 70% 1RM	↑	↑	↓	↑	—	↓
Eliasson 2006	LP: 4 x 6 @100% 1RM Con	↔	↔	—	↔	↔	—
	LP: 4 x 6 @100% 1RM Ecc	↔	↔	—	↑	↑	—

Arrows denote phosphorylation status ($p < 0.05$): ↑ Increased, ↓ Decreased, ↔ No change, — not measured. RM, Repetition Maximum; LP, Leg Press; LE, Leg Extension; KE, Knee Extension; Ecc, Eccentric; Con, Concentric; S, Squat.

Reference	Protocol	Akt Ser^{473}	mTOR Ser^{2448}	TSC2 Thr^{1462}	p70S6K Thr^{389}	rpS6 Ser$^{235/6}$	4E-BP1 Thr$^{37/46}$
Fry 2011	LE: 8 x 10 @ 70% 1RM	↑	↑	—	↑	↑	↑
Glover 2008	LP: 4 x 10 @ 10RM + KE: 4 x 10 @ 10RM	↑	↔	—	↑	↑	—
Holm 2010	KE: 10 x 36 @ 16% 1RM	↑	—	—	↔	—	↔
	KE: 10 x 8 @ 70% 1RM	↑	—	—	↔	—	↑
Hulmi 2009a	LP: 5 x 10 @ 70% 1RM	↔	↔	—	↔	↑	↓
Karlsson 2004	LP: 4 x 10 @ 80% 1RM	—	—	—	↔	↔	—
Koopman 2006b	LP: 8 x 10 @ 75% 1RM +8 x 10 @ 75% 1RM LE	—	—	—	↑	↑	↓
Kumar 2009	LE: 3 X 9 @ 60% 1RM, or 3 x 8 @ 75% 1RM, or 6 x 8 @ 90% 1 RM	—	—	—	↑	—	↑
Mascher 2008	LP: 4 X 10 @ 80% 1RM LP	↔	↑	—	↑	↑	—
Mayhew 2009	LP, KE, S: 3 x 8-12 each	↑	↔	—	↔	↔	↑

Arrows denote phosphorylation status ($p < 0.05$): ↑ Increased, ↓ Decreased, ↔ No change, — not measured. RM, Repetition Maximum; LP, Leg Press; LE, Leg Extension; KE, Knee Extension; Ecc, Eccentric; Con, Concentric; S, Squat,

Reference	Protocol	Akt	mTOR	TSC2	p70S6K	rpS6	4E-BP1
		Ser^{473}	Ser^{2448}	Thr^{1462}	Thr^{389}	$Ser^{235/6}$	$Thr^{37/46}$
Reitelseder 2010	LE: 10 X 8 @ 80% 1RM	↔	—	—	↔	—	↑
Terzis 2010	LP: 1 x 6RM or 3 x 6RM	↔	↑	—	↑	↑	—
Endurance Exercise							
Benziane 2008	CYC: 60 min @ 70% VO_{2peak}	↔	↑	—	↓	↑	—
Ivy 2008	CYC: 45 min @ 75% VO_{2peak} + 5 x 1 min sprints @ 90% VO_{2peak}	↔	↔	—	↑	↑	—
Mascher 2007	CYC: 60 min @ 75% VO_{2peak}	↑	↑	—	↔	—	—
Mascher 2011	CYC: 60 min @ ~70% 1L VO_{2peak}	↔	↑	—	↑	—	—
Rose 2009b	CYC: 30 min @ 30% VO_{2peak}	—	—	—	—	—	↓

Arrows denote phosphorylation status ($p < 0.05$): ↑ Increased, ↓ Decreased, ↔ No change, — not measured. RM, Repetition Maximum; LP, Leg Press; LE, Leg Extension; KE, Knee Extension; Ecc, Eccentric; Con, Concentric; S, Squat;

1.4 Skeletal Muscle Protein Turnover

Muscle protein turnover refers to the continuous and simultaneous processes of muscle protein synthesis (MPS) and muscle protein breakdown (MPB) (Phillips et al. 2005). The net balance between these two processes determines whether skeletal muscle tissue is increasing (hypertrophy) or decreasing (atrophy) total protein content (Burd et al. 2009, Cuthbertson et al. 2004). Muscle hypertrophy can only occur when there is an accumulation of muscle proteins during repeated periods of anabolism (i.e. resistance exercise, protein ingestion) that exceeds the loss of muscle proteins during intervening periods of catabolism (Burd et al. 2009, Cuthbertson et al. 2004) (Figure 1.7).

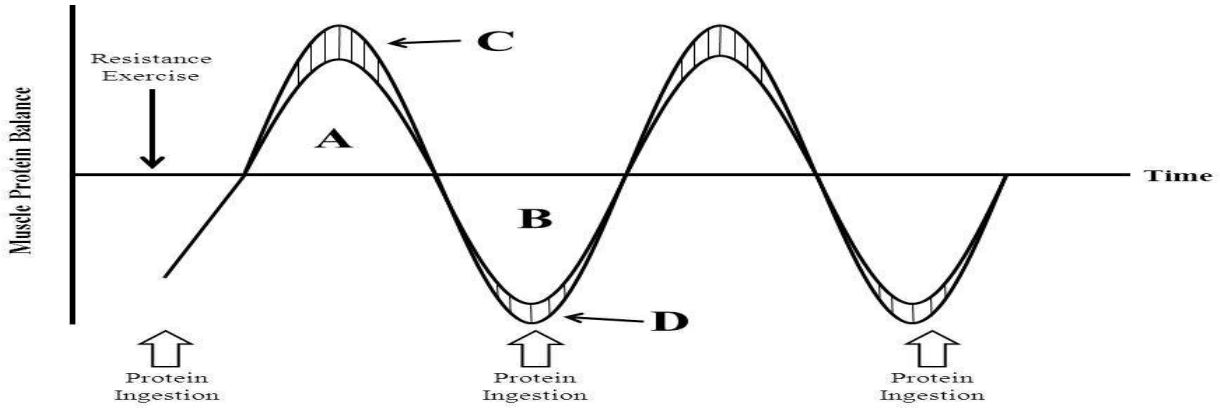

Figure 1.7 (A) Normal fed state gains (synthesis) and (B) losses (breakdown) in skeletal muscle protein balance following resistance exercise. Hatched areas at the peak and nadir points of oscillations indicate the beneficial effects of contractile activity and protein ingestion that result in fed-state gains (C) and reduced fasted-state losses (D) to enhance net protein balance. Figure adapted from (Phillips 2004).

When compared with other tissues such as the liver and gut, skeletal muscle protein turnover is relatively slow, contributing only 25 – 30% of the total body protein turnover at a rate of 1 - 2% per day (Wagenmakers 1998b). As muscle proteins are continually damaged and require repair during activities of daily living, turnover is necessary to maintain protein quality and function. In the resting, fasted state (or postabsorptive state) muscle protein net balance is negative, with a

positive balance achieved after consumption of protein. However, protein balance is also altered by stimuli such as injury, disease, fasting and diet. Importantly, modulating protein synthesis and degradation is the mechanistic underpinning for skeletal muscle plasticity that permits exercise-induced adaptation and can result in an enhanced muscle phenotype (Phillips 2004).

Amino acids are the functional subunits of proteins and are mediators of changes in protein turnover. Less than 2% of total amino acids in the human body exist in their free forms, with approximately half of this population present at the intracellular level in skeletal muscle (Wagenmakers 1998a). Amino acids enter this free amino acid pool via import into muscle from the circulation or *de novo* synthesis of essential amino acids as the product of muscle protein breakdown (Figure 1.8). Amino acids in the intramuscular free pool can also be oxidized, exported into blood or metabolised (TCA cycle). Exercise and dietary status can alter the overall number and flux of amino acids into and out of the free pool and therefore mediate subsequent changes in muscle protein synthesis and degradation.

The following section will discuss the processes regulating muscle protein turnover and how these changes in turnover can be quantified.

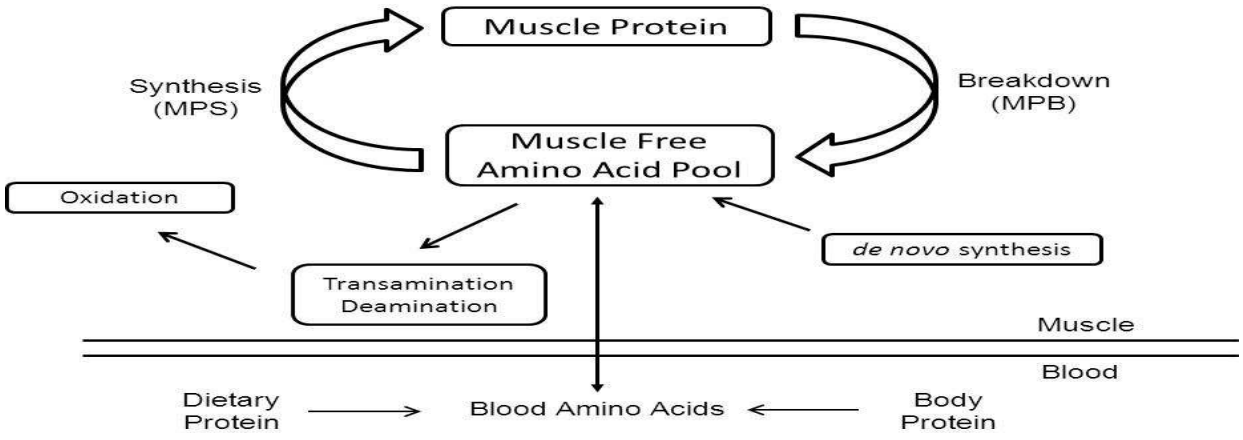

Figure 1.8 General overview of muscle protein turnover and the major sources and metabolic processes that influence the free amino acid pool in skeletal muscle. Schematic adapted from (Phillips 2004, Gibala 2001).

Muscle Protein Synthesis

The process of MPS includes the translation of messenger RNA (mRNA) to form protein. Translation of mRNA involves three phases: initiation, elongation and termination. Each of these three phases are coordinated by eukaryotic initiation factors (eIFs), elongation factors (eEFs), and release factors (eRFs), respectively (Kimball and Jefferson 2010, Proud 2007). The initiation phase has been shown to be a rate limiting step in the protein synthetic process (Jackson, Hellen and Pestova 2010, Sonenberg and Hinnebusch 2009) regulated, at least in part, by several downstream targets of the mTORC1 signalling cascade (Figure 1.6). Control of translation initiation is implicated in regulation of both global rates of protein synthesis and specific subsets of mRNAs. There are several informative reviews detailing the process of translation (Jackson et al. 2010, Kimball and Jefferson 2010, Sonenberg and Hinnebusch 2009). Briefly, translation initiation involves the binding of eukaryotic initiation factor 2 (eIF2) to initiator methionyl-tRNA (met-tRNAi), a process that involves a guanine nucleotide exchange that is mediated by eIF2B. This regulatory point in global translation initiation is mediated by eIF2α via its inhibitory effect on eIF2B. The final step in translation initiation is the formation of an 80S initiation complex (Kimball, Farrell and Jefferson 2002). The amino acid-charged transfer RNA (tRNA) then sequesters amino acids to the ribosome complex and commences the elongation step. This step is characterised by the sequential bonding and subsequent elongation of tRNA-bound amino acids through peptide bonds in accordance with the mRNA code. The final stage of translation is characterised by the termination of the polypeptide chain from the ribosome-protein complex as mediated by the presence of a stop codon. Modulation of MPS has been shown to be the result of increased mRNA translational efficiency, primarily at the stage of initiation (Holz et al. 2005, Hornberger, Sukhija and Chien 2006b, Welle, Bhatt and Thornton 1999).

Methods to Assess Skeletal Muscle Protein Synthesis

Several methods have been used to measure muscle protein synthesis in humans (Gore, Wolfe and Chinkes 2007, Zhang, Chinkes and Wolfe 2002, Rennie 1999). A common approach to quantify rates of skeletal muscle protein synthesis is determining the fractional synthetic rate (FSR) of muscle proteins through the use of stable isotope tracer methodology. The FSR of muscle protein is the rate at which a labelled amino acid is incorporated into muscle-bound

protein over time compared with the enrichment of the precursor pool from which the labelled amino acid originated prior to being incorporated into intact protein (Rennie, Smith and Watt 1994). This can be achieved by administering a primed continuous intravenous infusion of an isotopically labelled amino acid tracer. Phenylalanine is preferentially used as a tracer as it is neither oxidized nor synthesised in the body (Phillips et al. 1997). Therefore, with no *de novo* synthesis, the disappearance (Rd) or reappearance (Ra) of phenylalanine is commensurate with changes in protein synthesis and breakdown, respectively (Phillips et al. 1997, Rennie 1999 , Wolfe 1992). A prerequisite for the valid estimation of muscle protein FSR is that an isotopic steady state between the tracer and tracee is reached to ensure incorporation of the tracer into protein occurs in a linear fashion. Muscle biopsies obtained during an experimental trial are used for laboratory analysis where the protein is precipitated, hydrolysed and the amino acids, after derivatisation, are analysed to determine the tracer incorporated into the muscle-bound protein as a rate in per cent per unit time using gas chromatography-mass spectrometry (GC-MS) or gas chromatography-isotope ratio mass spectrometry (GC-IRMS) technology (Rennie et al. 1994). The increment in tracer enrichment is divided by the precursor pool tracer enrichment obtained from either plasma free amino acid, the tissue intracellular free amino acid (IC) or aminoacyl-tRNA pool (if obtainable), to determine the fraction of the muscle protein pool that has been synthesized per hour as shown in the following equation (Phillips et al. 1997, Wolfe and Chinkes 2005):

$$\text{FSR (\%h)} = \frac{Et_1 - Et_0}{E_p \times (t_1 - t_0)} \times 100$$

Et_0 = bound protein enrichment of chosen labelled amino acid at first specified time point

Et_1 = bound protein enrichment of chosen labelled amino acid at second (later) specified time point

$(t_1 - t_2)$ = time between muscle biopsy samples

E_p = average muscle intracellular enrichment of chosen labelled amino acid from t_0 to t_1

Recent studies have demonstrated that the measurement of mixed muscle protein synthesis following exercise and/ or nutrient supplementation may not accurately reflect alterations in the synthetic rates of the different protein fractions and therefore not represent the true phenotypic response (Louis et al. 2003, Rooyackers et al. 1996). Advances in laboratory technology in the past two decades have also led to the development of appropriate methods to reliably quantify physiological changes in the different muscle protein fractions (i.e. myofibrillar, sarcoplasmic and mitochondrial). The analysis of each distinct protein fraction is likely to provide a more accurate reflection of the specificity of adaptation responses following divergent exercise stimuli.

Muscle Protein Breakdown

The process of muscle breakdown eliminates dysfunctional proteins from the cell and generates free amino acids for protein synthesis or metabolism (Mitch and Goldberg 1996). Muscle contraction with exercise inevitably results in damage of some intracellular proteins. Intuitively, the proteolysis associated with exercise is unlikely to be entirely detrimental and is a requirement for muscle remodelling and adaptation. Compared to muscle protein synthesis, quantification of muscle protein breakdown *in vivo* is technically difficult due to greater methodological complexities (Zhang et al. 2002). The 'tracee release' model provides the most direct measure of mixed muscle protein breakdown and utilises a primed constant infusion of stable isotope to assess the rate at which a tracee (i.e. unlabelled essential amino acid) is released from intact muscle protein and dilutes the intracellular enrichment. The rationale behind this model is that essential amino acids can only enter the muscle intracellular free amino acid pool via transport through arterial blood or bound muscle proteolysis (Zhang et al. 2002). The regulation and activity of proteolytic pathways *in vivo* human muscle in response to exercise remain poorly defined. Current evidence suggests three major proteolytic systems work independently or synergistically to promote protein breakdown in skeletal muscle (Figure 1.9): the calcium-dependent calpain (Zeman et al. 1985, Murphy 2010), the lysosomal protease/cathepsins (Bird et al. 1980) and the ubiquitin-proteasome systems (Fagan, Waxman and Goldberg 1987).

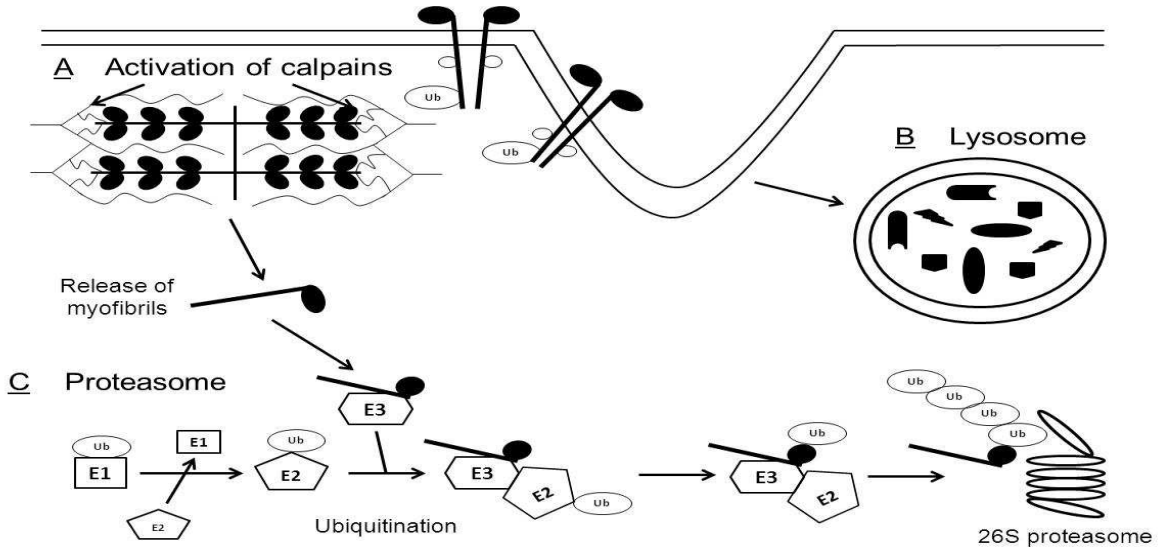

Figure 1.9 The three major proteolytic systems implicated in muscle protein degradation/ atrophy. The calcium-dependent calpain system (A) is associated with the breakdown of muscle proteins involved in the assembly and scaffolding of myofibrillar proteins. The lysosomal protease system (B) directs the degradation of membrane proteins, including receptors, ligands, channels and transporters. The ubiquitin-proteasome system (C) is the major pathway responsible for intracellular protein degradation in skeletal muscle (~ 80%) and involves the identification and subsequent 'tagging' of proteins to be degraded through the interaction of three classes of proteins termed E1 (ubiquitin activating), E2 (ubiquitin conjugating) and E3 (ubiquitin ligating) enzymes. Adapted from (Jackman and Kandarian 2004).

While these different proteolytic pathways are all likely to mediate muscle protein breakdown and remodelling responses, the time course of activation and potential role of each in the adaptation responses to exercise in human skeletal muscle is not clear. Moreover, the inherent methodological difficulty of measuring muscle protein breakdown *in vivo* has resulted in few studies investigating this component of muscle protein balance. Nonetheless, studies investigating the mRNA and protein expression patterns of these putative regulators of muscle protein breakdown show changes following exercise in human skeletal muscle.

1.5 Nutrient Training Interactions

Manipulating the availability of nutrients before, during and after exercise alters the adaptation response in skeletal muscle (Burke, Kiens and Ivy 2004, Hawley et al. 2011, Beelen et al. 2010). Indeed, acute dietary manipulation can exert profound effects on skeletal muscle gene expression (Arkinstall et al. 2004, Cameron-Smith et al. 2003), cell signalling (Creer et al. 2005, Wojtaszewski et al. 2003), muscle protein synthesis (Drummond et al. 2009, Phillips et al. 2005) and other processes involved in training adaptation (Hansen et al. 2005, Morton et al. 2009, Yeo et al. 2008). Nutrient ingestion during post-exercise recovery is important to replenish endogenous substrate stores and to facilitate repair and remodelling of skeletal muscle. Protein and carbohydrate are widely studied macronutrients with regard to their capacity to generate changes in exercise-induced adaptation and athletic performance. Each macronutrient has distinct functions, but can also work synergistically to generate an anabolic state within skeletal muscle when co-ingested after an exercise bout. This section will briefly outline the impact of carbohydrate and protein ingestion on adaptation responses to exercise in skeletal muscle.

Carbohydrate

Insulin and exercise stimulate glucose uptake in skeletal muscle by promoting GLUT4 translocation to the cell membrane. Glucose not immediately required for energy is converted to the polysaccharide glycogen and stored in skeletal muscle and the liver. Innovative imaging techniques have identified glycogen granules present in skeletal muscle in at least three distinct subcellular locations with one large compartment (~ 80%) between the myofibrils and two smaller compartments (~ 10%) located within the myofibrils and the sarcolemma (Nielsen et al. 2011, Prats, Gómez-Cabello and Hansen 2011). Nielsen and co-workers recently reported exhaustive arm and leg endurance exercise depletes intramyofibrillar glycogen stores significantly more than the intermyofibrillar and subsarcolemmal depots (Nielsen et al. 2011). Whether the same subcellular utilisation pattern occurs following high intensity resistance exercise remains to be established.

Glucose and intramuscular glycogen become increasingly important energy substrates with increasing exercise intensity (Holloszy and Kohrt 1996). Muscle glycogen content can be reduced by ~25% in the acute recovery period (i.e. 4 h) following a single bout of resistance exercise (Robergs et al. 1991, Churchley et al. 2007) compared with reductions of ~ 50% or greater after

high intensity endurance exercise (Wojtaszewski et al. 2003, Bergström and Hultman 1967, Vøllestad and Blom 1985, Vøllestad, Vaage and Hermansen 1984). Following exercise, muscle glycogen is typically restored to pre-exercise levels within 24 h (Piehl 1974b, Piehl 1974a). Rates of glycogen resynthesis are dependent on sufficient amounts of carbohydrate being ingested (Burke et al. 1995, Costill et al. 1981) and to a lesser extent the magnitude of post-exercise muscle glycogen depletion (Price et al. 1994). In this regard, both the amount and timing of post-exercise carbohydrate ingestion are the most important factors determining the rate of muscle glycogen synthesis (Beelen et al. 2010, Burke, Loucks and Broad 2006, Costill et al. 1981, Jentjens and Jeukendrup 2003). Post-exercise muscle glycogen synthesis rates in the fasted state are ~ 11 mmol·kg $dw^{-1}\cdot h^{-1}$ (Ivy et al. 1988b) and can increase up to 20 - 45 mmol·kg $dw^{-1}\cdot h^{-1}$ when carbohydrate is consumed immediately after exercise (Ivy et al. 1988a, Ivy et al. 1988b, Jentjens et al. 2001, Maehlum, Høstmark and L. 1977, van Loon et al. 2000a). Recent work investigated the effects of protein/ amino acid co-ingestion with carbohydrate show no increases in post-exercise muscle glycogen synthesis when sufficient carbohydrate (i.e.: 1.2g·$kg^{1}\cdot h^{-1}$) is ingested at regular intervals (every 15-30 min) (Beelen et al. 2010).

Over the last decade it has become apparent glycogen concentration is an important molecular signal modulating the adaptation response to exercise (Hargreaves 2004). Many skeletal muscle proteins/enzymes contain glycogen binding domains that directly regulate their activity, localisation, structure and function which is altered by changes in glycogen concentration (Machovič and Janeček 2006). Substrate metabolism is altered in a glycogen depleted state resulting in increases in the systemic release of amino acids from muscle protein breakdown (Blomstrand and Saltin 1999) along with elevations in fat metabolism and plasma free fatty acids (FFA) (Steensberg et al. 2002). The increase in circulating free fatty acids provides substrate for β-oxidation in the mitochondria and also act as a signalling intermediary to activate transcription factors and nuclear receptors that regulate proteins involved in the transport and breakdown of lipids (Kiens 2006). Given exercise induces dynamic changes in skeletal muscle glycogen concentration, altered glycogen availability has been shown to generate transient changes in mRNA levels of myogenic, catabolic and metabolic genes after an acute bout of exercise in humans (Churchley et al. 2007, Coffey et al. 2009a, Coffey et al. 2009b, Harber et al. 2010, Cluberton et al. 2005, Pilegaard et al. 2002, Pilegaard et al. 2000). Accordingly, it has been

proposed that commencing training with low muscle glycogen may improve subsequent adaptation to endurance training (Hawley et al. 2011).

Several studies have investigated the adaptive responses to exercise commenced with low or sub-optimal glycogen levels. Wojtaszeski and co-workers first observed elevated resting and exercise-induced AMPK activity with low (~ 160 mmol·kg·dw^{-1}) compared with high (~ 910 mmol·kg·dw^{-1}) muscle glycogen following endurance exercise (Wojtaszewski et al. 2003). Similarly, others have shown increases in mRNA and protein levels of p38 MAPK (Chan et al. 2004), heat shock protein 72 (HSP72) (Febbraio et al. 2002) and GLUT4 (Steinberg et al. 2006) when endurance exercise was commenced with low compared to normal muscle glycogen concentration. Hansen and colleagues were the first to examine the 'train-low, compete high' paradigm following a 10 week training study and showed resting muscle glycogen content, citrate synthase activity, and exercise time to exhaustion were enhanced when training sessions were commenced with low compared to normal muscle glycogen content (Hansen et al. 2005). Several other studies have investigated this paradigm (Morton et al. 2009, Yeo et al. 2010, Yeo et al. 2008, Hulston et al. 2010) and despite showing enhanced activation of several markers of endurance adaptation when training with low glycogen concentration, no conclusive benefit to endurance performance has been established.

There is a paucity of data examining adaptation responses to resistance exercise commenced with low muscle glycogen concentration. Creer and colleagues showed an attenuation in Akt phosphorylation during the acute recovery period when subjects commenced a bout of moderate-intensity resistance exercise with low (~ 175 dry weight (dw)$^{-1}\cdot$h^{-1}) versus high (~ 600 dw$^{-1}\cdot$h^{-1}) muscle glycogen (Creer et al. 2005). Moreover, Churchley and co-workers observed an attenuated mRNA abundance of genes associated with hypertrophy, carbohydrate metabolism and muscle atrophy at rest prior to undertaking resistance exercise with low (~ 193 dw$^{-1}\cdot$h^{-1}) compared to normal (~ 435 dw$^{-1}\cdot$h^{-1}) glycogen concentration (Churchley et al. 2007). The effects of commencing resistance exercise with low muscle glycogen concentration remains unclear and whether post-exercise nutrient ingestion following resistance exercise commenced with low glycogen concentration augments or inhibits any adaptation response has yet to be investigated.

Protein

Protein ingestion enhances muscle protein synthesis and promotes muscle hypertrophy (Beelen et al. 2010, Burd et al. 2009, Phillips et al. 2005). The anabolic capacity of dietary protein to stimulate muscle protein synthesis is initiated by the transfer and incorporation of amino acids from these dietary protein sources into skeletal muscle proteins. Muscle anabolism stimulated by protein feeding balances against the periods of net catabolism that occurs between feedings to generally ensure muscle mass remains constant in young healthy adults. Rennie and colleagues first demonstrated the anabolic capacity of protein in human skeletal muscle (Rennie et al. 1982), and this response was later shown to be attributable to ingestion of essential amino acids (EAA) (Smith et al. 1992). Elevated concentrations of EAA in plasma transported in to the muscle cell directly stimulate the muscle's translational machinery (Drummond et al. 2009, Proud 2007, Apró and Blomstrand 2010) and provides amino acid precursors for the synthesis of muscle proteins (Bohé et al. 2003, Fujita et al. 2006, Reitelseder et al. 2011). The stimulation of myofibrillar and sarcoplasmic protein synthesis by amino acid infusion or beverage ingestion at rest is transient and characterised by a peak in muscle protein synthesis approximately 90 min after ingestion before returning to basal levels 3 h after feeding (Atherton et al. 2010, Bohé et al. 2001, Moore et al. 2009b). The inevitable fall in muscle protein synthesis can occur despite continued amino acid availability, a mechanism referred to as the ‘muscle full’ paradigm where increases in protein synthesis are regulated by an intracellular set point rather than sustained elevation in plasma/ extracellular amino acid bioavailability (Atherton et al. 2010, Bohé et al. 2001). Regardless, amino acids delivered in excess to requirements of the muscle synthetic machinery generally undergo irreversible oxidation (Moore et al. 2009a).

The type of protein (e.g. whey, casein, soy), and digestion and absorption rates are important factors in the nutritional regulation of muscle protein synthesis in humans. Tang and colleagues have shown feeding-induced stimulation of muscle protein synthesis is greater after whey protein consumption compared to casein at rest and after resistance exercise (Tang et al. 2009). The authors proposed this enhanced response was also related to differences in the leucine content of each protein source. Several studies have demonstrated leucine to be a potent anabolic stimulus for skeletal muscle protein synthesis (Fujita et al. 2007, Koopman et al. 2005, Rieu et al. 2006).

Moreover, other branched chain amino acids such as isoleucine and valine are unable to induce comparable increases in muscle protein synthesis compared with leucine (Escobar et al. 2006, Garlick 2005). These findings provide support for a 'leucine trigger' paradigm where maximal rates of muscle protein synthesis are stimulated once a minimum leucine concentration (i.e. threshold) is achieved (Phillips 2011). Based on current evidence an oral dose of ~ 2 g of leucine contained in 20 g of high quality protein ingested following a resistance exercise bout appears sufficient to invoke maximal rates of muscle protein synthesis (Moore et al. 2009a). Therefore, ingestion of leucine in amounts greater than this 'saturating dose' (20 – 25 g whey protein containing 2.5 - 3.0 g leucine) is unlikely to increase the magnitude or duration of muscle protein synthesis during the early post-exercise recovery period (Tipton et al. 2009).

Insulin stimulates increased blood flow through binding of its surface receptor on the vascular endothelium which increases the synthesis of nitric oxide and causes the smooth muscle at capillary junctions to relax, thereby decreasing the resistance of the capillary to blood flow (Muniyappa and Quon 2007b). This increased blood flow may enhance delivery of amino acids to the muscle (Biolo et al. 1999, Fujita et al. 2006, Muniyappa and Quon 2007a). Early evidence implicated insulin as an anabolic hormone that directly increases muscle protein synthesis when administered alone (Gelfand and Barrett 1987). Of note, others have since demonstrated that only basal concentrations of insulin are required to permit the maximal stimulation of muscle protein synthesis when optimal levels of exogenous amino acids are available (Glynn et al. 2010, Greenhaff et al. 2008). Several studies have demonstrated hyperinsulinemia to inhibit muscle proteolysis (Biolo et al. 1999, Gelfand and Barrett 1987, Pozefsky et al. 1969), thereby stimulating muscle protein accretion resulting from an increase in net protein balance. Accordingly, the co-ingestion of amino acids with carbohydrate may promote muscle protein synthesis by eliciting an insulinotropic response and inhibiting protein breakdown (Beelen et al. 2010, van Loon et al. 2000b). However, Staples and co-workers have elegantly shown that when sufficient protein is ingested to maximally stimulate protein synthesis, the effect of insulin on muscle protein synthesis and breakdown is neither additive nor synergistic (Staples et al. 2011). Therefore, under specific conditions insulin may be permissive rather than stimulatory for the anabolic effect of amino acids but this physiological response remains contentious.

1.6 Specificity of Exercise-induced Adaptation in Skeletal Muscle

Exercise has traditionally been categorised into 'endurance/aerobic' and 'resistance/strength' modalities with each ultimately generating a specific phenotype necessary for enhanced performance. However, for many athletic performances, especially team sports, there is a requirement for high levels of aerobic capacity and strength/power and a necessity for a combination of endurance and resistance training for optimal performance. This section will review the present knowledge of several putative adaptive responses to resistance, endurance and concurrent training in human skeletal muscle that may determine the specificity of adaptation.

Hypertrophy/ Muscle Protein Synthesis

Chronic resistance training has long been recognised as the principal exercise stimulus for muscle hypertrophy and increased strength (Phillips et al. 1997, Rennie et al. 1982, Welle, Thornton and Statt 1995, Yarasheski, Zachwieja and Bier 1993) as a result of the exercise-induced neural adaptation and greater muscle fibre cross-sectional area (CSA) (~9 - 20%) (Aagaard et al. 2002, Kraemer and Ratamess 2004, Narici et al. 1989, Farup et al. 2012). Chesley and co-workers were among the first to demonstrate elevated rates of muscle protein synthesis after a bout of resistance exercise in trained young men (Chesley et al. 1992). Numerous studies have since reported that resistance exercise alone (~55 - 120%) (Phillips et al. 1997, Phillips et al. 1999, Welle et al. 1995, Yarasheski et al. 1993, Balagopal et al. 2001, Hasten et al. 2000, Holm et al. 2010, Kumar et al. 2009, Mayhew et al. 2009) and in combination with protein feeding (~ 200%) (Rasmussen et al. 2000, Tipton et al. 1999, West et al. 2011, Wilkinson et al. 2007) enhances muscle fractional synthetic rate. Moreover, rates of muscle protein synthesis peak early (1 – 5 h) above basal levels and can exceed basal rates (~40 - 150%) for 24 - 48 h following resistance exercise (Biolo et al. 1995b, Chesley et al. 1992, Phillips et al. 1997, Phillips et al. 1999).

Changes in muscle protein synthesis rather than muscle protein breakdown appears to be the most important factor modulating protein turnover and promoting skeletal muscle hypertrophy with resistance exercise (Phillips et al. 1997). The modest attenuation (~20 - 30%) of muscle protein breakdown with resistance exercise is generally observed only when exogenous essential amino acids are ingested during recovery (Biolo et al. 1997, Rasmussen et al. 2000, Tipton et al. 1999, Wilkinson et al. 2007). Several studies have reported essential amino acid ingestion provides an

additive stimulus for muscle protein synthesis after resistance exercise and is necessary for generating maximal rates of muscle protein synthesis (Biolo et al. 1997, Børsheim et al. 2002, Cuthbertson et al. 2006, Koopman et al. 2005, Miller et al. 2003., Moore et al. 2005, Rasmussen et al. 2000).

Acute activation of signalling proteins regulating translation initiation and elongation for protein synthesis including Akt (~ 2 fold), mTOR (~ 2 fold), ERK (~ 3 fold), p70S6K (~ 4 fold), rpS6 (~ 4 fold), 4E-BP1 (~ 5 fold) and eEF2 (~ 2 fold) are induced following resistance exercise (Coffey et al. 2005, Dreyer et al. 2006, Eliasson et al. 2006, Karlsson et al. 2004, Koopman et al. 2006b, Williamson et al. 2003, Terzis et al. 2010) Several studies have also demonstrated an additive effect on the magnitude (~3 - 20 fold) of phosphorylation with ingestion of protein following resistance exercise (Dreyer et al. 2008b, Karlsson et al. 2004, Koopman et al. 2007b). However, chronic changes in anabolic signalling (phosphorylation or total protein content) associated with elevated rates of muscle protein synthesis following resistance training are not well defined. Vissing and colleagues recently showed $mTOR^{Ser2448}$ (~ 200%) and $p70S6K^{Thr389}$ (~ 400%) phosphorylation were increased above resting levels after 10 weeks of resistance, but not endurance training (Vissing et al. 2011). Similarly, Leger and colleagues have also shown elevated phosphorylation of insulin/IGF pathway signalling proteins at rest following 8 weeks resistance training (Léger et al. 2006). Wilkinson and co-workers determined signalling phosphorylation status and rates of myofibrillar and mitochondrial synthesis at rest and after an acute bout of resistance exercise in subjects before and after 10 weeks of resistance training (Wilkinson et al. 2008). The resting phosphorylation status of key insulin/IGF signalling proteins for translation initiation were increased after the 10 week training program (Wilkinson et al. 2008). Elevated phosphorylation status at rest may be a factor that contributes to the higher resting protein synthesis rates with chronic resistance training (Phillips et al. 1999, Wilkinson et al. 2008, Phillips et al. 2002). Thus, it appears changes in muscle cell signalling and protein synthesis with repeated bouts of resistance exercise promote a specificity of training response within the initial weeks-months of training, and the protein synthesis generally occurs in the myofibrillar protein fraction (Kim, Staron and Phillips 2005, Wilkinson et al. 2008).

Studies that have examined the effects of endurance exercise on human skeletal muscle protein synthesis are limited compared with the abundance of data available on resistance exercise.

Intuitively, this may be due to the minor effect of endurance training on muscle hypertrophy and muscle mass compared with large effects on the mitochondria and aerobic metabolism (Figure 1.10). Moreover, a recent meta-analysis investigating adaptation responses to exercise shows endurance training has little effect on lower body hypertrophy, strength and power compared to resistance training (Wilson et al. 2012). While these findings indicate a limited anabolic effect, endurance exercise has the capacity to alter the activity of the muscles protein synthetic machinery.

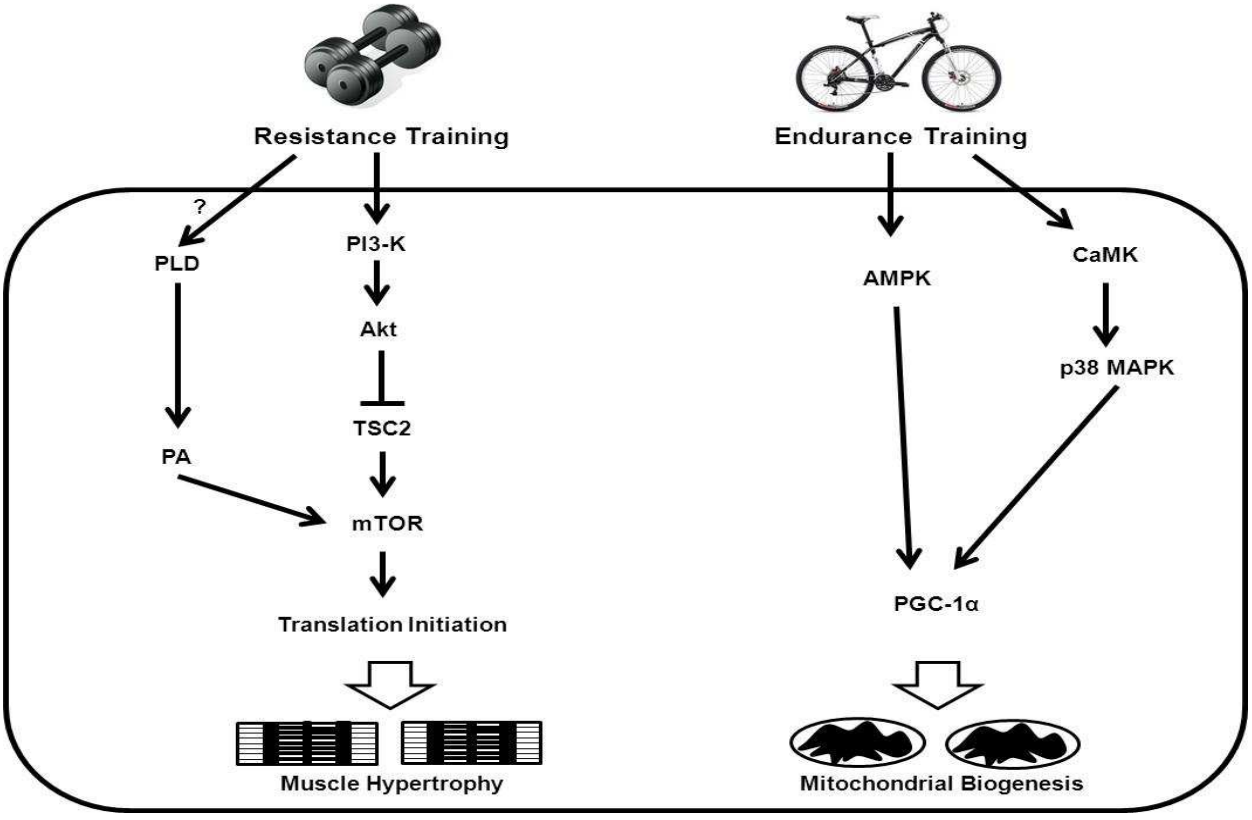

Figure 1.10 Intracellular signalling pathways mediating skeletal muscle adaptations to resistance and endurance exercise. Adapted from (Hawley 2009).

Mascher and colleagues were among the first to examine the phosphorylation responses of proteins in the insulin/IGF pathway during the acute recovery period following endurance exercise and their results show increases in phosphorylation of Akt^{Ser473} (~2 - 3 fold),

mTOR^{Ser2448} (~ 3 fold), and p70S6K$^{Ser424/Thr421}$ (~5 - 8 fold) after 1 h cycling at ~ 75% $VO2_{peak}$ (Mascher et al. 2007). Other studies have also shown similar exercise-induced changes in signal transduction (Coffey et al. 2009b, Wilkinson et al. 2008, Ivy et al. 2008) despite the use of differing post-exercise time-points for analysis. Regardless, it seems intuitive that contractile activity would promote translation initiation signalling during the early recovery period irrespective of exercise mode.

Several studies have investigated whole body protein balance and/or muscle protein synthesis following endurance exercise (Carraro et al. 1990, Durham et al. 2004, Harber et al. 2009, Harber et al. 2010, Howarth et al. 2009, Howarth et al. 2010, Levenhagen et al. 2002, Pikosky et al. 2006, Sheffield-Moore et al. 2004, Short et al. 2004). Levenhagen and co-workers have shown increased leg and whole body protein synthesis when protein and carbohydrate were co-ingested (10 g protein, 8 g CHO) immediately after 1 h submaximal cycling (Levenhagen et al. 2002). Harber and colleagues extended on these findings by demonstrating elevated rates of mixed muscle protein synthesis (~ 65%) in the acute recovery period following moderate intensity cycling (1 h, ~ 70% $VO2_{peak}$) (Harber et al. 2010). Thus, endurance exercise appears to stimulate muscle protein synthesis in the acute recovery period. In response to chronic endurance training, increases in resting muscle fractional synthetic rate have been reported after as little as 4 weeks (~ 15%) (Pikosky et al. 2006) and also after 4 months endurance training (~ 22%) (Short et al. 2004). This increase in protein synthesis has been suggested to reflect increases in the synthesis of mitochondrial-related proteins that subsequently contribute to aerobic muscle fibre type characteristics and an endurance phenotype (Baumann et al. 1987, Pikosky et al. 2006).

An important step to advance understanding of the adaptation response with endurance exercise was to delineate the specific protein fraction/s contributing to changes in mixed muscle protein synthesis. Miller and co-workers have shown increases in rates of sarcoplasmic and myofibrillar proteins 48 and 72 h post-exercise using a one-legged kicking endurance model (Miller et al. 2005). Breen and co-workers determined acute mitochondrial and myofibrillar protein synthesis in well trained cyclists when carbohydrate or carbohydrate plus protein beverages were ingested following prolonged (90 min, ~ 75% $VO2_{peak}$) cycling (Breen et al. 2011b). Interestingly, the results show selective increases in myofibrillar, but not mitochondrial, protein synthesis when protein was co-ingested with carbohydrate but the study was limited to post-exercise rates of

protein synthesis. In support of these findings, rates of myofibrillar protein synthesis were also recently reported to be elevated in the early (0.5 – 4.5 h) and late (24 – 28 h) recovery period following 30 min cycling at 60% W_{max} in young, recreationally trained males (Di Donato 2012). Thus, it appears endurance exercise is capable of stimulating myofibrillar protein synthesis in the acute recovery period in human skeletal muscle.

The requirement for myofibrillar protein synthesis following endurance exercise may relate to the large size (~ 60%) of the myofibrillar protein pool and subsequently being a preferential site of disposal for free amino acids, particularly following protein ingestion. Regardless, not all studies have reported increases in rates of myofibrillar protein synthesis with endurance exercise. Donges and colleagues recently reported no increase in myofibrillar protein synthesis after 40 mins cycling at 55% W_{max} (Donges et al. 2012). However, Wilkinson and colleagues (Wilkinson et al. 2008). reported that after 10 weeks of endurance training (45 min cycling; 75% $VO2_{peak}$; 3 times/week), an acute bout of endurance exercise elevated rates of mitochondrial, but not myofibrillar, protein synthesis in young healthy males. Another study investigating protein synthesis responses to chronic endurance training (6 weeks) has shown increases in mixed muscle protein content, but not mitochondrial DNA or protein synthesis compared to a non-exercising control group (Robinson et al. 2011).

Post-exercise stimulation of myofibrillar protein synthesis with endurance training may be integral for tissue repair and remodelling and/or may represent training-induced adaptation for enhanced cycling power output at high workloads that may be beneficial for performance (Breen et al. 2011b, Burd et al. 2009). Several studies have shown changes in fibre cross-sectional area (~4 - 10%) and muscle mass (~ 1%) following endurance training and while modest muscle hypertrophy has been reported, the studies have largely included elderly or untrained/sedentary populations (Harber et al. 2009, Harber et al. 2012, Hudelmaier et al. 2010, Konopka et al. 2010, Schwartz et al. 1991). These results are in contrast to studies of young, active subjects or following prolonged periods of endurance training where hypertrophy and increases in muscle mass have not been consistently observed (Short et al. 2004, Wilkinson et al. 2008, Glowacki et al. 2004). While there is data showing comparable changes in molecular and physiological adaptations governing muscle protein synthesis following an acute bout of endurance or consecutive resistance and endurance (concurrent) exercise, the overwhelming evidence shows

gains in muscle mass are more pronounced with chronic resistance exercise. Accordingly, it is likely the increase in myofibrillar protein synthesis with endurance exercise is specific to the extent/type of muscle overload and individual training status, and would diminish with chronic training (Wilkinson et al. 2008).

Muscle Protein Breakdown

Compared to the relatively well-characterised effects of exercise on muscle protein synthesis, changes in muscle protein breakdown following resistance and endurance exercise in human skeletal muscle are not well defined. This likely relates to the methodological difficulty of measuring muscle protein breakdown *in vivo* human skeletal muscle and the modest changes in protein breakdown compared to synthesis following exercise. Intuitively, muscle breakdown is an integral factor in maintaining skeletal muscle homeostasis and in the adaptation response to exercise.

Resistance exercise increases MuRF1 mRNA abundance and total protein in human skeletal muscle during the post-exercise recovery period (Glynn et al. 2010, Louis et al. 2007, Mascher et al. 2008, Yang, Jemiolo and Trappe 2006, Fry et al. 2012). Conversely, atrogin-1 expression can remain unchanged (Coffey et al. 2006, Glynn et al. 2010, Louis et al. 2007, Lundberg et al. 2012, Mascher et al. 2008, Yang et al. 2006) or is reduced (Kostek et al. 2007) following acute resistance exercise. The discordance in mRNA responses following exercise between atrogin-1 and MuRF1 is surprising considering their dual upstream regulation via the Akt-FOXO pathway although cross-talk from other pathways, such as p38MAP kinase and/or NF-κB (Cai et al. 2004, Li et al. 2005), may also be involved in the regulation of atrogin-1/MuRF1 gene expression. Regardless, it appears changes in the transcriptional activity of "atrogenes" are induced in response to heavy resistance exercise and may represent an important factor in determining the training-induced phenotype. Interestingly, Borgenvik and co-workers have recently shown a decrease in atrogin-1 mRNA levels at rest and following resistance exercise when branched chain amino acids are ingested (Borgenvik, Apró and Blomstrand 2012), indicating a nutrient-mediated effect on atrogene expression that may modulate remodelling/ hypertrophy in skeletal muscle. Several previous studies have also shown myostatin peptide (Hulmi et al. 2009b) and

mRNA abundance (Hulmi et al. 2009a, Jones et al. 2004, Kim, Cross and Bamman 2005, Louis et al. 2007, Mascher et al. 2008, Raue et al. 2006) is suppressed following an acute bout of resistance exercise. This attenuation may be dose-dependent considering myostatin mRNA levels are unchanged following low intensity resistance exercise (4 × 15-30 reps at 20%1RM) (Manini et al. 2011). Importantly, a clear association between changes in atrogene transcriptional activity/total protein and direct measures of proteasome activity or muscle protein breakdown in human skeletal muscle has yet to be established.

Elevated muscle protein breakdown has been reported following resistance exercise using either the tracer dilution (Biolo et al. 1995b, Biolo et al. 1999) or fractional breakdown rate methods (Phillips et al. 1997, Staples et al. 2011, Phillips et al. 1999). Phillips and colleagues have shown muscle protein breakdown increased ~ 35% following resistance exercise and returned to resting levels 24 h post-exercise (Phillips et al. 1997, Phillips et al. 1999). Interestingly, this response only occurred in untrained subjects indicating exercise-induced protein breakdown is attenuated with chronic resistance training (Phillips et al. 1999). Indeed, an increase in muscle protein breakdown following resistance exercise has not been consistently observed (Fry et al. 2011, Glynn et al. 2010). This discrepancy may be due to differences in the timing of post-exercise analyses given muscle protein breakdown following resistance exercise may peak early (~ 3 h) in the post-exercise recovery period (Phillips et al. 1997).

The effects of endurance exercise on muscle protein breakdown are not well characterised despite some evidence showing endurance exercise alters muscle proteolytic signalling in human skeletal muscle (Coffey et al. 2006, Harber et al. 2009, Harber et al. 2010, Louis et al. 2007). Several studies have examined the responses of MuRF1 and atrogin-1 mRNA to endurance exercise in human skeletal muscle. Coffey and colleagues have shown increases in atrogin-1 mRNA following cycling exercise (Coffey et al. 2006) and MuRF1 mRNA abundance can also be elevated after running (Harber et al. 2009, Louis et al. 2007) and cycling (Harber et al. 2010), a nutrient-sensitive effect that may be attenuated with protein-carbohydrate ingestion during recovery (Harber et al. 2010).

There is a paucity of studies investigating muscle protein breakdown following endurance exercise. Carraro and co-workers examined rates of muscle protein breakdown following 4 h treadmill walking at 40% $V_{O_{2peak}}$ using 3-methyl-histidine and showed muscle protein breakdown

was unchanged immediately post-exercise but increased ~ 85% at the 4 h post-exercise time-point (Carraro et al. 1990). Human skeletal muscle proteolysis is also elevated after 45 min of treadmill walking (40% VO_{2peak}) but returns to resting levels in the first hour of recovery (Sheffield-Moore et al. 2004). Only one study has investigated the effects of chronicendurance training on rates of muscle protein breakdown in human skeletal muscle (Pikosky et al. 2006). Pikosky and co-workers show rates of muscle fractional breakdown are increased following four weeks endurance training resulting in a negative net protein balance (Pikosky et al. 2006). These findings support the premise that endurance training does not generate substantial hypertrophy and/or may indicate a net loss of muscle mass might be expected with endurance training. However, further studies measuring the fractional breakdown rates of skeletal muscle following both acute endurance exercise bouts and chronic training are required to clarify the extent of breakdown and its effects on muscle mass.

Muscle Oxidative Capacity/ Mitochondrial Biogenesis

Resistance training is not an exercise modality that generates significant adaptations in oxidative/mitochondrial capacity. Indeed, conventional thinking regarding exercise-induced increases in mitochondrial biogenesis/density suggests such adaptations are exclusively achieved with endurance training (Holloszy et al. 1977, Gollnick et al. 1973, Henriksson and Reitman 1977). Cross-sectional analysis of skeletal muscle in resistance-trained individuals shows reduced mitochondrial content (Alway et al. 1988) and citrate synthase activity (Tesch, Komi and Häkkinen 1987) compared to untrained controls. Mitochondrial content has also been shown to be reduced in as little as 6 weeks of resistance training (Lüthi et al. 1986) while 6 months of resistance training can also decrease muscle mitochondrial volume per fibre area in humans (MacDougall et al. 1979). Collectively, this indicates resistance training reduces mitochondrial density and oxidative capacity of skeletal muscle but several studies have challenged this notion (Balakrishnan et al. 2010, Pesta et al. 2011, Tang, Hartman and Phillips 2006, Vanhatalo et al. 2011, Wang et al. 2011, Ross and Leveritt 2001, Staron et al. 1984).

Tang and colleagues have observed increases in citrate synthase, β-hydroxyacyl CoA dehydrogenase (~ 20%) and hexokinase (~ 42%) activity concomitant with muscle fibre

hypertrophy following 12 weeks of resistance training (Tang et al. 2006). The authors propose that as muscle fibre size increases the oxidative enzyme content also increase such that oxidative potential is improved with resistance training. Furthermore, chronic resistance training (10 - 14 weeks) has been reported to increase mitochondrial DNA copy number (Balakrishnan et al. 2010) and muscle-specific lipid oxidation capacity (Pesta et al. 2011), indicative of a greater mitochondrial content following training. However, numerous studies have also failed to show increases in mitochondrial enzymes and content following resistance training (Bell et al. 2000, Chilibeck, Syrotuik and Bell 1999, Green et al. 1999a, Green et al. 1999b, Ploutz et al. 1994, Tesch et al. 1987, Wang et al. 1993, Tesch, Thorsson and Colliander 1990).

Endurance training enhances capillary density in skeletal muscle and resistance training may also represent an exercise stimulus capable of promoting angiogenesis. This adaptive process is characterised by an expansion of the muscle's pre-existing capillary network that improves gas and nutrient exchange to/from the bloodstream and enhance exercise capacity (Saltin and Rowell 1980). An acute resistance exercise bout upregulate's vascular endothelial growth factor (VEGF) mRNA (~3 - 4 fold) (Gavin et al. 2007, Jozsi et al. 2000, Trenerry et al. 2007) and total protein abundance (Gavin et al. 2007), which is an important mediator of angiogenesis. Resistance training has also been shown to increase muscle capillary density (Green et al. 1999b, McCall et al. 1996, Campos et al. 2002), indicating resistance exercise-induced muscle hypertrophy may also be accompanied by concomitant increases in capillary density.

Whether any changes in the oxidative machinery with resistance training translate into improvements in aerobic capacity remains controversial (Bishop and Jenkins 1996, Bishop et al. 1999, Hickson, Rosenkoetter and Brown 1980, Hickson et al. 1988). Enhanced aerobic performance with resistance training has been associated with changes in muscle fibre recruitment rather than metabolic adaptations that maintain/increase force production and delay fatigue (Hickson et al. 1980). Indeed, numerous studies have shown no improvements in aerobic performance with resistance training (Bishop and Jenkins 1996, Bishop et al. 1999). These findings suggest potential performance benefits to endurance exercise with resistance training may be dependent on, and specific to, the components of the resistance training program.

Burd and co-workers have recently investigated muscle protein synthesis responses to resistance exercise when manipulating the time the muscle is under tension with contraction (Burd et al.

2012). Interestingly, the increased time under tension was also associated with increases in PGC-1α mRNA expression (~ 3 fold) and mitochondrial protein synthesis (~ 2.5 fold). Additional studies have also shown increases in rates of mitochondrial protein synthesis following an acute bout of resistance exercise (Donges et al. 2012, Wilkinson et al. 2008). However, the possibility exists that the increase in mitochondrial protein synthesis is the result of a generic, non-specific adaptation response following disruption to homeostasis with a novel or unaccustomed contractile stimulus. Such a thesis is supported by data from studies incorporating prolonged resistance training interventions (i.e. 6 months) or subjects with extensive resistance training history showing no increase in mitochondrial content or oxidative metabolism (MacDougall et al. 1979, Fry 2004). Indeed, Wilkinson and colleagues have shown initial increases in rates of mitochondrial protein synthesis following an acute bout of resistance exercise are attenuated after 10 weeks resistance training (Wilkinson et al. 2008). Thus, chronic resistance training induces muscle hypertrophy but, despite the potential for acute resistance exercise to upregulate post-exercise rates of mitochondrial protein synthesis, the capacity for resistance training to promote mitochondrial biogenesis and muscle oxidative capacity in skeletal muscle has not been established.

Endurance-based exercise results in increased aerobic capacity that enables prolonged periods of low-moderate contractile activity and this well-established exercise-induced adaptation response has been shown recently following 10 weeks of endurance (~ 10%) but not resistance training (Farup et al. 2012). The mechanistic underpinning of endurance exercise adaptations includes the transformation of muscle towards a phenotype with higher mitochondrial (Flück and Hoppeler 2003) and capillary density (Coggan et al. 1992, Andersen and Henriksson 1977), and systemic adaptations including enhanced cardiac output (Ekblom and L. 1968) and arterio-venous oxygen difference (Wilmore et al. 2001). Endurance adaptation also generates greater oxygen extraction and utilisation by exercising muscle and enhanced fatigue resistance, sparing muscle glycogen with greater contribution from fat oxidative metabolism (Coggan and Williams 1995). Holloszy was first to elucidate the increase in skeletal muscle oxidative capacity after endurance training via increases in mitochondrial content (Holloszy 1967). This work is supported by numerous studies demonstrating increased activity of oxidative enzymes such as citrate synthase and succinate dehydrogenase following endurance exercise in human skeletal muscle (Carter et al.

2001, De Bock et al. 2008, Fernström, Tonkonogi and Sahlin 2004, Howald et al. 1985, Short et al. 2003, Henriksson and Reitman 1977, Zamora et al. 1995).

The molecular bases of mitochondrial biogenesis has centred on the transcriptional co-activator peroxisome proliferator activated receptor gamma (PPARγ) coactivator-1α (PGC-1α) (Hood 2001, Puigserver et al. 1998, Scarpulla 2006) and to a lesser extent PGC-1β (Arany et al. 2007). Numerous studies have implicated PGC-1α as an important regulator of a wide variety of metabolic processes including brown fat thermogenesis, carbohydrate and fat metabolism, skeletal muscle fibre type transformation, and mitochondrial biogenesis (Lin, Handschin and Spiegelman 2005, Olesen, Kiilerich and Pilegaard 2010). Several molecular pathways are proposed to regulate PGC-1α activation including AMPK, p38 mitogen-activated protein kinase (Akimoto et al. 2005) or deacetylation by silent information regulator T1 (SIRT1) (Rodgers et al. 2005a) but a clear cause and effect has yet to be established (Figure 1.11). Recent evidence also suggests micro RNA-dependent regulation of PGC-1α in response to exercise as indicated by the down regulation of putative PGC-1α translation inhibitors miR-23 and miR-696 following endurance exercise (Safdar et al. 2009, Aoi et al. 2010).

PGC-1α transcriptional targets comprise several DNA binding transcription factors including the nuclear respiratory factors (NRF-1 and NRF-2) (Hood 2009) and peroxisome proliferator-activated receptors (PPARs) (Gilde and Van Bilsen 2003), and is also important for exercise-induced upregulation of skeletal muscle vascular endothelial growth factor (VEGF) expression (Arany et al. 2008 , Leick et al. 2009). Safdar and co-workers recently extended the current understanding of PGC-1α function in showing PGC-1α localises to mitochondria of skeletal muscle following endurance exercise with potential to facilitate nuclear-mitochondrial cross-talk in its co-ordination of mitochondrial biogenesis (Safdar et al. 2011). Thus, while it is certain that there is a highly complex network of molecular machinery controlling oxidative/mitochondrial adaptation, the extensive regulation of nuclear and mitochondrial genomes demonstrates PGC-1α's remains the best characterised "master-regulator" of exercise-induced mitochondrial biogenesis.

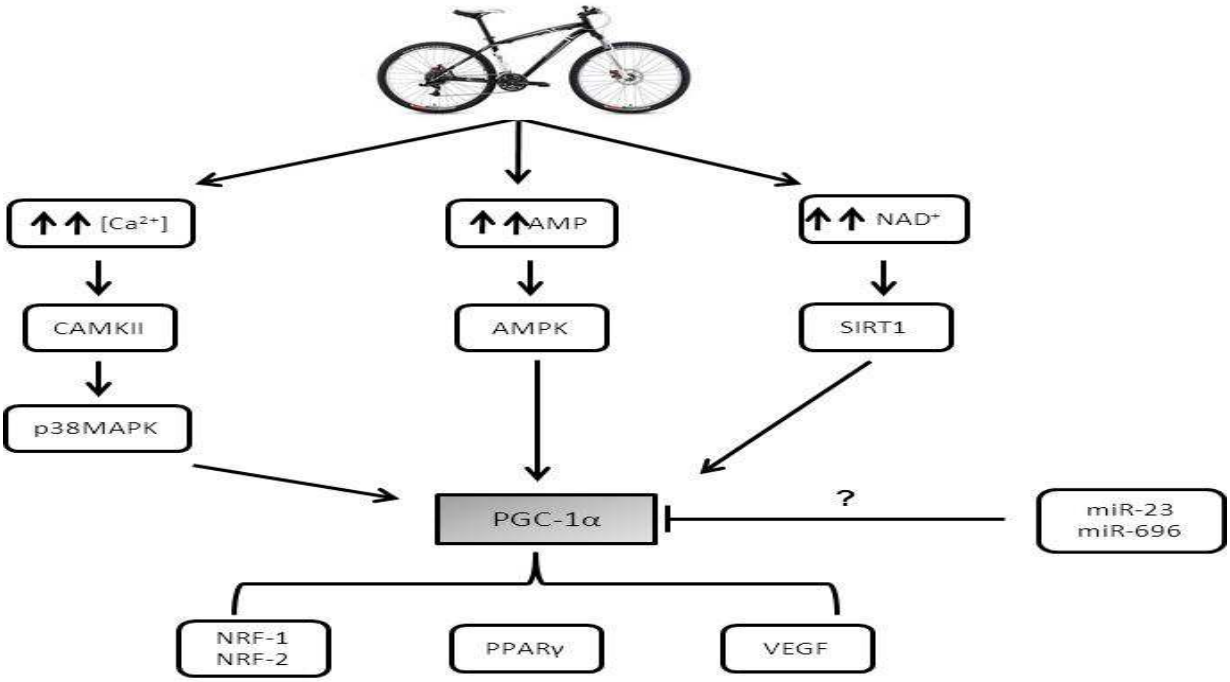

Figure 1.11 Putative regulation of PGC-1α expression in response to contraction in human skeletal muscle. Adapted from (Lanza and Nair 2010).

Higher PGC-1α mRNA and protein content has been repeatedly implicated in exercise-induced mitochondrial biogenesis following acute (~3 - 12 fold) (Burgomaster et al. 2008, Cartoni et al. 2005, Egan et al. 2010, Hellsten et al. 2007, Little et al. 2011, Mortensen et al. 2007, Perry et al. 2010, Russell et al. 2005) and chronic endurance training in human skeletal muscle (~ 6 fold) (Kuhl et al. 2006, Russell et al. 2003, Short et al. 2003). Perry and co-workers recently investigated the time course of changes in various oxidative/mitochondrial protein throughout a two week high intensity interval training program (Perry et al. 2010). This study was the first to show endurance training-induced increases in mitochondrial protein content is a result of the cumulative effects of repeated transient "bursts" in gene expression following each exercise bout (Perry et al. 2010). Importantly, alternative transcription factors and proteins are also likely to be essential for co-ordinating exercise-induced increases in mitochondrial content. For example, the cAMP response element–binding (CREB) protein contributes to mitochondrial function by directly acting on specific mitochondrial genes or by inducing PGC-1α expression (Cao et al.

2004, Herzig et al. 2001). c-Myc (Scarpulla 2008), yin yang 1 (YY1) (Cunningham et al. 2007), mitofusin (Zorzano 2009, Cartoni et al. 2005) and estrogen-related receptor (ERR) (Giguère 2008) have also been implicated in the regulation of mitochondrial biogenesis and function. Further work in human skeletal muscle is required to elucidate the role and extent these proteins may facilitate mitochondrial biogenesis with endurance exercise.

Studies measuring rates of mitochondrial protein synthesis following endurance exercise are currently limited but increases in mitochondrial protein synthesis have been observed in the acute recovery period following endurance exercise (Donges et al. 2012, Wilkinson et al. 2008). It also appears there is no additive effect of protein-carbohydrate ingestion after endurance exercise on mitochondrial synthesis (Beelen et al. 2011). Whether large amounts of protein are required (e.g. >20 - 30 g) to stimulate increases in mitochondrial protein synthesis with endurance exercise is unclear. Mitochondrial fractional synthesis rates have been shown to be elevated with high compared to low insulin infusion in human skeletal muscle (Stump et al. 2003) and a substantial insulinotrophic response may be required to mediate increases in mitochondrial protein synthesis. Indeed, the capacity of endurance exercise undertaken in the fasted state to clearly promote acute mitochondrial protein synthesis is equivocal and may reflect differences in the time-course of the adaptation response compared with the myofibrillar protein pool (Moore et al. 2009b, Rowlands et al. 2011). Regardless, adaptations to chronic endurance training are manifest through increases in mitochondrial content and oxidative capacity and future work will undoubtedly ascertain any capacity for nutrient strategies to enhance exercise-induced mitochondrial protein synthesis.

Muscle Substrate Metabolism

Few studies have investigated substrate utilization in response to resistance exercise and this likely relates to the relatively modest metabolic cost inherent with short duration, intermittent contractile activity. Early interest in characterising metabolic responses to resistance exercise posited particular metabolites may accumulate through vascular occlusion associated with high-force muscle contractions, and subsequently mediate changes in muscle protein synthesis/adaptation responses (Smith and Rutherford 1995). Tesch and co-workers first reported significant reductions in ATP, CP and glycogen following heavy resistance exercise in strength

trained athletes (Tesch et al. 1986). Others have also shown high-volume resistance exercise can significantly reduce glycogen (Koopman et al. 2006a, Pascoe et al. 1993, Pascoe and Gladden 1996, Robergs et al. 1991) and intramyocellular lipid concentrations (Koopman et al. 2006a). Moreover, these changes appear to be fibre-type specific with the potential for greatest reductions in glycogen and intramyocellular lipids in type IIx and type I muscle fibres, respectively (Koopman et al. 2006a). Dreyer and colleagues have shown leg glucose uptake is elevated during an acute bout of resistance exercise (10 × 10 leg extensions, 70% 1RM) and post-exercise whole body glucose oxidation was also reduced and fat oxidation increased compared to basal levels (Dreyer et al. 2008a). This glucose oxidation was proposed to be mediated, at least in part, by AMPKα2 phosphorylation of AS160 and the results also indicated AMPK may modulate post-exercise "fuel" selection to increase fat oxidation after resistance exercise (Dreyer et al. 2008a). Regardless, it appears heavy resistance exercise has a capacity to alter substrate metabolism during the post-exercise recovery period.

Howlett and colleagues have determined the effect of both endurance and resistance exercise modes on AS160-GLUT4 and found reduced AS160 phosphorylation immediately after resistance exercise accompanied by a decreased capacity for muscle AS160 to bind 14-3-3 proteins while endurance exercise increases AS160-14-3-3 binding affinity (Howlett et al. 2008, Howlett et al. 2007). Selective AS160 mediated GLUT4 translocation may therefore be related to the specificity in 14-3-3 protein binding to AS160 only with endurance exercise. Eccentric resistance exercise has also been shown to decrease GLUT4 protein content (~39 - 55%) in skeletal muscle that has been linked to exercise-induced immune or muscle damage responses (Asp et al. 1996, Asp, Kristiansen and Richter 1995, Kristiansen, Asp and Richter 1996, Kristiansen et al. 1997). However, others have shown GLUT4 protein to increase (~25 - 40%) following resistance exercise in human skeletal muscle (Derave et al. 2003, Holten et al. 2004, Tabata et al. 1999), suggesting GLUT4 responses may be contraction and intensity dependent. Nonetheless, resistance exercise has been shown to improve whole-body insulin sensitivity (Black, Swan and Alvar 2010, Breen et al. 2011a, Fluckey et al. 1994, Koopman et al. 2005) and glycemic control (Breen et al. 2011a, Fenicchia et al. 2004) for up to 24 h after cessation of exercise. This response appears to be volume-dependent with higher resistance exercise volumes necessary to elicit improvements in insulin sensitivity.

The prolonged duration typically associated with endurance exercise generates a different metabolic challenge for skeletal muscle compared to resistance exercise. The oxidation of carbohydrate and fat is necessary for energy production during endurance exercise and their relative contribution to generating ATP is dependent on factors such as intensity, duration and individual training status. Endogenous carbohydrates are stored primarily as glycogen in skeletal muscle and liver, and represent less than 5% of total energy storage (Spriet and Howlett 1999). Conversely, the majority of available energy is derived from fat in the form of triacylglycerol deposits in subcutaneous and deep visceral adipose tissue, and also depots of intramyocellular triacylglycerols within muscle fibres (Hoppeler et al. 1985).

The rate of muscle glycogen utilisation is generally highest during the first 30 min of exercise at intensities greater than ~ 60% $V_{O_{2peak}}$ (van Loon et al. 2001). Insulin sensitivity and glucose tolerance are enhanced following moderate-to-high intensity endurance exercise (Devlin et al. 1987, Mikines et al. 1988, Perseghin et al. 1996), an effect that can persist between 2 - 48 h (Mikines et al. 1988, Devlin et al. 1987, Perseghin et al. 1996) . This effect appears to be mediated by increases in the activity and content of GLUT4 and the intermediates of the insulin signalling pathway (Chibalin et al. 2000, Kim et al. 1999, Luciano et al. 2002). Indeed, increases in skeletal muscle GLUT4 mRNA and protein have been repeatedly observed following endurance exercise (Daugaard et al. 2000, Friedman et al. 1990, Houmard et al. 1995, Houmard et al. 1993, Kraniou, Cameron-Smith and Hargreaves 2006, Ren et al. 1994, Terada et al. 2001, Stuart et al. 2010, Garcia-Roves et al. 2003, Neufer and Dohm 1993).

Endurance exercise-mediated adaptations in substrate metabolism result in reduced glucose utilisation during both moderate (Coggan et al. 1990) and intense (Coggan et al. 1995) exercise which is compensated by a proportional increase in fat oxidation. The change in metabolic substrate utilisation is reflected by a lower respiratory exchange ratio at the same absolute and relative exercise intensities (Coggan and Williams 1995, Hurley et al. 1986, Stisen et al. 2006, Jeukendrup, Saris and Wagenmakers 1998). This training-induced adaptation may be attributed to increased mitochondrial volume and enzymatic activity to increase the capacity to oxidise fatty acids and subsequently allow a greater reliance on fat to fuel oxidative metabolism (Holloszy and Booth 1976, Hawley 2002). This likely occurs through increases in activity of enzymes such as citrate synthase, β-hydroxyacyl-CoA dehydrogenase, cytochrome c oxidase IV, aspartate

aminotransferase and pyruvate dehydrogenase (Gollnick et al. 1972, Hoppeler et al. 1973, Talanian et al. 2010).

Endurance exercise also mediates adaptations in fatty acid uptake and oxidation. Fatty acid translocase (FAT/CD36), plasma membrane-associated fatty acid binding protein (FABPpm) and carnitine palmitoyltransferase-1 are implicated in fatty acid transport and regulate endurance training induced adaptations in fatty acid oxidation (Tunstall et al. 2002, Arkinstall et al. 2004, Bonen et al. 1999, Bonen et al. 2000, Bradley et al. 2012, Kiens et al. 2004, Roepstorff et al. 2004, Talanian et al. 2010, Kiens et al. 1993, Bruce et al. 2006) (Figure 1.12).

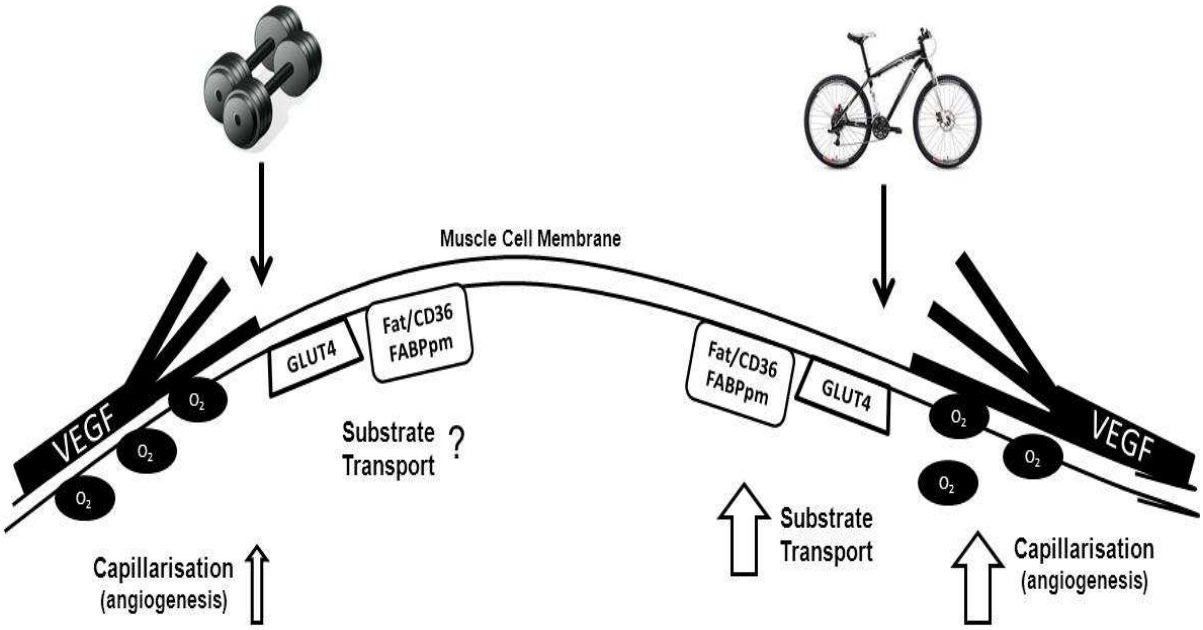

Figure 1.12 Signalling proteins mediating angiogenesis and substrate metabolic/ transport adaptations to chronic resistance and endurance exercise in human skeletal muscle. Clear arrows denote degree of adaptation. Adapted from (Mujika 2012).

Bonen and co-workers reported parallel increases in muscle oxidative capacity and FAT/ CD36 expression following endurance exercise in rats (Bonen et al. 1999). Increases in mitochondrial membrane FAT/CD36 content were also shown following 2 h cycling at 60% $VO2_{peak}$ in human skeletal muscle (Holloway et al. 2006). The authors proposed that increased FAT/CD36 content enhanced the ability to transport fatty acids into the mitochondria for fatty acid oxidation which spares intracellular fuel stores with endurance training. In support of this contention, Bradley and colleagues recently demonstrated increased plasma membrane FAT/CD36 and FABPpm protein content in human muscle after 2 h cycling at 60% $VO2_{peak}$ (Bradley et al. 2012). In contrast, no studies have reported changes in fatty acid transport proteins following resistance exercise in human skeletal muscle. Increases in rates of fatty acid oxidation (Dreyer et al. 2008a) and muscle oxidative capacity (Tang et al. 2006) with resistance exercise suggest changes in fatty acid transporter expression might be plausible. Intuitively, the lower metabolic cost associated with resistance exercise would be unlikely to mediate changes in these proteins to the same extent as endurance exercise. Thus, the alteration in substrate metabolism to spare muscle glycogen through selective increases in fat and decreases in carbohydrate oxidation, and upregulation of fatty acid transporter proteins, appears to be a specific adaptation response to endurance training.

Muscle Fibre Type

Human skeletal muscle is composed of different fibre types that have unique contractile and metabolic characteristics (Yan et al. 2011, Essén et al. 1975). 'Fast twitch' or type II muscle fibres are activated for short-duration anaerobic activities and are therefore associated with adaptations to resistance exercise (Wilson et al. 2012 , Garnett et al. 1979). Indeed, elite weight and power lifters have been shown to possess a greater type II fibre area than endurance athletes (Widrick et al. 2002), ranging from 60 - 80% of total fibres (Fry et al. 2003, Aagaard and Andersen 1998, Bergh et al. 1978). Type II fibres have sub-categories of type IIa that may have fast contractile characteristics for force generation but can also have similar oxidative profiles to type I fibres (Yan et al. 2011, Garnett et al. 1979), and type IIb fibres that are abundant in glycolytic enzymes but have lower mitochondrial and capillary density (Prince et al. 1981). Central to the specificity of adaptation with resistance exercise is that type II fibres have a greater capacity for exercise-induced hypertrophy (Schoenfeld 2000). However, studies investigating the

capacity for resistance training to induce a selective type I to II fibre shift are conflicting and may relate to the use of variations in the high velocity contractions and ballistic movements within different resistance exercise protocols (Wilson et al. 2012). For instance, 19 weeks of heavy resistance training was shown to increase type IIa fibre percentage without any change in type I fibres (Adams et al. 1993). In contrast, Paddon-Jones and co-workers observed a simultaneous increase in type IIb fibres (5.8 – 12.9%) and a decrease in type I fibres (53 – 39%) following 10 weeks of fast isokinetic resistance training (Paddon-Jones et al. 2001). The possible mechanisms that underpin this conversion are beyond the scope of this review, but may involve certain neurotrophic factors and hormones, or differences in neural input (Gundersen 1998, Wilson et al. 2012). In any case, adaptation to resistance exercise includes increases in type II fibre CSA that may be observed with or without concomitant reductions in type I percentage.

Several studies have examined the molecular responses in different fibre types following resistance exercise. Greater p70S6K phosphorylation has been observed in type II compared to I fibres (~ .9 vs. ~ 1.6 fold) following resistance exercise in human skeletal muscle indicative of higher rates of translation initiation to support hypertrophy (Koopman et al. 2006b, Tannerstedt, Apró and Blomstrand 2009). Verdijk and co-workers have also reported increases in muscle fibre hypertrophy in type II but not type I fibres following 12 weeks of resistance training (Verdijk et al. 2009). However, at the protein synthesis level mixed muscle protein synthesis is similar in type I compared with type II muscle fibres following an acute bout of resistance-based exercise (Koopman et al. 2011). Whether the absence of a fibre specific response early in the post-exercise period is sustained with chronic training is unclear but the authors postulated several mechanisms to explain the disparity in cell signalling and protein synthesis responses in type II fibres following an acute bout of resistance exercise. Firstly, the authors suggested AMPK activity may be higher in type II fibres during/ after resistance exercise and thus attenuate the protein synthesis response in these fibres following exercise. (Koopman et al. 2006a). The similarity in response between fibre types may also relate to the fed state of the participants prior to undertaking resistance exercise, or the recent finding that free phenylalanine concentrations are comparable between type I and II muscle fibres prior to exercise and during post-exercise recovery (Blomstrand and Essén-Gustavsson 2009).

Raue and colleagues recently reported divergence between type I and II fibres when evaluating the response of the transcriptome to resistance exercise (Raue et al. 2012). This work indicates that type II muscle fibres are responsive to resistance exercise and that ~ 97% of the transcriptome activity occurring in type II fibres was greater than that observed in mixed muscle samples (Raue et al. 2012). Given the technical difficulties of isolating and analysing a range of molecular responses in single fibres, a challenge that confronts exercise physiologists is finding a clearer resolution of fibre specific molecular responses with exercise *in vivo* human muscle. Nonetheless, fibre type transformation and fibre specific responses represent important factors for resistance training adaptation and subsequent increases in muscle strength and power in human skeletal muscle.

Endurance-based exercise is reliant on a significant contribution from type I 'slow-twitch' fibres due to their oxidative capacity (Prince et al. 1981) and slow force generation but with low susceptibility to fatigue (Witzmann, Kim and Fitts 1982, Essén et al. 1975, Garnett et al. 1979). Type I muscle fibres have greater mitochondria, myoglobin, oxidative enzymes and capillary-fibre ratio compared with type II fibres (Prince et al. 1981, Sullivan and Pittman 1987). Insulin-stimulated glucose transport is also greater in type I fibres promoting enhanced glucose uptake and metabolism (Daugaard et al. 2000, Henriksen et al. 1990, Song et al. 1999). Several studies have investigated the contribution of type I fibre composition to the endurance phenotype and shown elite long and middle distance runners possess slow twitch fibre percentages between 60 - 95% (Aagaard and Andersen 1998, Bergh et al. 1978, Costill et al. 1976, Coyle 2005, Fry et al. 2003, Saltin et al. 1977, Schantz and Dhoot 1987, Johnson et al. 1973). Moreover, there is generally some correlation between type I fibre composition and $V_{O_{2peak}}$ or performance in endurance events (Bergh et al. 1978, Foster et al. 1978).

Controversy exists with regard to the capacity for endurance training to mediate a type II to I fibre transformation. While some studies fail to support such a contention (Gollnick et al. 1973, McGuigan et al. 2002), others indicate increases in type I muscle fibre percentage following endurance exercise such as long distance running and cycling are plausible (Howald et al. 1985, Jansson, Sjödin and Tesch 1978). However, while no study to date has confirmed a transformation of type II to type I fibres with endurance exercise in human skeletal muscle it does result in greater oxidative characteristics in type IIa fibres (Yan et al. 2011).

Fibre-type specific signalling mechanisms implicate the calcineurin-NFAT signalling pathway and the transcriptional co-activator PGC-1α in mediating fibre type transformations in response to endurance training (Yan et al. 2011). Studies in rodents show lower percentages of type I fibres when calcinuerin-NFAT signalling is inhibited (McCullagh et al. 2004, Miyazaki et al. 2004, Serrano et al. 2001) but increases in type I fibres is seen in transgenic mice overexpressing calcineurin in skeletal muscle (Naya et al. 2000, Ryder et al. 2003). Increases in PGC-1α protein following endurance exercise have also led to the contention PGC-1α may be a key factor controlling development of type I fibres and an oxidative phenotype (Russell et al. 2003). Indeed, skeletal muscle-specific overexpression of PGC-1α in mice has promoted a type II to I fibre shift that was also associated with an enhanced oxidative phenotype and expression of mitochondrial markers, and improved endurance capacity (Calvo et al. 2008, Lin et al. 2002). However, studies demonstrating activation of the calcineurin-NFAT and PGC-1α related pathways potentially contributing to fibre type transformation *in vivo* human skeletal muscle are lacking.

Experiments in human skeletal muscle have demonstrated PGC-1α content is higher in type IIa compared to type I fibres following 6 weeks of endurance training (Russell et al. 2003). This finding was unexpected considering type I fibres have the greatest mitochondrial content and thus would be predicted to have the highest PGC-1α levels. However, the authors proposed this greater PGC-1α content in type IIa fibres to be part of a mechanism that increases mitochondrial biogenesis and subsequently mediates a potential switch to an oxidative phenotype (Russell et al. 2003). Nonetheless, increases in mitochondrial density of various magnitude has been shown in all fibre types following 6 weeks of endurance training (Howald et al. 1985).

Whether adaptation responses to chronic endurance training involve a type I fibre shift in human skeletal muscle requires further investigation. Challenges to establishing the endurance training adaptation include the potential fibre type transitions may require years rather than weeks or months, and may be dependent on training/activity history and individual genotype. Such knowledge would undoubtedly uncover important information central to adaptation processes induced by endurance exercise.

1.7 Concurrent Training

Many sports require various combinations of strength/power and endurance for successful performance. Studies examining the specificity of training adaptation have typically employed three exercise groups: resistance training only, endurance training only, and combined resistance and endurance training (concurrent training). Using this approach Hickson was first to determine adaptation responses to a combination of heavy resistance and high-intensity endurance training compared with either training regime undertaken in isolation (Hickson 1980). After 10 weeks of training the concurrent training group achieved similar increases in $VO2_{peak}$ (~ 20%) compared with the endurance only group while changes in strength were similar to resistance only during the initial 8 weeks but were attenuated during the last two weeks of training (Hickson 1980). The results indicate endurance training inhibits or 'interferes' with the development of strength within a concurrent paradigm when compared with training in isolation but resistance exercise does not appear to have a negative effect on aerobic capacity. Additional studies have generally confirmed that concurrent training relative to resistance training alone attenuates adaptation for hypertrophy, strength and power (Dolezal and Potteiger 1998, Häkkinen et al. 2003, Hickson 1980, Kraemer et al. 1995, Bell et al. 2000, Hunter, Demment and Miller 1987, Dudley and Djamil 1985). However, there are studies reporting little or no decrements in strength with combined resistance and endurance training (Balabinis et al. 2003, McCarthy et al. 1995, McCarthy, Pozniak and Agre 2002, Sillanpää et al. 2008, Sillanpää et al. 2009, Glowacki et al. 2004 , Lundberg et al. 2013) (Table 1.2). This disparity may result from a number of variables that differ between studies including the mode, frequency, duration and intensity of training, training history of participants and time between training sessions (Leveritt et al. 1999). Nonetheless, meta-analysis of literature comparing the adaptation response with concurrent training versus training in isolation has shown endurance training has a detrimental effect on hypertrophy and strength (Wilson et al. 2012).

Several potential mechanisms have been proposed to explain the 'interference' phenomenon during concurrent training. Craig and colleagues proposed 'acute' residual fatigue from endurance bouts compromises the muscle's ability to develop tension during subsequent resistance exercise which culminates in reduced strength development (Craig et al. 1991). Greater increases in strength have been observed when concurrent training was performed on alternate

days rather than on the same day, indicating greater recovery time and lower residual fatigue may enhance strength gains (Sale et al. 1990a). The inconclusive summation of 'end state' measures such as one repetition maximum lead researchers to investigate muscle morphology and fibre type. Concurrent training studies that quantified muscle fibre type transitions report little difference between concurrent and strength only training groups (Kraemer et al. 1995, Nelson et al. 1990, Sale et al. 1990b). More recently, mechanisms governing adaptation responses to concurrent training have begun to be elucidated often with a focus on comparisons of molecular responses to the divergent contractile activity with endurance and resistance exercise.

Adaptation Responses to Concurrent Training

The logical implication with concurrent training is that undertaking divergent contractile activity may result in adaptation interference because of the cross-talk inhibition of distinct molecular pathways or specificity of adaptive machinery, but data in humans is limited. Atherton and co-workers first proposed the role of an 'AMPK-Akt switch' in the specificity of training response when they observed selective activation of Akt-mTOR-p70S6K or AMPK-PGC-1α signalling pathways following resistance- compared with endurance-like electrical stimulation, respectively (Atherton et al. 2005). A modest number of studies have investigated the molecular responses of combined resistance and endurance exercise in human skeletal muscle. Wang and co-workers recently investigated anabolic and mitochondrial adaptation responses to an acute bout of endurance exercise undertaken in isolation or followed by a subsequent resistance exercise bout (Wang et al. 2011). The results showed successive endurance and resistance exercise bouts enhanced the expression of genes implicated in mitochondrial biogenesis and increased phosphorylation levels of signalling proteins regulating translation initiation (Wang et al. 2011).

While the results indicate concurrent endurance and resistance exercise may augment the exercise-induced stimulation of putative mediators of mitochondrial biogenesis, a single exercise order for the concurrent training session was used without comparison to the alternate exercise order. Coffey and co-workers examined the acute molecular responses and the effect of exercise order following consecutive resistance and endurance exercise (Coffey et al. 2009b). Using a cross-over design subjects completed two experimental trials comprising a resistance exercise (8

× 5 leg extensions, ~ 80% 1RM) followed by an endurance exercise bout (30 min cycling, ~ 70% $VO2_{peak}$), and on another occasion the reverse order. The authors reported comparable phosphorylation responses in signalling markers of translation initiation during the acute recovery phase regardless of exercise order/ contraction mode (Coffey et al. 2009b).

Table 1.2: Summary of adaptation responses in young, healthy subjects to chronic resistance, endurance and concurrent training in human skeletal muscle.

	Resistance Exercise	Endurance Exercise	Concurrent Exercise
Muscle Hypertrophy			
Myofibrillar Protein Synthesis	↑	↔	?
Muscle Cross Sectional Area	↑↑↑	↔	↑↑
Muscle Strength	↑↑↑	↑	↑↑
Muscle Power	↑↑↑	↔	↑↑
Oxidative Capacity			
Mitochondrial Biogenesis	↔	↑↑↑	?
$VO2_{peak}$	↔	↑↑↑	↑↑↑
Muscle Angiogenesis	↑	↑↑↑	?

Arrows denotes the degree to which an adaptation response may be generated: ↔, No change; ?, Unknown; ↑, Small effect; ↑↑ Moderate effect; ↑↑↑, Large effect.

	Resistance Exercise	Endurance Exercise	Concurrent Exercise
Substrate Metabolism			
Carbohydrate Transport	↑	↑↑↑	?
Fat Transport	?	↑↑↑	?
Muscle Glycogen	?	↑↑↑	?
Muscle Fibre Type			
Type I Fibre	↑	↑↑↑	↑↑
Type IIa Fibre	↑↑	↑	↔
Type IIb Fibre	↑↑	↔	↔

Arrows denotes the degree to which an adaptation response may be generated: ↔, No change; ?, Unknown; ↑, Small effect; ↑↑ Moderate effect; ↑↑↑, Large effect.

Conversely, mRNA responses of select anabolic, metabolic and catabolic markers showed a propensity for exercise order to influence the adaptation profile with consecutive exercise bouts resulting in attenuation in muscle anabolic mRNA expression when endurance exercise was undertaken before resistance exercise, while transcription of markers of inflammation and degradation were exacerbated when endurance exercise was undertaken after resistance exercise (Coffey et al. 2009b).

A key variable consistent in the studies of Wang and co-workers and Coffey and colleagues was a 15 min recovery time between the consecutive exercise bouts. The authors suggest they chose this recovery period to replicate 'real world' training practices used by some elite athletes (Coffey et al. 2009b) although no studies have directly investigated the effect of recovery time between exercise sessions which would undoubtedly influence the adaptation response to concurrent training. Lundberg and colleagues compared molecular responses to resistance exercise compared to a resistance exercise bout followed by an aerobic exercise bout performed 6 h later (Lundberg et al. 2012). Their results showed concurrent training induced a greater phosphorylation of anabolic signalling proteins and suppressed myostatin expression for longer compared to resistance exercise alone. Thus, these findings indicate a greater magnitude of translation initiation signalling when aerobic and resistance exercise bouts are combined on the same day. Whether this response can be maintained with chronic concurrent training remains to be determined.

A limitation in our current understanding of the adaptation response to concurrent training is the lack of data on muscle protein synthesis. Furthermore, concurrent training studies have been almost exclusively conducted in the fasted state, therefore the impact of nutrient status/ingestion on adaptation responses is also unknown. Tipton and co-workers were the first to report changes in muscle protein synthesis in a concurrent training paradigm following combined swimming and resistance exercise (Tipton et al. 1996). Rates of mixed muscle protein synthesis were unchanged when swimming or resistance exercise was undertaken alone, however when undertaken concurrently mixed muscle protein synthesis was increased above resting levels (Tipton et al. 1996). This stimulatory effect on muscle protein synthesis with concurrent training may be explained by the increased volume of total work performed but does indicate, when combined, divergent exercise modes are capable of inducing an acute anabolic response in the fasted state.

Donges and colleagues recently compared post-exercise rates of myofibrillar and mitochondrial synthesis after acute bouts of resistance, aerobic and concurrent exercise with 20 g whey protein ingested immediately post-exercise (Donges et al. 2012). Despite the concurrent training group undertaking half the resistance exercise only volume, rates of myofibrillar protein synthesis were similar. Similarly, rates of mitochondrial protein synthesis were comparable between the concurrent training and aerobic exercise groups even with the concurrent training bout incorporating 50% of work compared with cycling alone (Donges et al. 2012). These results indicate an acute bout of concurrent training has the capacity to promote myofibrillar and mitochondrial adaptation comparable with single mode exercise in untrained/sedentary subjects.

Molecular Bases of the 'Interference' Paradigm?

Determining the molecular mechanisms governing a putative "interference effect" with concurrent resistance and endurance training has so far proved futile. It is plausible that the capacity to quantify the key proponents of the specificity of training adaptation is too restrictive and/or signalling proteins and pathways other than those currently identified may underpin the attenuation of hypertrophy. Nonetheless, there are several potential mechanisms that may regulate adaptation responses with concurrent training in human skeletal muscle and may explain, at least in part, adaptation interference.

Glycogen Availability

Manipulating muscle glycogen concentration to commence endurance-based exercise in a low glycogen state has been shown to increase the activities of several oxidative/metabolic proteins in skeletal muscle that promote endurance adaptation (Hansen et al. 2005, Yeo et al. 2010, Yeo et al. 2008, Hulston et al. 2010, Morton et al. 2009). How alterations in glycogen availability might impact adaptations to concurrent training is unknown. Low or 'sub-optimal' glycogen availability with limited recovery between training sessions during concurrent training may diminish the muscle anabolic adaptation response due to an increase in metabolic stress. Creer and co-workers showed low muscle glycogen concentration impairs the Akt phosphorylation response during the early recovery period following an acute bout of resistance exercise undertaken in the fasted state

(Creer et al. 2005). Furthermore, endurance exercise commenced with low muscle glycogen concentration exacerbates net muscle protein breakdown as a result of a concomitant increase in protein degradation and decrease in protein synthesis (Howarth et al. 2010). These results infer sub-optimal glycogen availability attenuates anabolic/ hypertrophy related training adaptations. Should concurrent training be undertaken with limited recovery between sessions and prevent adequate resynthesis of muscle glycogen an interference effect may ensue. Coffey and co-workers measured glycogen concentration when resistance exercise was undertaken before or after endurance exercise (Coffey et al. 2009b). While overall glycogen concentration after each concurrent session was comparable, when resistance exercise was performed after endurance exercise, it was undertaken with significantly lower glycogen availability. Nonetheless, the phosphorylation status of translation signalling proteins during the 3 h recovery period were similar regardless of exercise order (Coffey et al. 2009b). Whether the acute anabolic signalling response is predictive of protein synthesis in a "train-low state" or whether a potential negative effect on muscle mass occurs with repeated train low sessions during chronic concurrent training is unclear. Moreover, nutrient ingestion during recovery when resistance training with sub-optimal glycogen concentrations may have the potential to 'rescue' the anabolic environment but this has not been determined.

AMPK-mTORC1 Signalling

The past decade has established mTORC1 as an essential regulator in the adaptation response to exercise and also mediates important regulatory steps in the anabolic response to nutrients and insulin/IGF (Rivas, Lessard and Coffey 2009, Zoncu et al. 2011). However, the energy-sensing AMPK has emerged as an antagonistic factor with potential to modulate mTORC1 activity. Specifically, AMPK can suppress mTORC1 signalling by directly phosphorylating TSC1/2, mTOR and raptor (Gwinn et al. 2008, Inoki et al. 2003). Previous studies have implicated this metabolic cross-talk in regulation of skeletal muscle adaptation responses to exercise. For instance, AMPK phosphorylation/activation in response to mechanical overload-induced skeletal muscle growth has been correlated with an attenuation in mTOR-mediated signalling (Aguilar et al. 2007, Bolster et al. 2002, Thomson and Gordon 2005, Thomson and Gordon 2006). Alternately, decreases in mTOR signalling and muscle protein synthesis during resistance

exercise have been associated with an increase in AMPK activity (Dreyer et al. 2006). However, a muscle-specific knock-out of LKB1, the primary upstream kinase for AMPK, has failed to induce an increase in muscle cell size (Sakamoto et al. 2005) and McGee and co-workers have shown skeletal muscle hypertrophy following chronic mechanical overload may be promoted by the α1 isoform of AMPK (McGee et al. 2008). This is in agreement with recent findings that the AMPKα1 catalytic isoform plays an important role in the regulation of skeletal muscle growth (Mounier et al. 2009).

It is well established that increases in cellular AMP/ATP ratio activate AMPK, causing ATP-consuming anabolic processes such as protein synthesis and cell growth to be "switched off" (Hardie et al. 2012, Inoki et al. 2003). Moreover, multiple studies have reported increases in AMPK phosphorylation and activity during endurance exercise in human skeletal muscle (Coffey et al. 2005, Fujii et al. 2000, Mascher et al. 2011, Stephens et al. 2002, Wang et al. 2011, Wilkinson et al. 2008, Wojtaszewski et al. 2003, Wojtaszewski et al. 2000, Yu et al. 2003). Thus, increases in AMPK activity with endurance exercise and its putative negative regulation of mTORC1-mediated signalling represent a possible mechanism to explain reduced muscle hypertrophy and strength gains with concurrent resistance and endurance training. The AMPK complex also has a glycogen binding domain and it seems plausible alterations in glycogen availability with endurance exercise may have the capacity to negatively regulate mTORC1 activation (Bolster et al. 2002, Gwinn et al. 2008). This putative negative AMPK-mediated regulation of mTOR signalling may also interfere with molecular cues involved in satellite cell activation/ proliferation (Hardie 2005, Williamson, Butler and Alway 2009).

Notably, studies investigating post-exercise AMPK-mTOR signalling in human skeletal muscle with different exercise modes fail to support AMPK inhibition of mTOR phosphorylation and show no evidence for reciprocity in cross-talk between proteins/pathways (Benziane et al. 2008, Coffey et al. 2009b, Coffey et al. 2005, Dreyer et al. 2006, Mascher et al. 2011, Wang et al. 2011). Indeed, concomitant increases in mTOR signalling and/ or muscle protein synthesis with AMPK phosphorylation/activation have been observed following endurance, resistance and concurrent exercise (Coffey et al. 2009b, Wang et al. 2011, Benziane et al. 2008, Dreyer et al. 2006, Mascher et al. 2011). Considering AMPK activation may be regulated by exercise intensity (Chen et al. 2003, Fujii et al. 2000, Wojtaszewski et al. 2000), it is possible the exercise sessions

employed in these studies were inadequate to significantly disrupt cell energy status to a level required to observe a regulatory effect of AMPK on mTORC1 signalling responses. If any causal relationship of AMPK inhibition on mTOR signalling exists following concurrent training, considering the anabolic effects of amino acids in muscle, the ingestion of protein following exercise may represent a strategy to reduce this potential negative regulation of muscle mass.

Satellite Cell Activation

The majority of studies investigating the effects of exercise on satellite cells have been confined to resistance exercise with limited data reporting satellite cell content following concurrent training. Verney and co-workers have investigated satellite cell responses to concurrent training in the human skeletal muscle of elderly subjects where upper body resistance and lower body endurance exercise was undertaken in a single session (3 times/week). They show 14 weeks of training increased satellite cell content in response to both endurance (vastus lateralis) and resistance exercise (deltoid) by ~ 38% and this increase was selectively observed in type II fibres (Verney et al. 2008). Acute satellite cell responses were recently examined following a single bout of concurrent training and showed a modest increase in satellite cell number during the (9 h overnight) post-exercise recovery period (Snijders et al. 2012). However, in contrast to previous findings no differences in satellite cell activation were observed between type I and II muscle fibres. This is likely explained by the time-course of analysis (9 h) but may also relate to the elderly status of the subject cohort. While these studies show concurrent training may increase satellite cell content in human skeletal muscle, it is not known how this response compares to each exercise mode undertaken in isolation. Babcock and colleagues determined the satellite cell response to an acute resistance, endurance or concurrent training bout and quantified the satellite cell contribution to changes in muscle fibre characteristics with concurrent training. Interestingly, increases in satellite cell content with resistance exercise in isolation were ablated following the concurrent training bout, an effect that was evident in mixed, type I and type II fibres (Babcock et al. 2012). The mechanisms orchestrating this attenuation in satellite cell response with concurrent training are unknown but the authors suggest may be the result of differences in the hormonal milieu or AMPK activation generated with addition of an endurance exercise stimulus (Babcock et al. 2012). Whether these differences in the acute satellite cell response continue with chronic

concurrent training and attenuate hypertrophy is unknown. Nonetheless, this novel finding indicates combined resistance and endurance exercise may 'interfere' with, and inhibit, addition of myonuclei required for muscle fibre hypertrophy.

Summary

Adaptation responses and performance outcomes following concurrent training suggest a reduced capacity for the optimal development of hypertrophy/ strength compared with single-mode resistance exercise, but little attenuation of endurance adaptation. Debate exists regarding the potential mechanisms governing attenuated hypertrophy with concurrent training and may involve alterations in muscle glycogen availability, AMPK-mTORC1 crosstalk and satellite cell activation. Nonetheless, recent studies employing acute measurements of molecular markers regulating muscle anabolism indicate concurrent training may be as effective at stimulating adaptation responses as single mode resistance training, at least in the short-term, (Donges et al. 2012, Lundberg et al. 2012, Wang et al. 2011) but these findings remain equivocal (Coffey et al. 2009a, Coffey et al. 2009b). Interestingly, no studies to date have compared the effect of protein ingestion with a placebo treatment on muscle protein synthesis with concurrent training. Therefore, issues pertaining to the time-course for an interference effect or a specificity of training adaptation and whether protein ingestion has the capacity to enhance or inhibit the adaptation response remain unclear.

1.8 Aims of the Thesis

The adaptive responses to exercise in human skeletal muscle are highly specific to the type of contractile activity performed. Indeed, resistance training stimulates increases in myofibrillar protein synthesis and promotes muscle hypertrophy and strength, while endurance exercise induces increases in mitochondrial content that underlie increases in aerobic capacity. Further, these adaptive processes are enhanced by nutrient (protein/carbohydrate) ingestion/availability during the early recovery period. Many cell signalling pathways in human skeletal muscle are exercise and nutrient responsive and have been implicated in the adaptation response in human skeletal muscle. However, there is limited knowledge regarding the activation of the signalling machinery to divergent exercise modes performed in isolation or consecutively, and their contribution to the specificity of training adaptation in skeletal muscle. Whether these adaptation responses are influenced by altered substrate availability is also unknown. Accordingly, the primary aim of the investigations undertaken for this thesis was to determine the mechanistic underpinning of the specificity of acute exercise-induced adaptation responses.

The aim of the first study (Chapter 2) was to investigate the early (60 min) time course of Akt-mediated cell signalling after resistance and endurance exercise and to provide new information regarding the optimal timing for post-exercise muscle biopsy sampling. The hypothesis tested was that the divergent exercise modes would generate differences in the timing and/ or magnitude of signalling events in accordance with the respective contractile stimulus. Thus, the prolonged, moderate-intensity contraction, and greater glycogen depletion associated with endurance cycling would initiate early Akt-mediated signalling for glucose uptake and glycogen resynthesis compared with the activation of markers for translation initiation associated with protein synthesis following resistance exercise.

In the second experimental chapter (Chapter 3), glycogen concentration was manipulated to determine the effect of initiating resistance exercise with sub-optimal glycogen concentration on acute anabolic responses (i.e.: cell signalling and myofibrillar protein synthesis) in the fasted state or with protein/ carbohydrate supplementation. Considering muscle protein synthesis is an energy-requiring process, the hypothesis tested was that low muscle glycogen concentration (compared to normal) would suppress the muscle anabolic response to resistance exercise but that

nutrient provision in the early recovery period would restore muscle anabolism to a state that may promote hypertrophy.

Finally, the third study (Chapter 4) examined the acute effects of protein ingestion compared with placebo on myofibrillar and mitochondrial protein synthesis following a bout of consecutive resistance exercise and cycling. The hypothesis tested in this study was that protein ingestion would enhance anabolic and metabolic signalling and subsequent protein synthesis during the early recovery period following the concurrent exercise bout.

Chapter Two

Early Time Course of Akt Phosphorylation after Endurance and Resistance Exercise

Adapted from: Camera, D.M., Edge, J., Short, M.J., Hawley, J.A., Coffey, V.G. (2010) *Medicine in Sports and Sports and Exercise,* 42:1843-52

2.1 Introduction

Mechanotransduction is an intricate series of events that converts contraction-mediated stimuli into biological responses in skeletal muscle (Coffey and Hawley 2007b). In this regard, cell signalling networks are a complex yet integral part of the adaptation process responding to numerous inputs and generating a multiplicity of outputs resulting in altered biological function (Coffey and Hawley 2007b). Contractile activity associated with exercise training represents a stimulus capable of inducing transient alterations in cell signal transduction and metabolism that, when repeated over time, acts to promote the selective attainment of specific biochemical and morphological adaptations in skeletal muscle (Coffey and Hawley 2007b). Specifically, high-intensity, short-duration contraction promotes muscle hypertrophy and strength, whereas prolonged, low-intensity contractile activity is associated with increased mitochondrial density and enhanced resistance to fatigue (Coffey and Hawley 2007b). Many signalling pathways are exercise responsive and have been implicated in adaptation in human skeletal muscle. Indeed, signalling mechanisms in muscle are modulated with the onset of contraction and during the initial minutes or hours after cessation of exercise (Coffey et al. 2009a, Coffey et al. 2009b, Coffey et al. 2005, Mascher et al. 2007, Rose et al. 2009b). However, the current understanding of the various signalling responses and interactions that may be involved in orchestrating exercise-induced adaptation is far from complete. For example, the insulin–insulin-like growth factor (IGF) signalling pathway has been implicated in regulating exercise-induced adaptation in skeletal muscle given its putative capacity to direct diverse cell processes such as glucose transport, glycogen resynthesis, hypertrophy, translation, and ubiquitin-mediated protein degradation (Coffey and Hawley 2007b). An important focal point in the insulin–IGF pathway is the serine-threonine kinase Akt (also known as protein kinase B/PKB), which is proposed to regulate many cell signalling responses and adaptation machinery stimulated by vigorous exercise (Coffey and Hawley 2007b). Previous investigations examining Akt-mediated signalling in skeletal muscle after exercise have shown disparate responses (Benziane et al. 2008, Coffey et al. 2005, Deldicque et al. 2008, Mascher et al. 2007), possibly reflecting differences in research design such as exercise mode, intensity, and/or duration. Regardless, the apparent lack of clarity makes it difficult to ascertain the precise role of Akt-mediated signalling in promoting or inhibiting activity of important exercise-induced mechanisms that may contribute to the specificity of training adaptation in skeletal muscle. Accordingly, the primary aim of the present

study was to determine the early time course of phosphorylation for cell signalling proteins after resistance and endurance exercise and to provide new information regarding the optimal timing for post-exercise muscle biopsy sampling. It was hypothesised that the prolonged, moderate-intensity contraction, and greater glycogen depletion associated with endurance cycling would initiate early Akt-mediated signalling for glucose uptake compared with the anabolic Akt-mTOR-S6K phosphorylation response to resistance exercise occurring later for translation and subsequent protein synthesis. Thus, the divergent exercise modes would generate a disparity in the timing and/or magnitude of phosphorylation events in human skeletal muscle.

2.2 Methods

Overview

Sixteen healthy male subjects with a training history in recreational fitness or team sports volunteered for this study. Subjects were randomly assigned to either a cycling (n = 8; mean ± SE: age = 29.0 ± 2.3 yr, body mass = 77.1 ± 5.2 kg, peak oxygen uptake ($V\dot{O}_{2peak}$) = 54.3 ± 1.3 $mL \cdot kg^{-1} \cdot min^{-1}$) or a resistance (n = 8; mean ± SE: age = 28.4 ± 1.6 yr, body mass = 81.8 ± 5.6 kg, one-repetition maximum (1RM) leg extension = 120.0 ± 10.4 kg) exercise group. The diverse exercise modes used in the present study would be expected to generate a significant disparity in total work (cycling ~660 kJ vs. resistance exercise <130 kJ) (Bloomer 2005). In addition, the study was restricted to between-group comparisons because of the prohibitive number of muscle biopsies (i.e. 10) with a cross-over design. The experimental procedures and the possible risks associated with the study were explained to each subject, who all gave written informed consent before participation. The study was approved by the Human Research Ethics Committee of the RMIT University (Melbourne, Australia) and the Massey University (Palmerston North, New Zealand).

Study Design

Preliminary testing

$V\dot{O}_{2peak}$. $V\dot{O}_{2peak}$ was determined during an incremental test to volitional fatigue on a Lode cycle ergometer (Groningen, The Netherlands). The protocol has been described in detail previously

(Hawley and Noakes 1992). In brief, subjects commenced cycling at a workload equivalent to 1.5 $W \cdot kg^{-1}$ for 150 s. Thereafter, the workload was increased by 25 W every 150 s until volitional fatigue, defined as the inability to maintain a cadence > 70 rpm. Throughout the test, which typically lasted 10 – 12 min, subjects breathed through a mouthpiece attached to a metabolic cart (Vista TurboFit; VacuMed, Ventura, CA) to record oxygen consumption.

Maximal Strength. Quadriceps strength was determined during a series of single repetitions on a standard pulley leg extension machine (Fitness Works, Auckland, New Zealand) until the maximum load lifted was established (1RM). Repetitions were separated by a 3-min recovery and were used to establish the maximum load or weight that could be moved through the full range of motion once, but not a second time. Exercise range of motion was 85º, with leg extension end point set at -5º from full extension.

Diet and Exercise Controls. Before both experimental trials (described subsequently), subjects were instructed to refrain from alcohol consumption and vigorous physical activity for a minimum of 48 h. Subjects were provided with standardized prepacked meals that consisted of 3 g of carbohydrate per kilogram of body mass, 0.5 g of protein per kilogram of body mass, and 0.3 g of fat per kilogram of body mass consumed as the final caloric intake the evening before reporting for an experimental trial.

Experimental Trials. On the morning of an experimental trial, subjects reported to the laboratory after an approximately 10-h overnight fast. After subjects rested in the supine position for approximately 15 min, local anaesthesia (2 – 3 mL of 2% Xylocaine (lignocaine)) was administered to the skin, subcutaneous tissue, and fascia of the vastus lateralis muscle (~ 15 cm above the patella) in preparation for the series of muscle biopsies. A resting (basal) biopsy was taken using a 5-mm Bergstrom needle modified with suction, and approximately 150 mg of muscle was removed, blotted to remove excess blood, and immediately frozen in liquid nitrogen. Four additional incisions were made in preparation for subsequent post-exercise biopsies. Subjects then completed a bout of either cycling or resistance exercise (described in detail subsequently), and a second biopsy was taken immediately after the cessation of exercise. Subjects then rested in the supine position for 60 min, and further biopsies were taken after 15, 30, and 60 min post-exercise recovery. Muscle biopsies were taken from a separate site (distal to proximal) from the same leg. Samples were stored at -80ºC until subsequent analysis. In addition,

a catheter was inserted into the antecubital vein for blood sampling, and blood samples (~2 mL) were taken at equivalent time points with muscle biopsies.

Cycling Exercise. Subjects performed 60 min of continuous cycling at a power output that elicited approximately 70% of individual VO_{2peak}. Subjects were fan cooled and allowed ad libitum access to water throughout the ride. Visual feedback for pedal frequency, power output, and elapsed time was provided to subjects.

Resistance Exercise. After a standardized warm-up (1 × 5 repetition at 50% and 60% 1RM), subjects performed eight sets of five repetitions leg extension at approximately 80% 1RM. Each set was separated by a 3-min recovery period during which the subject remained seated on the leg extension machine. Contractions were performed at a set metronome cadence, and strong verbal encouragement was provided during each set.

Analytical Procedures

Blood Glucose, Lactate, and Insulin. Whole blood samples were collected in EDTA-containing tubes and immediately analysed for glucose and lactate concentration using an automated glucose/lactate analyser (YSI 2300, Yellow Springs, OH). Blood samples were then centrifuged at 1000g (4ºC) for 15 min, and aliquots of plasma were stored at -80ºC until analysis. Plasma insulin concentration was determined using an immunoassay (EIA) kit (ALPCO Diagnostics, Salem, NH).

Muscle glycogen. A small piece of frozen muscle (~ 20 mg) was freeze dried and powdered to determine muscle glycogen concentration. Freeze-dried muscle was extracted with 500 µL of 2 M hydrochloric acid, incubated at 100ºC for 2 h, and then neutralised with 1.5 mL of 0.67 M sodium hydroxide for subsequent determination of glycogen concentration via enzymatic analysis with fluorometric detection (Jasco FP-750 spectrofluorometer, Easton, MD) at excitation 365 nm/emission 455 nm. Glycogen concentration was expressed as millimoles of glycogen per kilogram of dry weight.

Western Blots. Muscle samples were homogenized in ice-cold buffer (1:8 mg muscle:mL buffer) containing 50 mM of Tris–HCl, pH 7.5, 1 mM of EDTA, 1 mM of EGTA, 10% glycerol, 1% Triton X-100, 50 mM of NaF, 5 mM of sodium pyrophosphate, 1 mM of DTT, 10 $\mu g \cdot mL^{-1}$ of

trypsin inhibitor, 2 $\mu g \cdot mL^{-1}$ of aprotinin, 1 mM of benzamidine, and 1 mM PMSF using a motorized pellet pestle (Sigma-Aldrich, St. Louis, MO) with 5-s pulses. The lysate was kept on ice at all times and was then centrifuged at 12,000g for 20 min at 4ºC. The supernatant was transferred to a sterile tube and was subsequently aliquoted for determination of protein concentration using a BCA protein assay (Pierce, Rockford, IL). Lysate was then resuspended in Laemmli sample buffer, with 50 Kg of protein loaded and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes blocked with 5% non-fat milk, washed with 10 mM of Tris–HCl, 100 mM of NaCl, and 0.02% Tween 20, and incubated with primary antibody (1:1000) overnight at 4ºC. Membranes were incubated with secondary antibody (1:2000), and proteins were detected via enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK; Pierce Biotechnology, Rockford, IL) and quantified by densitometry. All sample time points for each subject were run on the same gel, and data are expressed relative to α-tubulin. Polyclonal anti-phospho- Akt^{Ser473} (cat no.9271), anti-phospho-mTOR^{Ser2448} (2971), anti-phospho-glycogen synthase (GS)^{Ser641} (3891), antiphospho- 4E-BP1^{Thr70} (9455), and anti-phospho-Ser/Thr Akt substrate (9611) and monoclonal anti-phospho-Akt^{Thr308} (4056), anti-phospho-tuberin (TSC2)^{Thr1462} (3617), antiphospho- GSK-3β^{Ser9} (9323), and anti-phospho-4E-BP1$^{Thr37/46}$ (2855) were from Cell Signalling Technology (Danvers, MA). Polyclonal anti-phospho-eIF2Bε^{Ser539} (44-530G) was from Biosource (Carlsbad, CA), and p70S6K^{Thr389} (04-392) was from Millipore (Temecula, CA). Anti-phospho- AMPKα^{Thr172} was raised against AMPK-α peptide (KDGEFLRpTSCGAPNY) as described previously (Clark et al. 2004). Monoclonal anti-α tubulin control protein antibody was from Sigma-Aldrich (T6074).

Statistical Analysis

All data were analysed by two-way ANOVA (two factor: time × exercise) with Student–Newman–Keuls post hoc analysis. Statistical significance was established when $P < 0.05$ (SigmaStat for Windows, Version 3.11). Phosphorylation at rest was not different between exercise groups for any of the proteins of interest. Consequently, data are expressed relative to rest in arbitrary units ± SEM.

2.3 Results

Blood Lactate, Insulin, and Glucose

Peak blood lactate concentration after cycling (CYC) occurred immediately post-exercise ($P < 0.05$; Table 2.1). Blood lactate remained elevated above rest after 15 min (146%, $P < 0.001$) and 30 min (79%, $P < 0.01$) of recovery from CYC. After resistance exercise (REX), blood lactate concentration was significantly elevated above rest immediately post-exercise (106%, $P < 0.05$). Blood lactate concentrations after CYC were higher compared with REX at each corresponding time point during the 60-min recovery period ($P < 0.01$; Table 2.1). There were no differences in plasma insulin and blood glucose concentration (Table 2.1).

Table 2.1 Blood lactate, insulin, and glucose concentration measured at rest and during 60 min post-exercise recovery (mean ± SE).

	CYC					REX				
	Rest	0 min	15 min	30 min	60 min	Rest	0 min	15 min	30 min	60 min
Lactate ($mmol \cdot ^{-1}$)	1.57 ± 0.2	5.93 ± 0.6[a]	3.87 ± 0.4[a,b]	2.82 ± 0.5[a,b,c]	2.45 ± 0.5[b,c]	1.29 ± 0.2	2.68 ± 0.3*,[a]	1.87 ± 0.2*	1.34 ± 0.1*,[b]	1.20 ± 0.1*,[b]
Insulin ($\mu IU \cdot mL^{-1}$)	4.16 ± 0.9	4.17 ± 1.0	4.17 ± 1.0	5.88 ± 0.9	3.85 ± 0.7	3.83 ± 0.8	10.60 ± 4.2	7.39 ± 2.1	4.75 ± 1.2	7.17 ± 3.5
Glucose ($mmol \cdot L^{-1}$)	5.32 ± 0.1	5.11 ± 0.2	5.21 ± 0.2	4.92 ± 0.2	4.78 ± 0.2	5.46 ± 0.2	5.27 ± 0.2	5.21 ± 0.2	5.20 ± 0.2	4.95 ± 0.1

Significantly different vs. [a]rest, [b]0 min, and [c]15 min post-exercise; *different vs. CYC ($P < 0.05$). CYC, cycling; REX, resistance exercise.

Muscle Glycogen

Muscle glycogen concentration decreased approximately 56% after 60 min of CYC (214 $mmol \cdot kg \cdot^{-1}$ of dry weight, $P < 0.001$; Figure 2.1), whereas REX reduced muscle glycogen approximately 26% (103 $mmol \cdot kg \cdot^{-1}$ of dry weight, $P < 0.01$). Of note, pre-exercise muscle glycogen concentration was not different, but greater glycogen utilization during 60 min CYC compared with REX resulted in significantly lower post-exercise glycogen concentration (REX vs. CYC ~93%, $P < 0.01$; Figure 2.1).

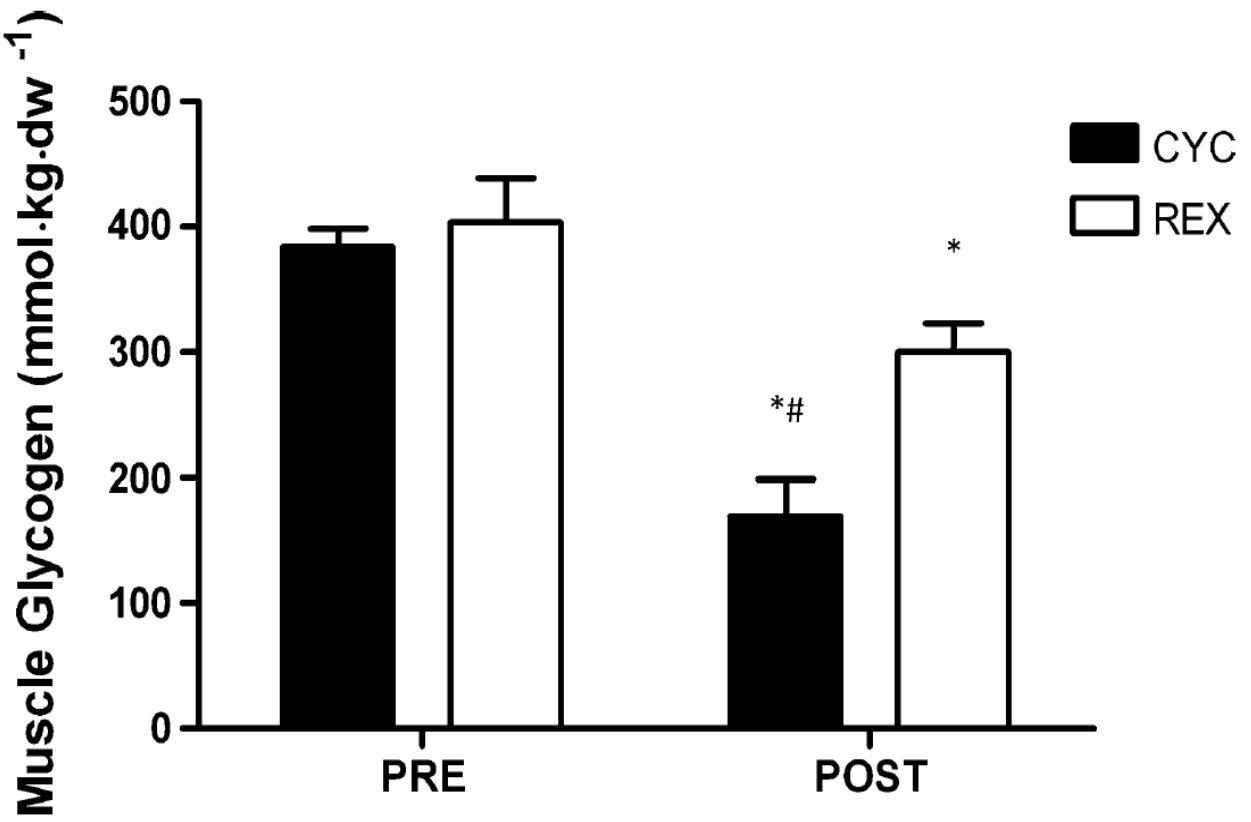

Figure 2.1 Muscle glycogen concentration before (PRE) and immediately after (POST) cycling (CYC; 60 min at ~70% VO_{2peak}) or resistance exercise (REX; 8 × 5 leg extensions at ~ 80% 1RM). Values are means ± SE. dw, dry weight; *different vs. rest, #different vs. REX ($P < 0.05$).

Signalling Responses

Akt/tuberous sclerosis complex (TSC) 2/mammalian target of rapamycin (mTOR). There were comparable changes in Akt^{Thr308} and Akt^{Ser473} phosphorylation during the 60-min post-exercise time course after both CYC and REX (Figures. 2.2A and B). Moreover, despite only minor changes during the initial recovery period (0 – 15 min), there were significant differences for 30 and 60 min post-exercise compared with rest. Specifically, CYC increased Akt^{Thr308} phosphorylation approximately 250% – 300% and Akt^{Ser473} phosphorylation approximately 130% after 30 and 60 min of recovery, respectively ($P < 0.05$). Similarly, REX induced a significant increase in $Akt^{Thr308/Ser473}$ phosphorylation above rest (~100% – 200%) at equivalent time points ($P < 0.05$; Figures. 2.2A and B).

Changes in $TSC2^{Thr1462}$ phosphorylation after CYC were increased above rest at all time points peaking 30 min post-exercise (~ 130%, $P < 0.001$), whereas REX did not alter TSC2 phosphorylation (Figure 2.2C). Consequently, phosphorylation of $TSC2^{Thr1462}$ during recovery from CYC was higher compared with corresponding time points after REX ($P < 0.01$; Figure 2.2C). There were variable responses in $mTOR^{Ser2448}$ phosphorylation that resulted in significant effects for time and exercise mode ($P < 0.05$; Figure 2.2D).

CYC generated a significant increase in $mTOR^{Ser2448}$ phosphorylation above rest immediately post-exercise (~ 100%, $P < 0.05$) then rapidly abated during the initial 15-min recovery but was elevated 60 min post-exercise (~ 90%, $P = 0.051$). In contrast, $mTOR^{Ser2448}$ phosphorylation after REX was largely unaffected during the initial recovery period (0 – 15 min) but increased 30 min post-exercise (~ 100%, $P < 0.05$). The divergent $mTOR^{Ser2448}$ phosphorylation immediately post-exercise resulted in a significant difference between exercise modes ($P < 0.05$; Figure 2.2D).

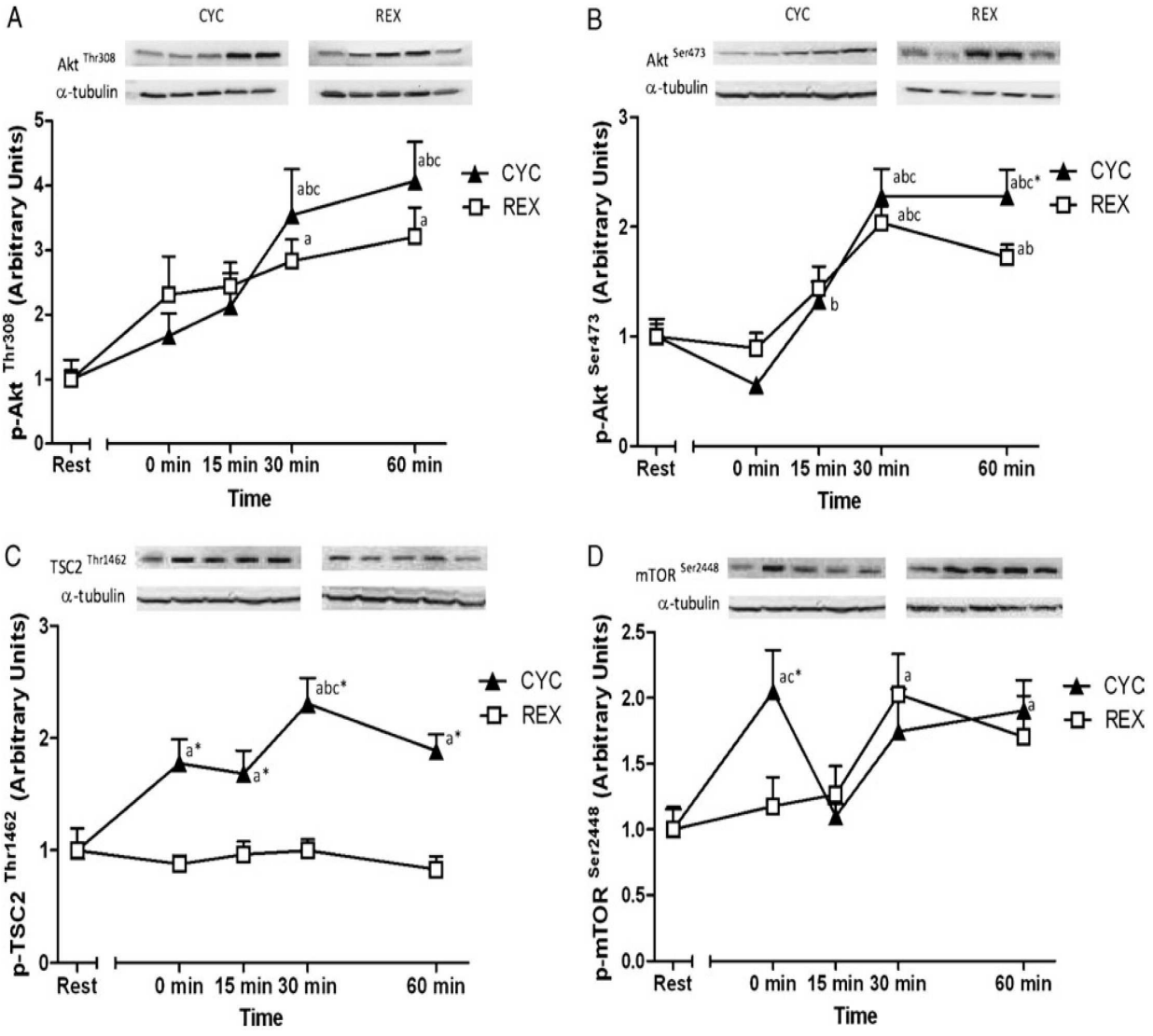

Figure 2.2 Akt^{Thr308} (A), Akt^{Ser473} (B), tuberous sclerosis complex 2 (TSC2)^{Thr1462} (C), and mammalian target of rapamycin (mTOR)^{Ser2448} (D) phosphorylation in skeletal muscle at rest and during 60 min post-exercise recovery after cycling (CYC; 60 min at ~ 70% VO_{2peak}) or resistance exercise (REX; 8 × 5 leg extensions at ~ 80% 1RM). Values are expressed relative to α-tubulin and presented in arbitrary units (mean ± SE, n = 8). Different vs. ^{a}rest, ^{b}0 min, and ^{c}15 min; *different between exercise mode at equivalent time point ($P < 0.05$).

p70 S6 kinase (S6K)/eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1). There were similar increases in $S6K^{Thr389}$ phosphorylation for each exercise mode during the acute post-exercise recovery period (Figure 2.3A). However, the increase in S6K phosphorylation above rest failed to reach significance throughout the 60-min recovery period after CYC (62% – 140%), whereas phosphorylation of $S6K^{Thr389}$ was only different from rest 60 min post-exercise after REX (~ 176%, $P = 0.050$; Figure 2.3A).

There were comparable post-exercise responses between exercise modes for phosphorylation of 4E-$BP1^{Thr37/46}$ but a greater magnitude of response during recovery from CYC (Figure 2.3B). There was a significant dephosphorylation at $4EBP1^{Thr37/46}$ compared with rest immediately post-exercise after CYC (~ 92%, $P < 0.01$). During the subsequent 30-min recovery period, 4E-$BP1^{Thr37/46}$ phosphorylation rapidly increased and was elevated at 30 min (95%, $P < 0.05$) and 60 min of recovery from CYC (77%, $P < 0.05$). In contrast, REX failed to generate a significant change in $4EBP1^{Thr37/46}$ phosphorylation during the recovery period, resulting in a difference from CYC at 30 and 60 min post-exercise (~60%, $P < 0.05$; Figure 2.3B).

Changes in $4EBP1^{Thr70}$ phosphorylation were discordant compared with 4E-$BP1^{Thr37/46}$ (Figure 2.3C). Specifically, 4E-$BP1^{Thr70}$ phosphorylation decreased after CYC and remained suppressed during the 60-min recovery period (52% – 58%), approaching significance 15 min post-exercise (-72%, $P = 0.056$; Figure 2.3C). Conversely, there was a modest increase in phosphorylation at 4E-$BP1^{Thr70}$ that was sustained throughout the recovery period after REX, but these increases failed to reach statistical significance (40% – 52%). The divergent 4E-$BP1^{Thr70}$ phosphorylation responses resulted in a significant exercise mode effect at all post-exercise time points ($P < 0.01$; Figure 2.3C).

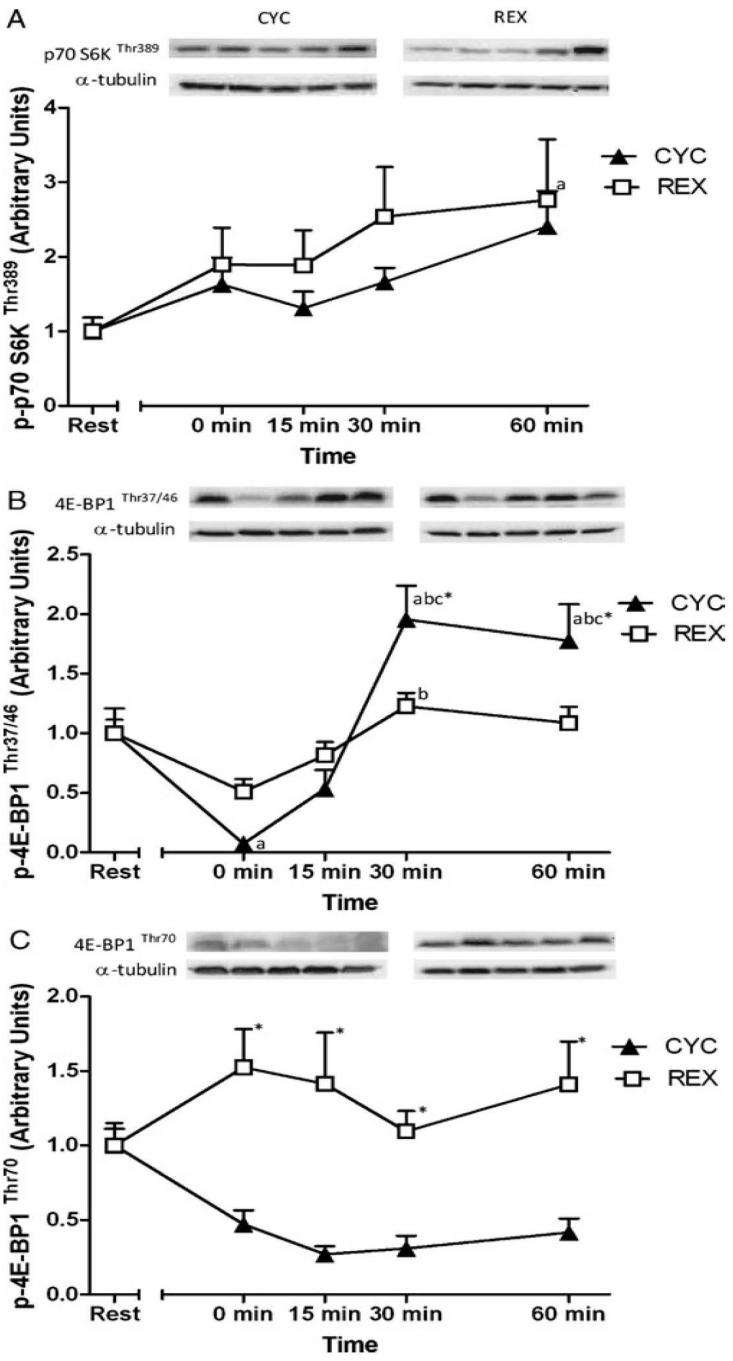

Figure 2.3 p70S6K^{Thr389} (A), eukaryotic initiation factor 4E-binding protein 1 (4E-BP1)$^{Thr37/46}$ (B), and 4E-BP1^{Thr70} (C) phosphorylation in skeletal muscle at rest and during 60 min post-exercise recovery after cycling (CYC; 60 min at ~ 70% VO_{2peak}) or resistance exercise (REX; 8 × 5 leg extensions at ~ 80% 1RM). Values are expressed relative to α-tubulin and presented in arbitrary units (mean ± SE, n = 8). Different vs. [a]rest, [b]0 min, and [c]15 min; *different between exercise mode at equivalent time point ($P < 0.05$).

Glycogen synthase kinase (GSK) 3β/glycogen synthase (GS)/eukaryotic translation initiation factor (eIF) 2Bε. GSK-3β^{Ser9} phosphorylation increased above rest immediately post-exercise and 30 –60 min after CYC (~80% – 100%, $P < 0.05$; Figure 2.4A). The increase (51%) in GSK-3β^{Ser9} phosphorylation immediately after REX failed to reach significance and was not different from rest throughout recovery. Consequently, phosphorylation of GSK-3β during recovery from CYC was higher 30 – 60 min post-exercise compared with REX (~ 60%, $P < 0.05$; Figure 2.4A).

CYC induced a significant dephosphorylation of GS^{Ser641} immediately post-exercise (-59%, $P < 0.05$), which was sustained throughout the 60-min recovery period (~-74%, $P < 0.05$; Figure 2.4B). Conversely, GS^{Ser641} phosphorylation after REX was largely unchanged during the post-exercise period (30% – 45%). As a result, phosphorylation of GS^{Ser641} during recovery from REX was higher compared with equivalent time points after CYC ($P < 0.01$; Figure 2.4B).

CYC generated a significant increase in eIF2Bε^{Ser539} phosphorylation above rest immediately post-exercise (55%, $P < 0.05$), and this increase was also different from the corresponding REX time point ($P < 0.05$; Figure 4C). Phosphorylation at eIF2Bε^{Ser539} after CYC then rapidly abated during the initial 15-min recovery (-59%, $P < 0.05$) and remained suppressed for the remainder of the recovery period. Despite a modest attenuation in eIF2Bε^{Ser539} phosphorylation throughout recovery from REX, these changes were not significantly different from rest.

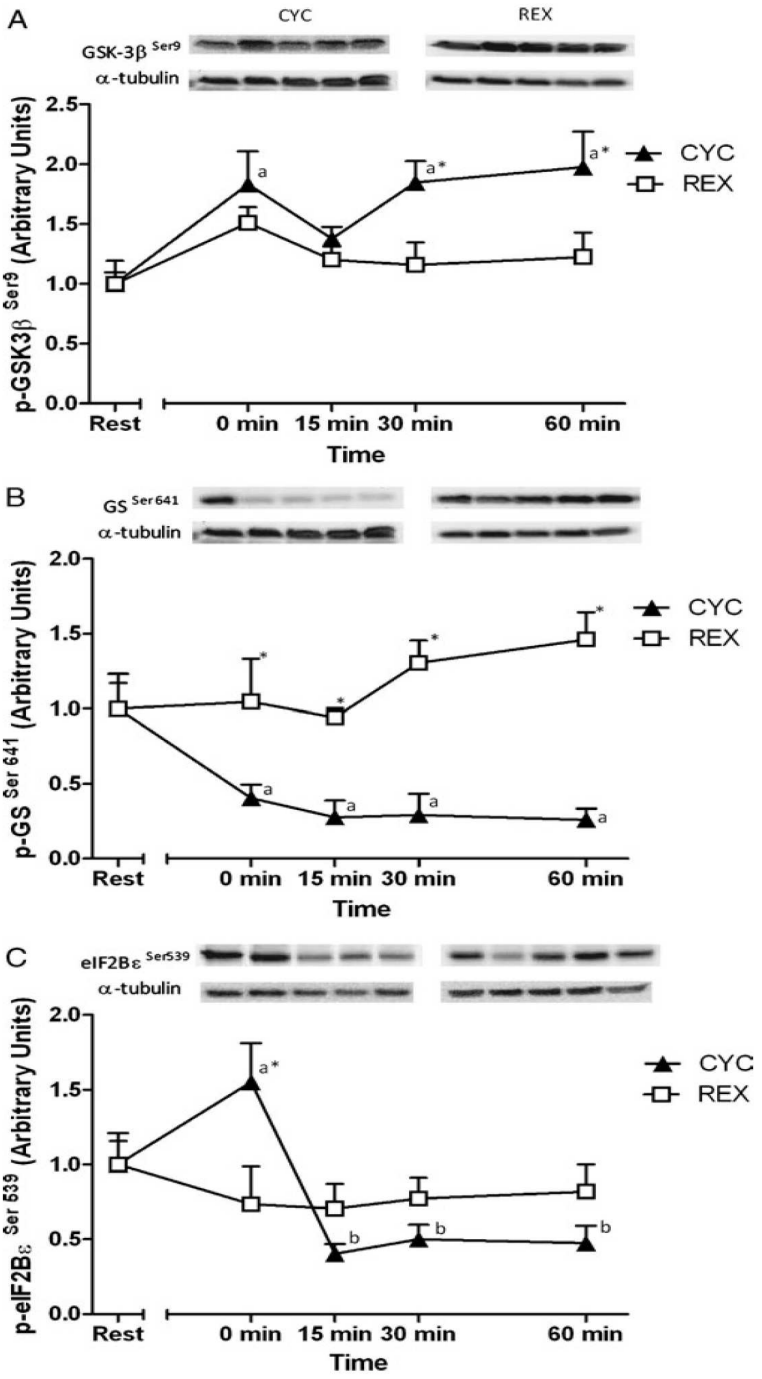

Figure 2.4 Glycogen synthase kinase-3β (GSK-3β)^{Ser9} (A), glycogen synthase (GS)^{Ser641} (B), and eukaryotic translation initiation factor 2Bε (eIF2Bε)^{Ser539} (C) phosphorylation in skeletal muscle at rest and during 60 min post-exercise recovery after cycling (CYC; 60 min at ~ 70% VO_{2peak}) or resistance exercise (REX; 8 × 5 leg extensions at ~ 80% 1RM). Values are expressed relative to α-tubulin and presented in arbitrary units (mean ± SE, n = 8). Different vs. ^{a}rest, ^{b}0 min, and ^{c}15 min; *different between exercise mode at equivalent time point ($P < 0.05$).

AMP-activated protein kinase (AMPK)/Akt substrate (AS) 160 kDa. $AMPK^{Thr172}$ phosphorylation after CYC was elevated above rest throughout the 60-min recovery period and was highest 30 min post-exercise (~ 195%, $P < 0.001$), whereas REX failed to induce any significant changes in post-exercise $AMPK^{Thr172}$ phosphorylation (Figure 2.5A). Phosphorylation of $AMPK^{Thr172}$ during recovery from CYC was higher 0 and 30 min post-exercise compared with equivalent time points after REX ($P < 0.05$; Figure 2.5A).

After modest increases in phosphorylation of AS160 during the initial post-exercise period (0 – 15 min), there was a rapid elevation 30 and 60 min after CYC (240% – 270%, $P < 0.001$; Figure 2.5B). REX failed to induce a change in AS160 phosphorylation during the post-exercise recovery period. Phosphorylation of AS160 after CYC was also higher compared with REX 15 – 60 min after exercise (104% – 140%, $P < 0.01$; Figure 2.5B).

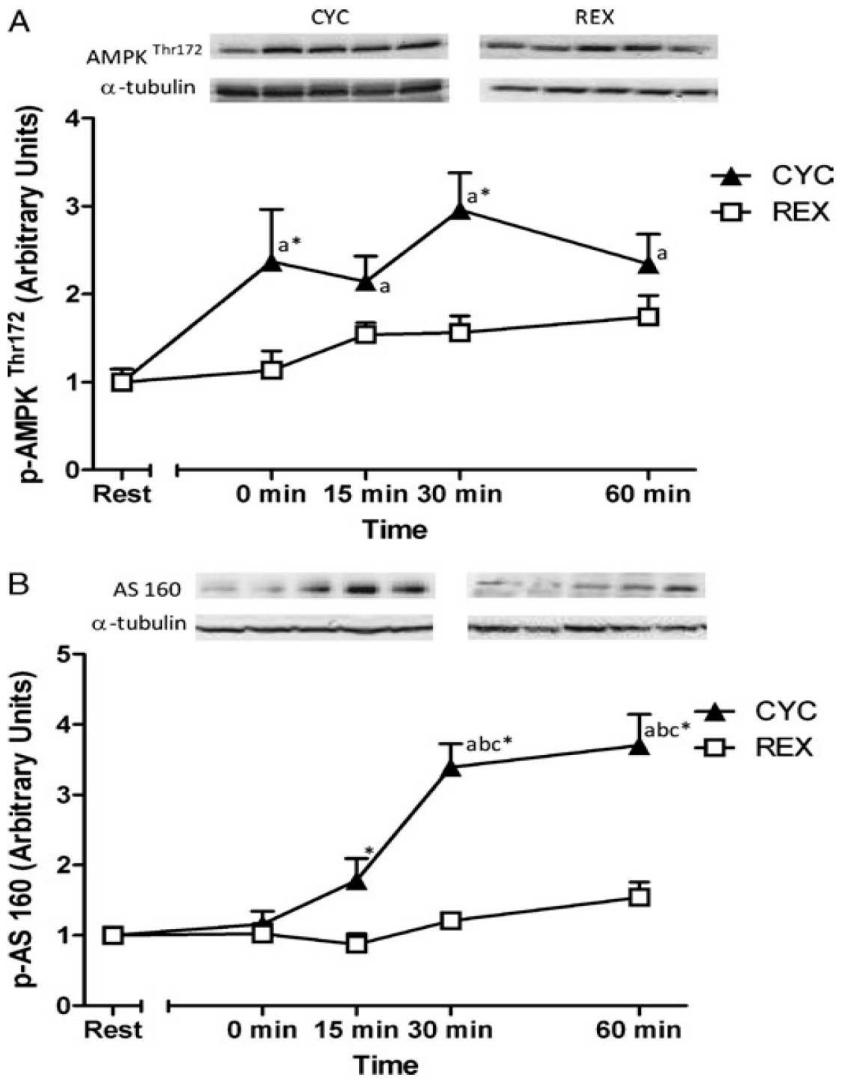

Figure 2.5 The 5'-adenosine monophosphate-activated protein kinase (AMPK)^{Thr172} (A) and the Akt substrate 160-kDa (AS160) (B) phosphorylation in skeletal muscle at rest and during 60 min post-exercise recovery after cycling (CYC; 60 min at ~ 70% VO_{2peak}) or resistance exercise (REX; 8 × 5 leg extensions at ~ 80% 1RM). Values are expressed relative to α-tubulin and presented in arbitrary units (mean ± SE, n = 8). Different vs. [a]rest, [b]0 min, and [c]15 min; *different between exercise mode at equivalent time point ($P < 0.05$).

2.4 Discussion

Cell signalling transduction networks are essential pathways for converting contraction-mediated signals to physiological responses. The aim of the present study was to establish the early time course of putative exercise-mediated phosphorylation responses after endurance and resistance exercise. These results show a similar time course for Akt- mTOR- S6K phosphorylation during the initial 60-min post-exercise recovery period despite differences in contractile activity with diverse exercise modes. Conversely, a distinct phosphorylation status to promote glucose transport and glycogen synthesis after endurance but not resistance exercise was observed. These results indicate that peak phosphorylation for many of the proteins of the insulin–insulin-like growth factor pathway occurs 30 – 60 min after exercise in the fasted state.

The first finding of the present study was the highly coordinated changes in the Akt^{Thr308} and Akt^{Ser473} phosphorylation time course after divergent exercise (Figures 2.2A and B), which occurred independent of blood glucose or insulin concentration (Table 2.1). Previous studies in humans have reported increased (Coffey et al. 2005, Creer et al. 2005, Dreyer et al. 2006, Howlett et al. 2008, Mascher et al. 2007, Sakamoto et al. 2004), decreased (Deldicque et al. 2008, Terzis et al. 2008), or unchanged (Benziane et al. 2008, Coffey et al. 2005, Creer et al. 2005) Akt phosphorylation after exercise in the fasted state. Accordingly, although the possibility exists that the apparent lack of agreement in exercise-induced Akt phosphorylation responses in previous studies may be attributed in part to differences in exercise intensity, duration, and/or subject muscle glycogen and training status, these results indicate that the timing of muscle biopsy sampling is an essential consideration. Moreover, exercise mode appears to have little impact in generating divergence in the phosphorylation status of Akt, at least during the first hour post-exercise. Work in rodents has shown an earlier peak activation of Akt in skeletal muscle (~5 – 10 min) after cessation of contractile activity (Bolster et al. 2003, Sakamoto et al. 2002). However, these findings are similar to those of Mascher et al. (Mascher et al. 2007) and Dreyer et al. (Dreyer et al. 2006), showing elevated Akt^{Ser473} phosphorylation 60 min post-exercise after endurance and resistance exercise in humans, respectively.

Akt may directly activate mTOR and subsequent downstream targets through phosphorylation at the $mTOR^{Ser2448}$ residue (Navé et al. 1999) or indirectly by phosphorylation and subsequent inhibition of tuberous sclerosis complex 2 (TSC2) at ^{Thr1642} (Inoki et al. 2002). There is a paucity

of information relating to the exercise-induced TSC2 phosphorylation response *in vivo* skeletal muscle, and it can only be speculated that the low- to moderate-intensity and prolonged duration of contraction associated with endurance but not resistance exercise initiates a yet to be defined mechanism for the $TSC2^{Thr1462}$ phosphorylation observed here. In contrast, exercise-induced $mTOR^{Ser2448}$ phosphorylation corresponded more closely with that of Akt (Figure 2.2D). Thus, endurance and resistance exercise appear capable of enhancing mTOR phosphorylation, and the diverse contractile stimuli both appear to initiate pathways to promote translation in skeletal muscle. There are numerous cell functions regulated by mTOR, and the immediate post-exercise increase in phosphorylation after cycling may reflect the putative role of mTOR in control of mitochondrial oxidative function (Cunningham et al. 2007). Moreover, Mascher et al. (Mascher et al. 2007) and Benziane et al. (Benziane et al. 2008) have also shown an increase in mTOR phosphorylation immediately after 60 min of cycling at approximately 70% – 75% VO_{2peak}.

Regulation of translation for protein synthesis via Akt-mTOR includes two parallel effectors: S6K and 4E-BP1 (Ruvinsky et al. 2005, Besse and Ephrussi 2008). S6K phosphorylation is proposed to enhance translation of mRNAs encoding ribosomal proteins and elongation factors, whereas 4E-BP1 can bind to eIF4E and prevent cap-dependent mRNA translation (Wang et al. 2005). This data provides further support for the contraction-mediated increase in S6K phosphorylation during the early recovery phase after exercise in human skeletal muscle (Coffey et al. 2005, Dreyer et al. 2006, Spiering et al. 2008, Terzis et al. 2008). Although there is evidence that elevated S6K phosphorylation may persist 2 – 6 h after resistance exercise in the fasted state (Coffey et al. 2005, Dreyer et al. 2006, Glover et al. 2008a, Spiering et al. 2008), the increase in translational activity appears to be initiated within the first 60 min of recovery.

4E-BP1 inhibits translation initiation by binding to eIF4E, preventing formation of the multiprotein eIF4E-G scaffolding complex (eIF4F), which binds the 40S ribosomal subunit to mRNA (Hayashi and Proud 2007). There were comparable patterns of $4EBP1^{Thr37/46}$ phosphorylation throughout the 60-min recovery period in the present study but a greater magnitude of effect after cycling (Figure 2.3B). The decrease in $4E\text{-}BP1^{Thr37/46}$ phosphorylation inhibiting translation immediately post-exercise is not surprising, given the results of Rose et al. (Rose et al. 2009b) showing depression of translation signalling during endurance exercise, indicative of the suppression of the energy consuming process of protein synthesis while other

cellular demands are met. The divergent phosphorylation status of 4E-BP1^{Thr70} compared with 4E-BP1$^{Thr37/46}$ during the 60-min post-exercise period was unexpected. To the best of my knowledge, this is the first study to investigate 4E-BP1^{Thr70} phosphorylation and to compare 4E-BP1 phosphorylation sites after exercise in humans. Undoubtedly, the regulation of 4E-BP1 activity is complex, and phosphorylation has been proposed to occur in a hierarchical manner with 4EBP1$^{Thr37/46}$ phosphorylation required for subsequent 4EBP1^{Thr70} modification (Wang et al. 2005). However, it is unclear how the changes in phosphorylation correspond with in vivo activity in humans after exercise. Moreover, whether the discordance between phosphorylation sites reflects differences in eIF4E binding in skeletal muscle and/or characterizes alternate mechanisms through which each exercise mode modulates translation requires further investigation.

Collectively, the data regarding phosphorylation of putative Akt-mTOR-regulated signalling proteins proximal to translation initiation or elongation emphasize the complexity of translational machinery for exercise-induced protein synthesis in skeletal muscle. How the adaptation response of these key signalling components is modified by various volumes and intensity of contractile activity remains equivocal. The early signalling responses may represent the cumulative effect of recovery from contraction-induced disruption to muscle homeostasis and translation initiation for early response genes. Regardless, these findings highlight differences in site-specific phosphorylation and divergence between exercise modes for 4E-BP1^{Thr70}. In addition, despite differences in the magnitude of effect, the exercise-induced increase in 4EBP1$^{Thr37/46}$ and S6K^{Thr389} phosphorylation is inhibited or delayed during the early recovery period (0 – 30 min) but is eventually enhanced after endurance and resistance exercise, respectively.

Phosphorylation of GSK-3 is reported to result in hypophosphorylation (activation) of glycogen synthase (GS) and/ or eIF2Bε (Jefferson, Fabian and Kimball 1999, McManus et al. 2005). In the present study, a lack of coordination in exercise-specific GSK-3β^{Ser9} phosphorylation when compared against phosphorylation of Akt was observed. This disparity may be due to GSK3 being part of the winglesstype MMTV integration site family (Wnt) signalling pathway. The Wnt pathway is exercise responsive and inhibits GSK3 activity (McManus et al. 2005) and Wnt signalling may have been differentially modulated by the divergent contractile stimuli. The changes in GS^{Ser641} phosphorylation largely corresponded with GSK- 3β^{Ser9} with sustained

dephosphorylation of GS^{Ser641} during the 60-min post-exercise period after cycling but not resistance exercise (Figure 2.4B). Because a critical regulatory of glycogen resynthesis increased GS activity (dephosphorylation), following a bout of endurance cycling would be expected given the prolonged, constant load contractile activity and greater glycogen depletion (Figure 2.1). Taken together, the changes in GSK-3 and GS phosphorylation provide a time course for the molecular signalling mechanisms regulating resynthesis of muscle glycogen during the early recovery period from endurance exercise (Mascher et al. 2007, Richter et al. 2003, Sakamoto et al. 2004). Conversely, glycogen resynthesis during initial recovery from heavy resistance exercise may represent a lesser priority.

Phosphorylation of GSK-3β^{Ser9} may also enhance translation by derepressing its inhibition of eIF2Bε (Jefferson et al. 1999, Welsh et al. 1997). The increase in eIF2Bε^{Ser539} phosphorylation (inactivation) immediately after cycling was analogous with changes in 4E-BP1$^{Thr37/46}$, indicating the suppression of global translational signalling during endurance exercise (Figure 2.4C). Similarly, eIF2Bε^{Ser539} phosphorylation rapidly diminished thereafter (15 min), characterizing the enhanced translation status during recovery from endurance exercise also seen with 4E-BP1$^{Thr37/46}$ (Figure 2.3C). To the best of my knowledge, this is the first study to investigate the eIF2Bε^{Ser539} response to an endurance exercise bout, although the results for resistance exercise are similar to that of Glover et al. (Glover et al. 2008a), showing decreased eIF2B phosphorylation after an acute bout of resistance exercise in humans.

As a metabolic regulator and energy-sensing protein, it is not surprising that AMPK phosphorylation was more pronounced after prolonged exercise duration (Figure 2.5A). The effect of resistance exercise on AMPK has received less scrutiny, but Dreyer et al. (Dreyer et al. 2006) and Koopman et al. (Koopman et al. 2006b) have shown increased AMPK activity in the early recovery period from resistance exercise in the fasted state. AMPK has been implicated in repressing anabolic processes in skeletal muscle via inhibition of mTOR-mediated signalling to initiate translation (Atherton et al. 2005, Dreyer et al. 2006). Intriguingly, the time-course data in the present study show that the increase in AMPK^{Thr172} occurred concomitantly with phosphorylation events for enhancing translation. Benziane et al. (Benziane et al. 2008) have also shown increased AMPK^{Thr172} coinciding with elevated mTOR^{Ser2448} after 60 min cycling. Likewise, Mascher et al. (Mascher et al. 2007) examined Akt-mTOR signalling after prolonged

cycling and also observed increased mTOR phosphorylation when AMPK activity would be expected to be elevated. As such, there is accumulating evidence that AMPK^{Thr172} phosphorylation may have limited capacity to subdue translational signalling, at least in the early post-exercise period in humans (Rose et al. 2009a).

Finally, divergent responses between exercise modes were evident for AS160 phosphorylation likely because of exacerbated glycogen depletion and subsequent stimulation of glucose transport machinery with endurance exercise (Howlett et al. 2008). AS160 phosphorylation may be regulated by Akt- and AMPK-mediated signalling pathways to release inhibition on the glucose transporter Glut4 (Kramer et al. 2006, Treebak et al. 2007). These current results are similar to those of Sriwijitkamol et al. (Sriwijitkamol et al. 2007) and Howlett et al. (Howlett et al. 2007) showing increased Akt and AMPK phosphorylation associated with elevated AS160 phosphorylation after prolonged (40 – 60 min) moderate-intensity cycling but not resistance exercise, respectively. Dreyer et al. (Dreyer et al. 2008a) have previously shown an increase in AS160 phosphorylation and glucose uptake 60 min after resistance exercise in humans. However, an important distinction between the study of Dreyer et al. (Dreyer et al. 2008a) and the present investigation was significantly greater volume of exercise. The current results show equivalent Akt responses yet divergence in the AMPK-AS160 time course with resistance exercise versus cycling indicate that regulation of AS160 phosphorylation may have been an AMPK- rather than an Akt-mediated response. Nonetheless, the relative role of AMPK and Akt in regulation of AS160 remains contentious, and further research is required to determine the contribution of each signalling pathway in modulating exercise-induced AS160 activation.

In conclusion, these findings offer insight of the IGF pathway and AMPK phosphorylation time course during the early recovery period after diverse exercise in skeletal muscle and extend the current knowledge regarding the exercise-specific signalling responses in humans (Wilkinson et al. 2008). Specifically, this work provides new information showing similar Akt-mTOR-S6K phosphorylation concomitant with divergent AMPK-AS160 and GS phosphorylation that was only enhanced after cycling. It is proposed that some of the uncertainty in establishing the contraction-induced signalling responses in human skeletal muscle has been due to differences in the timing of post-exercise muscle biopsies. Moreover, these results indicate that endurance and resistance exercise are equally capable of ‘‘switching on’’ translational signalling. Consequently,

it appears unlikely that alterations in translation significantly contribute to the specificity of training adaptation in skeletal muscle. Indeed, to better understand acute exercise-specific adaptive profiles and any incompatibility of adaptation machinery when comparing divergent exercise, elucidating changes at the transcriptional level may be more prudent. Regardless, given the limitations in the maximum number of muscle biopsies from any single individual when undertaking in vivo human research, these findings provide new information for determining the optimal post-exercise time point when investigating cell signalling after exercise in the fasted state.

Chapter Three

Low muscle glycogen concentration does not suppress the anabolic response to resistance exercise

Adapted from: Camera, D.M., West, D,W., Burd, N.A., Phillips, S.M., Garnham, A.P., Hawley, J.A., Coffey, V.G. (2012) *Journal of Applied Physiology*, 113:206-14

3.1 Introduction

Skeletal muscle glycogen concentration exerts numerous regulatory effects on cell metabolism in response to contraction (Hawley et al. 2011). Indeed, commencing endurance-based exercise with low muscle glycogen availability has been shown to increase the maximal activities of several oxidative enzymes in skeletal muscle that promote endurance adaptation (Hansen et al. 2005, Yeo et al. 2008). Although the anabolic effects of resistance-based exercise on skeletal muscle are well established (Coffey and Hawley 2007b), little is known regarding the effects of altered muscle glycogen concentration availability on the acute protein synthetic response to resistance exercise and whether the summation of these responses may enhance or attenuate training-induced adaptation.

The complex regulatory process of protein synthesis after muscle contraction and/or protein ingestion includes activation of the Akt-mTOR-S6K signalling pathway to initiate translation (Glass 2005, Philp et al. 2011). Numerous studies have addressed the signalling responses to resistance exercise under a variety of nutritional states (i.e., fasted/fed) (Coffey et al. 2009b, West et al. 2011). However, the effects of muscle glycogen availability have yet to be clearly elucidated. Work by Creer and colleagues (Creer et al. 2005) showed an attenuation in Akt phosphorylation during recovery when subjects commenced a bout of moderate-intensity resistance exercise with low (~175 mmol/kg·dry·wt) vs. high (~600 mmol/kg·dry·wt) muscle glycogen. Furthermore, contraction-induced translational signalling may be suppressed when energy-sensing AMPK activity is increased (Atherton et al. 2005, Thomson, Fick and Gordon 2008). Wojtaszeski and co-workers (Wojtaszewski et al. 2003) have observed elevated resting and exercise-induced AMPK activity when muscle glycogen levels were low (~160 mmol/kg·dw) compared with high (~910 mmol/kg·dw). Moreover, work from this laboratory also previously demonstrated low muscle glycogen concentration has the capacity to alter basal transcription levels of select metabolic and myogenic genes (Churchley et al. 2007). Thus the increased metabolic perturbation when exercising in a low glycogen state might be expected to inhibit the anabolic response to resistance exercise.

It is well accepted that protein intake following resistance exercise is critical for optimizing many of the training-induced adaptations in skeletal muscle (Hawley et al. 2006). Ingestion of high-quality protein has been shown to enhance translation initiation signalling and maximally

stimulate muscle protein synthesis rates after resistance exercise (Koopman et al. 2007b, Moore et al. 2009a). Carbohydrate (CHO) ingestion provides substrate for muscle glycogen resynthesis, but has no additive effect on rates of muscle protein synthesis after resistance exercise (Koopman et al. 2007a). The capacity for protein-carbohydrate co-ingestion in the early post-exercise period to rescue any putative attenuation of muscle protein synthesis when resistance exercise is performed with low glycogen availability has not been investigated. Accordingly, the primary aims of this study were to determine the effect of 1) decreased muscle glycogen concentration on the acute anabolic response after resistance exercise performed in the fasted state; and 2) the effect of protein/CHO supplementation on muscle cell signalling and myofibrillar protein synthesis rates following exercise commenced with low muscle glycogen. It was hypothesized that low muscle glycogen concentration would suppress the muscle anabolic response to resistance exercise but that nutrient provision in the early recovery period after exercise would restore muscle anabolism to a state that may promote hypertrophy.

3.2 Methods

Subjects

Sixteen healthy physically fit male subjects who had been participating in regular concurrent resistance and endurance training (~3×/ wk; > 1 yr) volunteered for this study. Subjects were randomly assigned to either a nutrient [n = 8, age 22.9 ± 2.6 yr, body mass 80.6 ± 8.8 kg, peak oxygen uptake (VO_{2peak}) 49.8 ± 5.4 $ml \cdot kg^{-1} \cdot min^{-1}$, unilateral leg press one repetition maximum (1RM) ~141.7 ± 4.6 kg] or placebo group [age 22.5 ± 4.4 yr, body mass 78.2 ± 4.7 kg VO_{2peak} 47.2 ± 6.9 $ml \cdot kg^{-1} \cdot min^{-1}$, unilateral leg press 1RM ±141.8 ± 0.8 kg; values are mean ± SD]. The experimental procedures and possible risks associated with the study were explained to each subject, who all gave written informed consent before participation. The study was approved by the Human Research Ethics Committee of RMIT University.

Preliminary Testing

VO_{2peak}. Peak oxygen uptake was determined during an incremental test to volitional fatigue on a Lode cycle ergometer (Groningen, The Netherlands). The protocol has been described in detail previously (Hawley and Noakes 1992). In brief, subjects commenced cycling at a workload

equivalent to 2 W/kg for 150 s. Thereafter, the workload was increased by 25 W every 150 s until volitional fatigue (defined as the inability to maintain a cadence >70 revolutions/min). Throughout the test, which typically lasted 12–14 min, subjects breathed through a mouthpiece attached to a metabolic cart (Parvomedics, Sandy, UT) to determine oxygen consumption.

Maximal strength. One repetition of maximal dynamic strength (1RM) for each leg was determined on a plate loaded 45° leg press machine (CalGym, Caloundra, Australia). Subjects completed the test with feet placed at the bottom edge of the foot plate and range of motion was 90° knee flexion/extension.

Familiarisation to exercise training sessions. To familiarise subjects to one-legged cycling (described subsequently), each subject completed three familiarisation sessions before the experimental trial. These sessions consisted of 2 × 10 min bouts of one-legged cycling, with a 2 min recovery period between repetitions. The power output was gradually increased so that by the final session subjects were performing one-legged cycling at ~ 75% of their two-legged $V_{O_{2peak}}$ (Pernow and Saltin 1971).

Diet/exercise control. Before the exercise depletion session (described subsequently), subjects were instructed to refrain from exercise training and vigorous physical activity and alcohol and caffeine consumption for a minimum of 48 h. A CHO-based diet (~ 9 g/kg body mass) was consumed 36 h before the one-legged exercise depletion session. All food and drinks were supplied to subjects pre-packaged with a food checklist to record their daily intake.

One-legged glycogen depletion protocol. Subjects began a one-legged cycling depletion session at a power output that elicited ~ 75% of two-legged $V_{O_{2peak}}$. The duration of each work bout was 10 min, with 2 min rest between work bouts. Subjects maintained this work-to-rest ratio until volitional fatigue. At this time, power output was decreased by 10 W and subjects cycled at this (lower) work rate with the same work-to-rest ratio until fatigue. After a 10-min rest, subjects then completed 90-s one-leg maximal sprints on a Repco RE7100 Ergo (Altona North, Australia), with 60 s of recovery between work bouts. This protocol was continued until volitional fatigue, defined as the inability to maintain 70 rpm. To further lower whole body glycogen stores and minimize glycogen resynthesis in the LOW leg, subjects completed 30 min of arm cranking on a

Monark Rehab Trainer 881E (Vansbro, Sweden). Following the exercise depletion session, subjects were fed a low CHO (~1 g/kg body mass) evening meal.

Experimental Testing Session

On the morning of an experimental trial, subjects reported to the laboratory after a ~10-h overnight fast. After resting in the supine position for ~ 15 min, catheters were inserted into the antecubital vein of each arm and a baseline blood sample (~ 3 ml) was taken (Figure 3.1). A primed constant intravenous infusion (prime: 2 µmol/kg; infusion: 0.05 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) of L-[*ring*-$^{13}C_6$] phenylalanine (Cambridge Isotopes Laboratories) was then administered. Under local anaesthesia (2 – 3 ml of 1% Xylocaine) a resting biopsy from the *vastus lateralis* of both legs was obtained 1.5 h after commencement of the tracer infusion using a 5-mm Bergstrom needle modified with suction. At this time, two separate sites on each leg (~ 5 cm distal from each other) were prepared for subsequent biopsies. Subjects then completed a standardized unilateral warm-up (1 × 5 repetitions at 50% and 60% 1RM) on a leg-press machine before the resistance exercise testing protocol was commenced. Resistance exercise consisted of eight sets of five repetitions at ~ 80% of 1RM for each leg. The glycogen depleted leg (LOW) began the protocol, with ~ 60 s rest before the rested normal leg (NORM) completed the same set. Each set was separated by a 3-min recovery period during which the subject remained seated on the machine. The training volume and intensity and recovery interval were selected to provide sufficient anabolic/ hypertrophy stimulus and minimize metabolic perturbation and has been used previously (Coffey et al. 2009b). If the LOW leg could not complete the repetitions, the NORM leg replicated the number of repetitions to ensure the exercise was work matched and the weight was decreased 5% for subsequent sets. Immediately after the cessation of exercise and 2 h post-exercise, subjects ingested a 500 ml placebo (water, artificial sweetener) or protein-CHO beverage (20 g whey protein, 40 g maltodextrin). The nutrient beverage was enriched with a small amount of tracer (to 6.5% of L-[*ring*-$^{13}C_6$] phenylalanine) according to the measured phenylalanine content of the beverage. Subjects rested throughout a 240-min recovery period, and additional muscle biopsies were taken 60 and 240 min post-exercise and the samples were stored at -80°C until analysis. Blood samples were collected in EDTA tubes at regular intervals during the post-exercise recovery period.

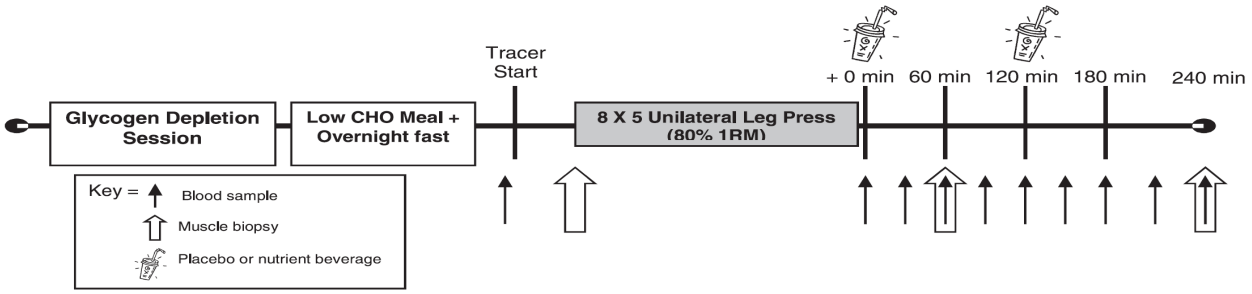

Figure 3.1 Schematic representation of the experimental trial. Subjects reported to the laboratory the evening before an experimental trial and performed a 1-legged glycogen-depletion protocol to fatigue before consuming a low carbohydrate (CHO) meal. After an overnight fast, a constant infusion of L-[*ring*-$^{13}C_6$] phenylalanine was commenced, and subjects completed 8 sets of 5 unilateral leg press repetitions at 80% one repetition maximum (1RM). Immediately after resistance exercise (REX) and 2 h later, subjects consumed a 500-ml bolus of a protein/CHO beverage (20 g whey + 40 g maltodextrin) or placebo. Muscle biopsies from both legs (*vastus lateralis*) were taken at rest and at 1 and 4 h after REX.

Analytical Procedures

Blood glucose and plasma insulin concentration. Whole blood samples were immediately analysed for glucose concentration using an automated glucose analyser (YSI 2300, Yellow Springs, OH). Blood samples were then centrifuged at 1,000 g at 4°C for 15 min, with aliquots of plasma frozen in liquid N_2 and stored at -80°C. Plasma insulin concentration was measured using a radioimmunoassay kit according to the manufacturer's protocol (Linco Research).

Plasma amino acids and enrichment. Plasma amino acid concentrations were determined by HPLC from a modified protocol (Moore et al. 2005). Briefly, 100 µl of plasma was mixed with 500 µl of ice cold 0.6 M PCA and centrifuged at 15,000 rpm for 2 min at 4°C. The PCA was neutralised with 250 µl of 1.25 M potassium bicarbonate ($KHCO_3$), and the reaction was allowed to proceed on ice for 10 min. Samples were then centrifuged at 15,000 rpm for 2 min at 4°C, and the supernatant was separated from the salt pellet and subsequently derivatised for HPLC analysis. Plasma [*ring*-$^{13}C_6$] phenylalanine enrichments were determined as previously described (Glover et al. 2008b).

Muscle glycogen. A small piece of frozen muscle (~ 20 mg) was freeze-dried and powdered to determine muscle glycogen concentration (Lowry and Passonneau 1971). Freeze-dried muscle was extracted with 500 µl of 2 M hydrochloric acid (HCl), incubated at 100°C for 2 h, and then neutralised with 1.5 ml of 0.67 M sodium hydroxide for subsequent determination of glycogen concentration via enzymatic analysis with fluorometric detection (Jasco FP-750 spectrofluorometer, Easton, MD) at excitation 365 nm/emission 455 nm.

Western blots. Muscle samples were homogenized in buffer containing 50 mM Tris·HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM DTT, 10 µg/ml trypsin inhibitor, 2 µg/ml aprotinin, 1 mM benzamidine, and 1 mM PMSF. Samples were spun at 18,000 g for 30 min at 4°C, and the supernatant was collected for Western blot analysis while the pellet was processed to extract the myofibrillar enriched proteins (described below). After determination of protein concentration using a BCA protein assay (Pierce, Rockford, IL), lysate was resuspended in Laemmli sample buffer, separated by SDS-PAGE, and transferred to polyvinylidine fluoride membranes blocked with 5% non-fat milk, washed with 10 mM Tris·HCl, 100 mM NaCl, and 0.02% Tween 20, and incubated with primary antibody (1:1,000) overnight at 4°C on a shaker. Membranes were incubated with secondary antibody (1:2,000), and proteins were detected via enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK; Pierce Biotechnology) and quantified by densitometry (Chemidoc, BioRad, Gladesville, Australia). All sample (50 µg) time points for each subject were run on the same gel. Polyclonal antiphospho- Akt^{Ser473} (no.9271), mTOR^{Ser244}8 (no. 2971), glycogen synthase (GS)^{Ser641} (no.3891), monoclonal anti-phospho-S6 ribosomal protein$^{Ser235/6}$ (no.4856), AMPKThr172 (no. 2535), and AS160 (no.2670) were from Cell Signalling Technology (Danvers, MA). Polyclonal anti-phospho-p70S6K^{Thr389} (no. 04–392) was from Millipore (Temecula, CA). When commercially available, positive controls (Cell Signalling Technology) were included confirming the band of interest. Data are expressed relative to α-tubublin (no. 3873, Cell Signalling Technology) in arbitrary units.

RNA Extraction and Quantification

Skeletal muscle tissue RNA extraction was performed using a TRIzol-based kit according to the manufacturer's directions (Invitrogen, Melbourne, Australia, Cat. No. 12183–018A). Briefly, ~ 15 mg of skeletal muscle tissue was removed from RNAlater-ICE solution and homogenized in TRIzol. After elution through a spin cartridge, extracted RNA was quantified using a QUANT-iT analyser kit (Invitrogen, Cat. No. Q32852) according to the manufacturer's directions.

Reverse Transcription and Real-Time PCR

First-strand complementary DNA (cDNA) synthesis was performed using commercially available TaqMan Reverse Transcription Reagents (Invitrogen) in a final reaction volume of 20 µl. All RNA samples and control samples were reverse transcribed to cDNA in a single run from the same reverse transcription master mix. Serial dilutions of a template RNA (AMBION; Cat. No. AM7982) were included to ensure efficiency of reverse transcription and for calculation of a standard curve for real-time quantitative polymerase chain reaction (RT-PCR). Quantification of mRNA (in duplicate) was performed on a BioRad iCycler (BioRad). Taqman-FAM-labelled primer/ probes for atrogin (Cat. No. Hs01041408) and myostatin (Cat. No. Hs00976237) were used in a final reaction volume of 20µl. PCR conditions were 2 min at 50 °C for UNG activation, 10 min at 95°C, then 40 cycles of 95°C for 15 s and 60°C for 60 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cat. No. Hs Hs99999905) was used as a housekeeping gene to normalize threshold cycle (CT) values. The relative amounts of mRNAs were calculated using the relative quantification (CT) method (Livak and Schmittgen 2001).

Myofibrillar Protein Synthesis

Myofibrillar enriched proteins were isolated according to a modified protocol (35). Briefly, the myofibrillar pellet was solubilised in 0.3 M NaOH, precipitated in 1 M PCA, washed in ethanol, and hydrolysed overnight with 6 M HCl while being heated to 120°C. Liberated myofibrillar and plasma amino acids (for determination of L-[*ring*-$^{13}C_6$] phenylalanine enrichment) were purified using cation-exchange chromatography (Dowex 50WX8–200 resin; Sigma-Aldrich) and

converted to their N-acetyl-n-propyl ester derivatives for analysis by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS: Hewlett Packard 6890; IRMS model Delta Plus XP, Thermo Finnagan, Waltham, MA). Intracellular free amino acids (IC) were extracted from a separate piece of wet muscle (~ 20 mg) with ice-cold 0.6 M PCA. Muscle was homogenized, and the free amino acids in the supernatant were purified by cation-exchange chromatography and converted to their heptafluorobutyric (HFB) derivatives before analysis by GC-MS (models 6890 GC and 5973 MS; Hewlett-Packard, Palo Alto, CA) as previously described (Moore et al. 2009a).

Calculations

The rate of myofibrillar protein synthesis was calculated using the standard precursor-product method: FSR (%/h) = [(E2b - E1b)/ (EIC × t)] × 100, where E2b - E1b represents the change bound protein enrichment between two biopsy samples, EIC is the average enrichment of intracellular phenylalanine between the two biopsy samples, and t is the time between two sequential biopsies.

Statistical Analysis

All data were analysed by two-way ANOVA (two factor: time × glycogen concentration) with Student-Newman-Keuls post hoc analysis. Statistical significance was established when $P < 0.05$ (SigmaStat for windows Version 3.11). Based on the *a priori* hypothesis that anabolic responses to nutrient administration are significantly elevated compared with placebo as shown previously (Burd et al. 2009, Fujita et al. 2007), no direct comparisons between nutrient and placebo interventions were made. Data for Western blotting and mRNA abundance were log-transformed prior to analysis. Log-transformed delta values between data time points were also directly compared and converted to Cohen effect sizes (ES). The default confidence interval was 90% to calculate ES making the same assumptions about sampling distributions that statistical packages use to derive P values (Hopkins et al. 2009). The magnitude of the ES was interpreted by using conventional threshold values of 0.2 as the smallest effect, 0.5 as a moderate effect, and 0.8 as a large ES (Hopkins et al. 2009). All data are expressed as arbitrary unit + SD.

3.3 Results

One-Legged Depletion Session and Muscle Glycogen

The time spent completing the one-legged depletion session at an intensity of ~ 75% of two-legged VO_{2peak} was 100 ± 3 min. Subjects also completed an average of 6 ± 2 one-legged maximal effort sprint repetitions. As intended, the combination of the exercise depletion protocol and dietary manipulation generated divergent muscle glycogen levels that were higher in NORM than LOW at rest for the placebo (382 vs. 176 mmol/kg·dw; $P < 0.001$) and nutrient groups (383 vs. 184 mmol/kg·dw; $P < 0.05$; Figure 3.2). Glycogen concentration was decreased from rest in the NORM leg in both groups at 1 and 4 h post-exercise ($P < 0.05$). However, no significant change from rest was evident in the LOW leg for either group. Muscle glycogen increased between 1 and 4 h post-exercise in the LOW leg in the nutrient group (~ 84 mmol/kg·dw; $P < 0.01$).

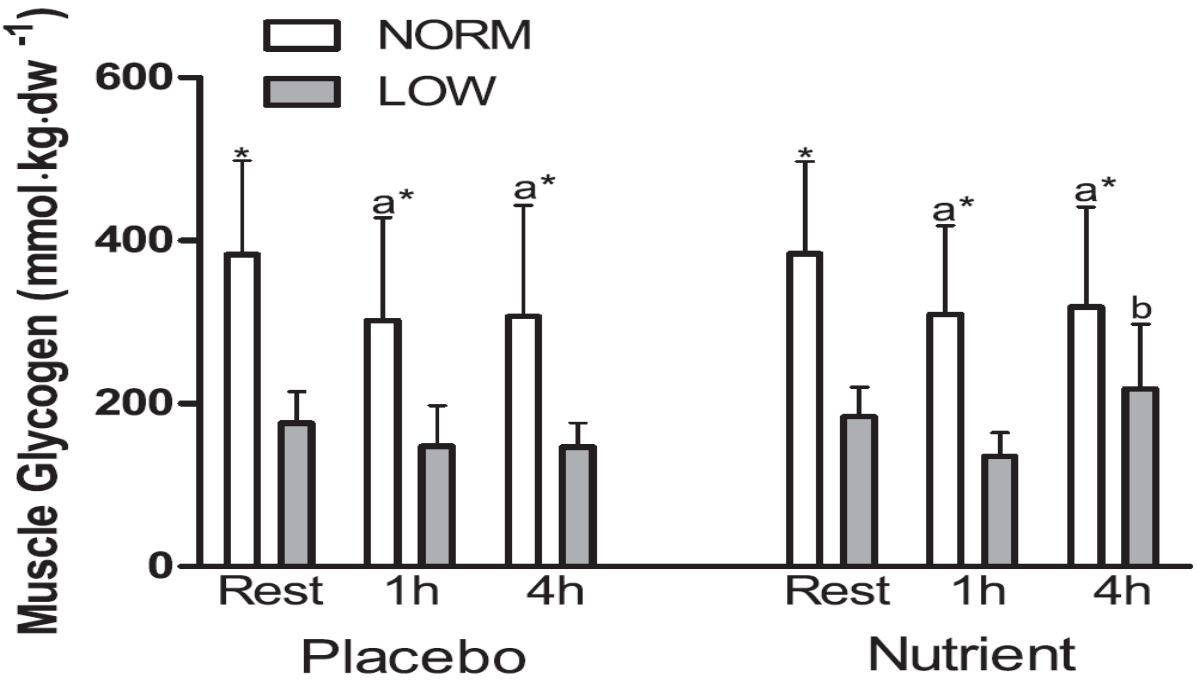

Figure 3.2 Muscle glycogen concentration at rest and during 4 h recovery after resistance exercise (8 × 5 leg unilateral leg press at ~ 80% 1RM) and ingestion of either 500 ml placebo or nutrient beverage immediately post and 2 h post-exercise in NORM and LOW glycogen legs. Values are mean ± SD. dw, dry weight. Significantly different ($P < 0.05$) vs. (a) rest, (b) 1 h and (*) between treatments (NORM vs. LOW) at equivalent time point.

Plasma Insulin, Glucose, and Essential Amino Acids

There were significant effects for plasma insulin and glucose concentration in the nutrient but not the placebo group ($P < 0.001$; Figure 3.3A and B). Peak blood insulin and glucose concentrations occurred at 30 and 150 min post-exercise ($P < 0.001$). Plasma essential amino acids (EAA) were elevated about rest 150 min and 180 min ($P < 0.05$) post-exercise in the nutrient group only (Figure. 3.3C).

Plasma Tracer Enrichments

Plasma L-[*ring* $^{13}C_6$] phenylalanine enrichment at rest and 60, 120, 180, and 240 min post-exercise for nutrient and placebo treatments were 0.042, 0.045, 0.055, and 0.049, and 0.058, 0.054, 0.062, 0.057, 0.065, and 0.054 tracer-to-tracee ratio: t/T, respectively. Linear regression analysis indicated that the slopes of the plasma enrichments were not significantly different from zero, demonstrating that isotopic plateau was achieved.

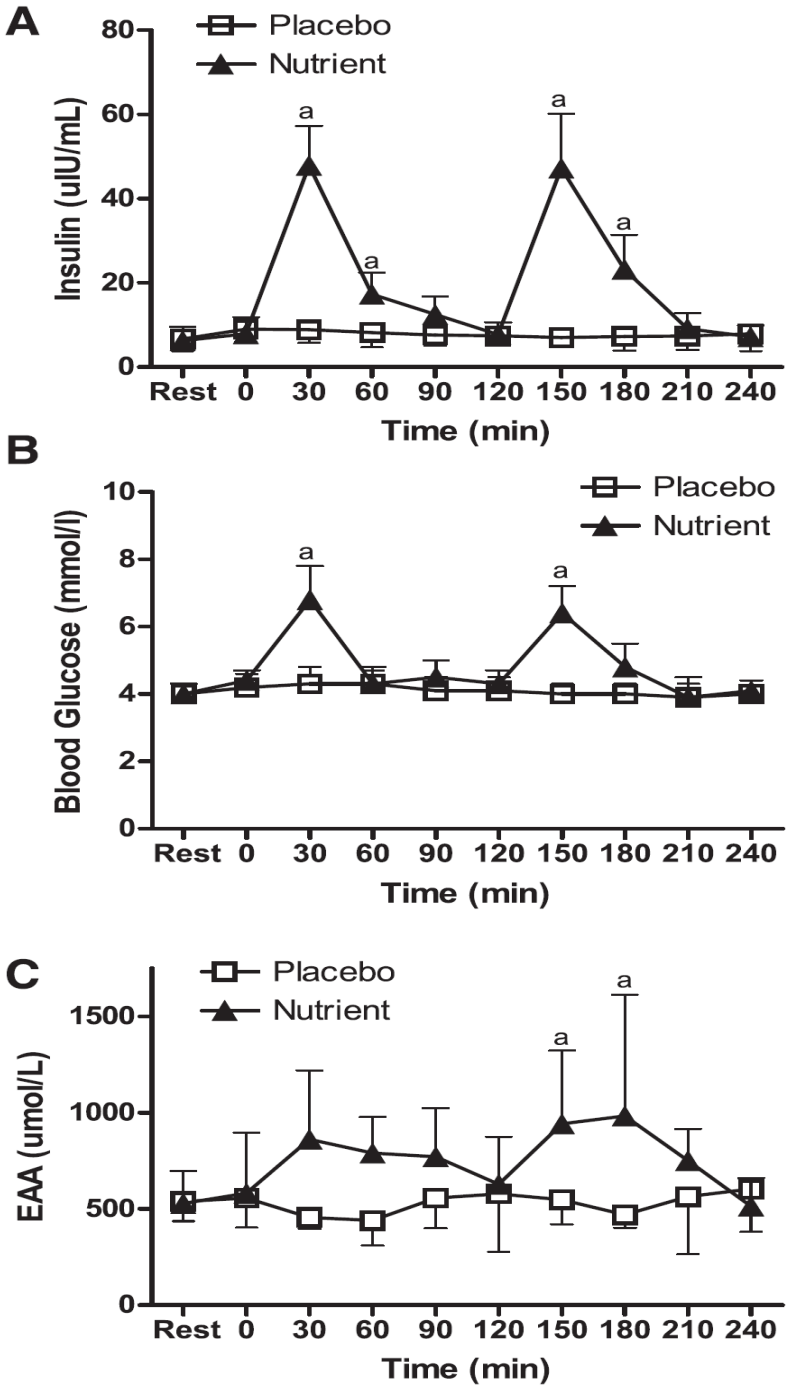

Figure 3.3 Plasma insulin (A), blood glucose (B), and plasma essential amino acid concentration (C) at rest and during 240 min recovery following resistance exercise (8 × 5 leg unilateral leg press at ~ 80% 1RM) and ingestion of either 500 ml placebo or nutrient beverage immediately post and 2 h post-exercise. Values are mean ± SD. Significantly different ($P < 0.05$) vs. (a) rest.

Akt-mTOR-p70S6K-rpS6. There were significant effects for Akt^{Ser473} phosphorylation for time and glycogen status ($P < 0.05$, Figure 3.4A). Resting Akt phosphorylation was higher in LOW than NORM in the placebo group and increased ~ 2-fold 1 h post-exercise in NORM only ($P < 0.05$, ES 0.75) before returning to baseline at 4 h. Phosphorylation at rest was also higher in the LOW compared with the NORM leg ($P = 0.058$) and increased ~ 10-fold in LOW and ~ 21-fold in NORM 1 h after resistance exercise in the nutrient group ($P < 0.001$, ES > 1). Akt phosphorylation remained above resting levels following 4 h recovery in the NORM leg only of the nutrient group ($P < 0.05$, ES > 1).

There were significant effects for time and glycogen concentration for mTOR^{Ser2448} phosphorylation ($P < 0.05$, Figure 3.4B). mTOR phosphorylation increased ~ 1-fold above rest at 1 and 4 h post-exercise in the NORM but not the LOW leg in the placebo group ($P < 0.05$, ES ~ 0.5). There was also disparity between legs in the nutrient group that increased in the NORM compared with LOW leg at 1 and 4 h recovery ($P < 0.05$, ES > 1). Phosphorylation in the nutrient group did increase above resting levels ~ 4-fold and ~ 1-fold in the LOW leg ($P < 0.01$), an effect that was more pronounced in the NORM leg (~ 11-fold and ~ 4-fold, respectively; $P < 0.01$).

p70S6K^{Thr389} phosphorylation was higher at rest in the LOW leg compared with NORM in placebo ($P < 0.05$, Figure 3.4C). The post- exercise phosphorylation response increased above rest at 1 and 4 h (~ 5-fold) in NORM but not the LOW leg ($P < 0.01$; ES ~ 1). The comparison of p70S6K phosphorylation between legs at rest in the nutrient group approached significance and was increased above resting levels in both legs at 1 h (LOW: ~ 45 fold, NORM: ~ 82 fold, ES > 1; $P < 0.001$) and 4 h (LOW: ~ 14 fold, NORM: ~ 16 fold; $P < 0.001$) during recovery from resistance exercise.

There were significant effects for rpS6$^{Ser235/6}$ phosphorylation in the nutrient but not placebo group ($P < 0.05$, Figure 3.4D). There were ~ 4- and ~ 6-fold increases in rpS6$^{Ser235/6}$ phosphorylation in the LOW leg with placebo at 1 and 4 h, respectively ($P < 0.05$), and this effect was mirrored in NORM with ~ 15-fold increases at 1 h ($P < 0.001$, ES 0.9) and 4 h ($P < 0.01$, ES 0.5). Resting rpS6$^{Ser235/6}$ phosphorylation was significantly elevated in the LOW compared with

NORM leg ($P < 0.05$) in the nutrient group and increased in both legs 1 h after resistance exercise and remained elevated following 4 h recovery ($P < 0.001$, Figure 3.4D).

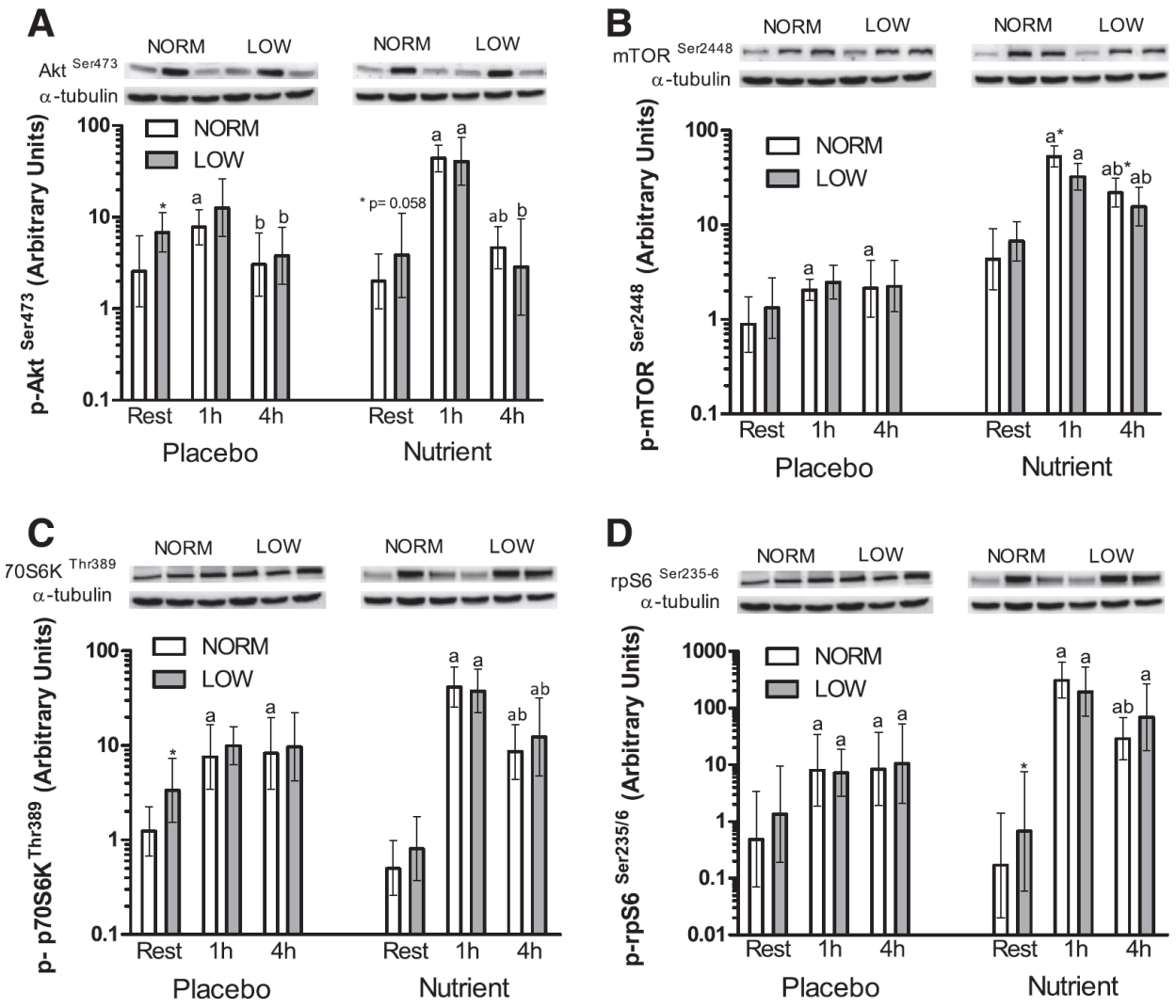

Figure 3.4 Akt^{Ser473} (A), mammalian target of rapamycin (mTOR)^{Ser2448} (B), p70S6K^{Thr389} (C), and ribosomal protein S6 (rpS6)$^{Ser235/6}$ (D) phosphorylation in skeletal muscle at rest and during 4 h post-exercise recovery following resistance exercise (8 × 5 leg unilateral leg press at ~ 80% one 1RM). Images are representative blots and values are expressed relative to α -tubulin and presented in arbitrary units (mean ± SD, n = 8). Significantly different ($P < 0.05$) vs. (a) rest, (b) 1 h, and (*) between treatments (NORM vs. LOW) at equivalent time point.

GS-AS160-AMPK. There were significant effects for GS^{Ser641} phosphorylation for time and glycogen status in placebo and nutrient groups (Figure 3.5A). GS phosphorylation was markedly higher in NORM than LOW at all time points in the placebo condition ($P < 0.001$). Following resistance exercise phosphorylation decreased ~3- to 4-fold 1 h post-exercise in both legs ($P < 0.01$) before increasing at 4 h in the LOW but not NORM leg ($P < 0.01$, ES 0.4). Similarly, phosphorylation was higher at all points in NORM compared with LOW in the nutrient group ($P < 0.05$). There was a decrease from resting levels in the LOW and NORM legs at 1 and 4 h post-exercise ($P < 0.05$) but GS phosphorylation only increased between 1 and 4 h in the LOW leg (~ 5-fold, $P < 0.01$, ES 0.6).

There were significant effects for time in placebo and nutrient groups for phospho-AS160 ($P < 0.05$, Figure 3.5B). AS160 increased in the placebo condition at 1 h (~ 2-fold $P < 0.01$, ES 0.9) and 4 h (~ 1-fold $P < 0.05$, ES 0.9) after resistance exercise in the LOW leg only. AS160 phosphorylation in the nutrient group was increased at 1 h recovery in the LOW leg (~ 4-fold, $P < 0.001$) and both 1 and 4 h post-exercise in the NORM leg (~ 8-fold, $P < 0.001$, ES 0.8; ~ 1 fold, $P < 0.05$, ES 0.5, respectively).

$AMPK^{Thr172}$ phosphorylation was not different at any time in placebo or nutrient groups (Figure 3.5C).

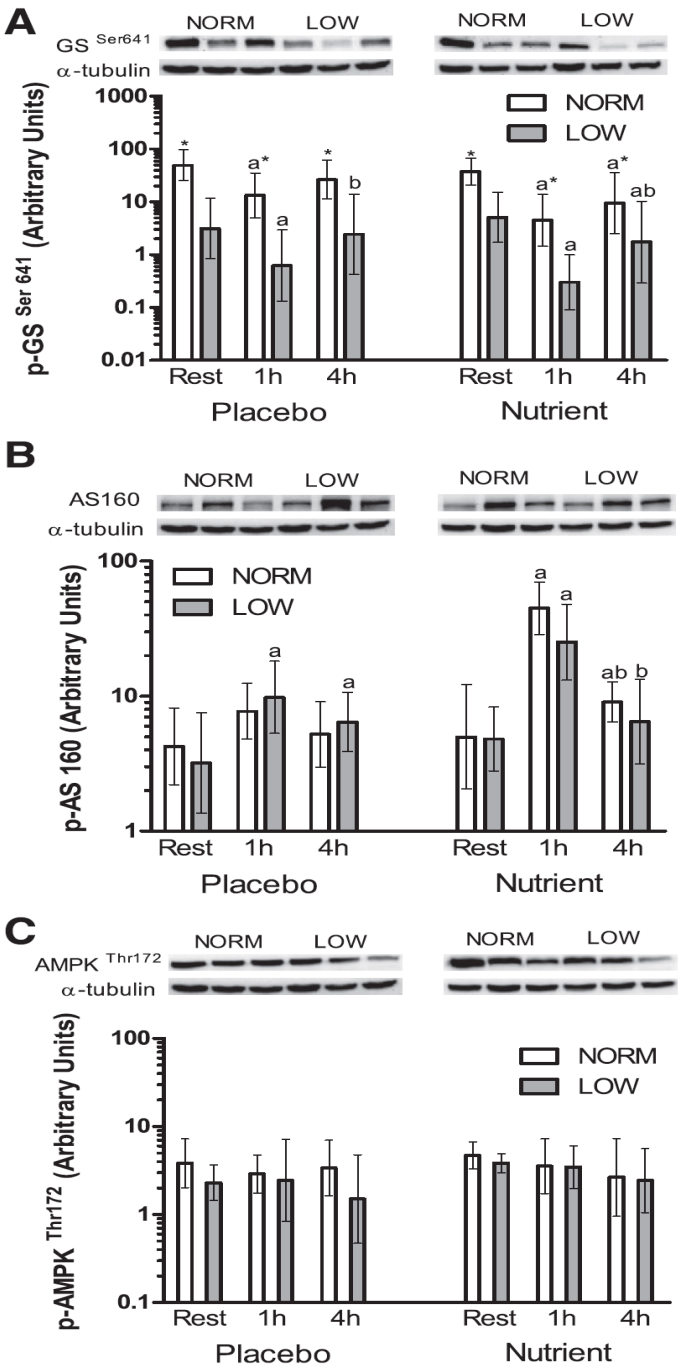

Figure 3.5 Glycogen synthase (GS)^{Ser641} (A), Akt substrate 160 kDa (AS160) (B), and 5'-adenosine monophosphate-activated protein kinase (AMPK)^{Thr172} (C) phosphorylation in skeletal muscle at rest and during 4 h post-exercise recovery following resistance exercise (8 × 5 leg unilateral leg press at ~ 80% 1RM). Images are representative blots and values are expressed relative to α–tubulin and presented in arbitrary units (mean ± SD, n = 8). Significantly different ($P < 0.05$) vs. (a) rest, (b) 1 h and (*) between treatments (NORM vs. LOW) at equivalent time point.

Myofibrillar Protein Synthesis

There were no differences in the rates of myofibrillar protein synthesis rates during the 1- to 4-h recovery period between LOW and NORM in placebo (0.049 ± 0.017 vs. 0.045 ± 0.021 %·h^{-1}) or nutrient (LOW vs. NORM: 0.068 ± 0.018 vs. 0.070 ± 0.022 %·h^{-1}) conditions (Figure 3.6).

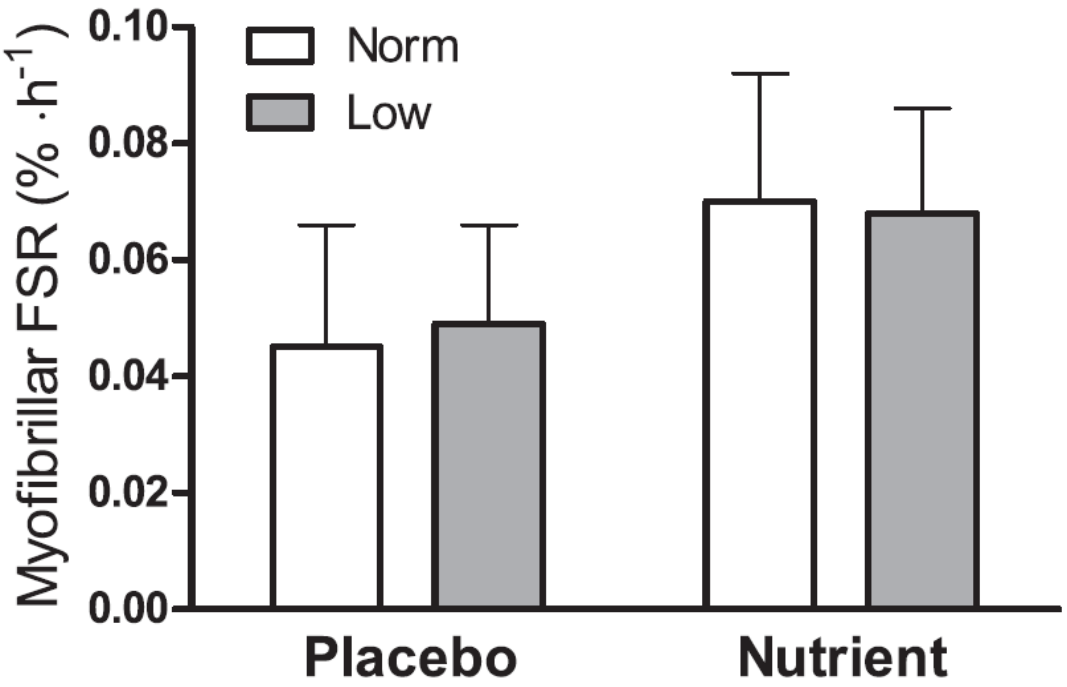

Figure 3.6 Myofibrillar protein fractional synthetic rates (FSR) during 4 h of recovery after resistance exercise (8 × 5 leg unilateral leg press at ~ 80% 1RM) and ingestion of either 500 ml placebo or nutrient beverage immediately post and 2 h post-exercise in NORM and LOW glycogen legs. Values are means ± SD.

mRNA expression

Atrogin-myostatin. Atrogin mRNA abundance decreased in the placebo group between rest and 4 h post-exercise in the LOW (~ 1.2-fold; $P < 0.05$) and NORM leg (~ 1.8-fold; $P < 0.01$) and was also different between 1 and 4 h in NORM (~1.4-fold; ES 0.5, $P < 0.01$) (Figure 3.7A). Likewise, atrogin-1 mRNA decreased from rest following 4 h recovery in the LOW (~ 0.5-fold; $P < 0.001$) and NORM leg (~ 2.6-fold, ES 0.6; $P < 0.001$) in the nutrient group. Atrogin was also different between 1 and 4 h post-exercise in the LOW (~ 0.5-fold, $P < 0.001$) and NORM leg (~ 2.2-fold, ES 0.25; $P < 0.001$). The mRNA abundance of atrogin was higher in the LOW leg compared with NORM leg at the 4 h post-exercise time point in the nutrient condition ($P < 0.01$). Myostatin mRNA decreased in the placebo group from rest to 4 h in LOW(~ 1.8-fold; $P < 0.01$) and NORM (~ 1.4-fold; $P < 0.001$) and between 1 and 4 h recovery in NORM only (~ 0.8 fold; ES 0.33, $P < 0.01$) (Figure 3.7B). In the nutrient condition, decreases in myostatin mRNA expression were only observed in the NORM leg that was reduced ~ 2.4-fold between rest and 1 h (ES ~ 1, $P < 0.01$) and ~ 1-fold 1 – 4 h during recovery from resistance exercise (ES 0.8, $P < 0.05$). Myostatin mRNA was higher in the LOW leg compared with NORM leg at 4 h post-exercise in the nutrient group ($P < 0.01$).

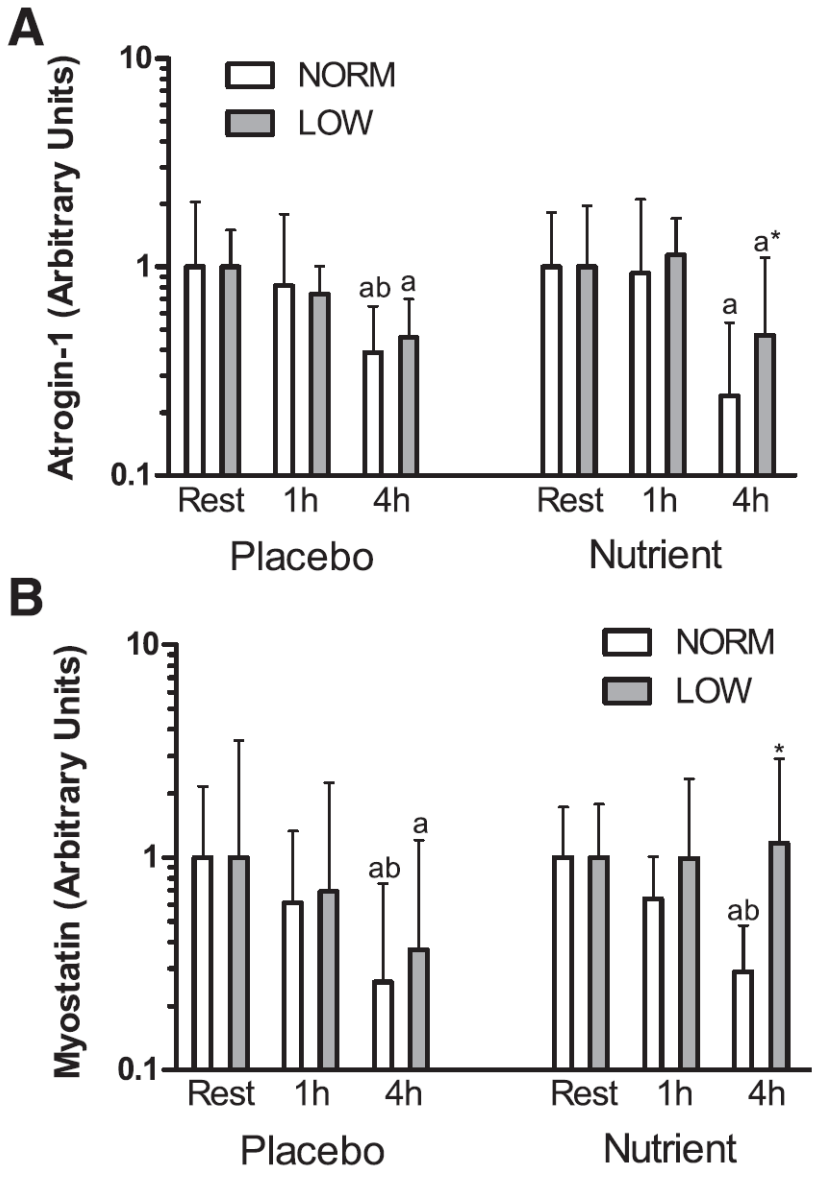

Figure 3.7 Atrogin (A) and myostatin (B) mRNA abundance at rest and during 4 h post-exercise recovery following resistance exercise (8 × 5 leg unilateral leg press at ~ 80% 1RM). Values are expressed relative to GAPDH and presented in arbitrary units (mean ± SD, n = 8). Significantly different ($P < 0.05$) vs. (a) rest, (b) 1 h and (*) between treatments (NORM vs. LOW) at equivalent time point.

3.4 Discussion

It is generally accepted that skeletal muscle adaptation to repeated bouts of contractile activity are specific to the mode, intensity, and duration of the exercise stimulus (Coffey and Hawley 2007b), but it is unclear how changes in skeletal muscle glycogen availability may modulate nutrient-training interactions to promote or inhibit the adaptive response to resistance exercise. Here it is reported for the first time that commencing a bout of strenuous resistance exercise with low muscle glycogen concentration has negligible effects on anabolic cell signalling and rates of muscle protein synthesis during the early (4 h) post-exercise recovery period. As expected, ingestion of a protein/CHO beverage enhanced the anabolic response to resistance exercise but failed to augment differences between the normal and low glycogen legs.

Exercising in a low glycogen state presents a unique metabolic challenge to skeletal muscle with few studies having investigated the interaction of glycogen content and nutrient provision or their effect on the adaptation response to resistance exercise. Hence the primary novel finding of the present study was that rates of myofibrillar protein synthesis between the NORM and LOW glycogen legs during 1 – 4 h recovery after resistance exercise were not different (Fig. 3.6). This finding was unexpected given that acute energy deficit has previously been reported to attenuate rates of mixed muscle protein synthesis by ~ 19% (Pasiakos et al. 2010), although the metabolic perturbation with low glycogen in the current study may have had less impact on cell energy status and thus failed to modulate the myofibrillar protein synthetic response to low volume high-intensity resistance exercise. Nonetheless, the one leg depletion protocol in combination with a low carbohydrate meal used in this work was successful in creating divergence in resting muscle glycogen concentration. Muscle glycogen content can be reduced by ~ 25 % following a single bout of resistance exercise (Robergs et al. 1991) compared with reductions of ~ 50% or greater after high intensity endurance exercise (Wojtaszewski et al. 2003). The distinct metabolic demands with endurance exercise may make the adaptation response in mitochondrial and CHO/fat metabolism more sensitive when training with low glycogen, although any benefit to endurance performance has yet to be established (Hulston et al. 2010, Yeo et al. 2010, Yeo et al. 2008). In the present study, glycogen availability in the LOW leg may have been sufficient to complete the short periods of contractile activity with long (3 min) recovery between sets without compromising myofibrillar protein synthesis rates during recovery. Moreover, it is possible that greater difference in glycogen availability is necessary to generate differences in metabolic

processes that might alter muscle protein synthesis. However, even an endurance exercise bout commenced with low glycogen has only modest effects on muscle protein metabolism (Blomstrand and Saltin 2001, Howarth et al. 2010).

The ingestion of carbohydrate post-exercise does not increase muscle protein synthesis in humans per se but it was hypothesized carbohydrate co-ingested with protein may have promoted the anabolic response when muscle glycogen was compromised. In the present study, the nutrient ingestion protocol resulted in divergent plasma glucose, insulin, and amino acid profiles during the 4-h recovery period (Fig. 3.3). However, no effect of carbohydrate co-ingestion on anabolic signalling and rates of myofibrillar protein synthesis were observed despite moderate muscle glycogen repletion during the early phase of recovery. Although insulin has been suggested as a potential anabolic hormone that contributes to skeletal muscle accretion (Biolo, Declan Fleming and Wolfe 1995a), recent evidence shows insulin to play only a permissive role in muscle anabolism, at least in young men (Koopman et al. 2007a). Despite the availability of carbohydrate for restoring muscle glycogen and the associated increase in plasma insulin levels during recovery in the low glycogen leg, there was no difference in myofibrillar protein synthesis compared with the normal leg. Nonetheless, these results provide further evidence of the well-established capacity for amino acids to augment the muscle protein synthesis response after resistance exercise following an overnight fast.

Another novel finding of this study was that divergent glycogen concentrations following the depletion protocol were associated with differences in pre-exercise phosphorylation status of key muscle cell signalling proteins that were generally ameliorated after the resistance exercise bout. Acute changes in translation initiation and glucose metabolism are stimulated by nutrient and contractile overload and mediated, at least in part, through the activation of the Akt-mTOR-S6K kinases (Glass 2005, Philp et al. 2011). Elevated resting Akt^{Ser473} phosphorylation was observed in the LOW glycogen leg (Fig. 3.4A) but this disparity did not extend to the post-exercise recovery period with similar responses between legs. In contrast, Creer and colleagues (Creer et al. 2005) reported similar Akt phosphorylation at rest and an attenuated post-exercise response with low muscle glycogen. The discrepancies between studies may reflect differences in protocols employed for generating divergent glycogen concentration and the training status of the subjects, but is most likely related to the timing of post-exercise biopsies. Nonetheless, it seems

plausible that Akt-mediated signalling would be enhanced to promote glucose transport and glycogen resynthesis at rest due to low muscle glycogen, but strong contractile stimuli upregulates the metabolic response uniformly regardless of glycogen status.

As might be expected, differences in markers of glucose uptake glycogen synthesis and were observed at rest and post-exercise (Fig. 3.5). Glycogen synthase^{Ser641} dephosphorylation (activation) was significantly greater in LOW compared with NORM at every time point in the nutrient and placebo groups (Fig. 3.5A). Moreover, GS was significantly dephosphorylated in the LOW glycogen legs of both groups 1 h after the resistance exercise bout. Considering the results from Chapter 2 where there was no change in GS phosphorylation after resistance exercise, this may indicate that low glycogen concentration is a critical factor for the capacity of low-volume, high-intensity resistance exercise to exert any significant effect on glycogen synthase activity and (re)synthesis. Post-exercise increases in AS160 phosphorylation were apparent with protein/ CHO ingestion but were not different between NORM and LOW glycogen legs (Fig. 3.5B). Conversely, AS 160 phosphorylation increased post-exercise only in LOW from the placebo group. This suggests any sensitivity AS 160 may exhibit to low glycogen availability following resistance exercise is eliminated upon adequate nutrient ingestion.

There was no effect of glycogen status on mTOR^{Ser2448} phosphorylation after resistance exercise in the placebo group, whereas nutrient ingestion elevated mTOR above rest 1 h after exercise to a greater extent in the NORM compared with the LOW leg (Fig. 3.4B). The increased phosphorylation of mTOR with protein/CHO ingestion likely represents a synergistic effect mediated through the insulin signalling cascade and capacity for amino acids to directly activate mTOR through a putative interaction between the Rag- and Rheb-GTPases (Sancak et al. 2008). Although the disparity in the magnitude of mTOR phosphorylation may indicate a modest suppression due to low glycogen, there was still a ~ 4-fold increase in mTOR phosphorylation in the LOW leg that was sufficient to initiate activation of downstream proteins more proximal to translation initiation. Moreover, the sustained elevation in mTOR phosphorylation 4 h after resistance exercise in the nutrient group was similar between NORM and LOW legs.

The AMPK complex has a glycogen binding domain that may influence AMPK's role as a cell energy sensor while also having the capacity to negatively regulate mTOR activation (Bolster et al. 2002, Gwinn et al. 2008). Previous work demonstrated increased mTOR phosphorylation and

muscle protein synthesis rates concomitant with elevated AMPK activity following exercise, indicating that any putative effect of AMPK on muscle protein synthesis in humans after resistance exercise may only be modest (Dreyer et al. 2008a). Regardless, no change in AMPK phosphorylation was observed between legs in the placebo or nutrient groups that might explain the moderate difference in mTOR phosphorylation 1 h post-exercise. Moreover, a similar phosphorylation status of regulatory targets of mTOR proximal to translation initiation indicative of comparable activation was also shown despite disparity in glycogen concentration. This is in agreement with numerous previous studies investigating translational signalling that show increases in p70S6K and rpS6 phosphorylation during the early recovery period following exercise and the augmented response with nutrient provision after an overnight fast (Deldicque et al. 2010, Dreyer et al. 2008b, Koopman et al. 2007b).

Consistent with the changes in cell signalling, muscle mRNA responses of select genes associated with muscle proteolysis and catabolism were relatively unchanged by muscle glycogen concentration. Muscle atrophy F-Box (MAFbx; also known as atrogin-1) belongs to the ubiquitin proteasome pathway involved in tagging contractile protein for degradation by cellular proteasomes (Bodine et al. 2001a, Gomes et al. 2001), whereas myostatin is a putative negative regulator of muscle growth (Thomas et al. 2000). The decrease in atrogin-1 mRNA abundance at 4 h post-exercise in NORM and LOW legs in the placebo and nutrient groups (Fig. 3.7A) is similar to previous work from this laboratory showing a decrease in atrogin mRNA 3 h after resistance exercise (Churchley et al. 2007). Likewise, the post-exercise decrease in myostatin mRNA expression (Fig. 3.7B) is in accordance with previous studies that have examined mRNA changes following resistance exercise (Harber et al. 2009, Louis et al. 2007). Interestingly, there was higher atrogin-1 and myostatin expression in the LOW glycogen leg compared with the normal glycogen leg after 4 h recovery in the nutrient group. To my knowledge, this is the first study to investigate the interaction of glycogen concentration and nutrients on catabolic genes after resistance exercise. The possibility exists that the abundance of exogenous CHO/amino acids and low muscle glycogen generated a signal to “switch on” processes regulating muscle remodelling/ adaptation after exercise rather than preserving muscle protein by suppressing breakdown without post-exercise nutrient provision in the placebo/fasted condition. However, it should be noted that the mRNA abundance of atrogin and myostatin were not elevated above resting levels and probably represents only a modest effect on catabolic processes.

In conclusion, and in contrast to the original hypothesis, commencing a bout of strenuous resistance exercise with low muscle glycogen availability failed to attenuate anabolic signalling and rates of myofibrillar protein synthesis compared with when the same exercise bout was undertaken with normal glycogen availability. Protein-CHO supplementation also failed to mediate any divergence in muscle protein synthesis between NORM and LOW during recovery. Moreover, whereas some disparity between legs was observed, undertaking exercise with low glycogen did not induce an increase in select mRNA markers of catabolic activity. Although it cannot be ruled out the possibility that alternative resistance training bouts employing different contraction volumes and intensities might generate a more pronounced effect of glycogen concentration on post-exercise muscle cell signalling and muscle protein synthesis rates, these findings indicate that commencing resistance exercise with low muscle glycogen does not impair this anabolic response in the early recovery period. This is imperative when considering the potential for suboptimal muscle glycogen situations when undertaking multiple high intensity exercise bouts in a day. Nonetheless, whereas low glycogen availability may promote the aerobic training phenotype, this work provides new information to show that modulating glycogen concentration neither promotes nor inhibits the acute adaptation response after resistance exercise.

Chapter Four

Effects of Protein Ingestion on Muscle Protein Synthesis and mRNA Expression Following Consecutive Resistance and Endurance Exercise

Adapted from: Camera, D.M., West, D,W., Phillips, S.M., Rerecich, T., Stellingwerff, T., Hawley, J.A., Coffey, V.G. (2013) *Journal of Applied Physiology* (In Review)

4.1 Introduction

Contraction-induced adaptations in skeletal muscle are largely determined by the mode, volume and intensity of exercise (Coffey and Hawley 2007b). Repeated bouts of endurance exercise generates multiple adaptations in skeletal muscle including, but not limited to, increased capillary and mitochondrial density (Holloszy 1967, Saltin and Gollnick 1983), whereas chronic resistance training generally promotes a phenotype of increased myofibrillar protein accretion and cross sectional area of type II fibres (D'Antona et al. 2006, Phillips et al. 1999). Exercise-nutrient interactions are also critical in determining skeletal muscle adaptation and may have the capacity to modulate the specificity of training response (Hawley et al. 2011). Indeed, manipulating carbohydrate availability and/or muscle glycogen stores alter the endurance exercise adaptation response (Bergström et al. 1967, Ivy et al. 1988a), while protein/ amino acid (leucine) supplementation interacts synergistically with resistance exercise to increase muscle protein synthesis (Phillips et al. 2005, Rennie et al. 1982). However, a limited number of studies have investigated the acute adaptation response to the combined effects of endurance and resistance ('concurrent') exercise and, in particular, the interaction with protein ingestion/supplementation.

The cellular mechanisms regulating the specificity of training adaptation within a concurrent training paradigm is undoubtedly complex given the capacity of single mode endurance and resistance training to generate divergent phenotypes (D'Antona et al. 2006, Wilkinson et al. 2008) and the potential confounding factors of exercise order and recovery between bouts. Wilson and colleagues have reported that endurance exercise inhibits hypertrophy/strength in a volume and frequency dependent manner within a concurrent training paradigm (Wilson et al. 2012). Various cell signalling responses related to translation initiation and mRNA expression of mitochondrial/metabolic and myogenic adaptation have also been observed from past work in this lab following a concurrent exercise bout in the fasted state (Coffey et al. 2009a, Coffey et al. 2009b). Interestingly, comparable increased rates of myofibrillar and mitochondrial synthesis were recently shown following concurrent resistance and endurance exercise when compared to each mode in isolation in sedentary middle-aged men (Donges et al. 2012). Therefore, while the molecular profile generated by an acute bout of concurrent training has yet to be clearly established, the possibility exists that successive resistance and endurance exercise may have the capacity to promote both myofibrillar and mitochondrial protein synthesis.

Consumption of high-quality protein in close temporal proximity to resistance exercise enhances translation initiation signalling and maximally stimulates rates of muscle protein synthesis (Koopman et al. 2007b, Moore et al. 2009a). Likewise, protein feeding following endurance exercise can increase the transcriptional profile of mitochondrial-related genes (Rowlands et al. 2011). To date no study has determined the effect of protein ingestion following concurrent exercise on the acute myofibrillar and mitochondrial protein synthesis rates in skeletal muscle. Accordingly, the primary aim of the present investigation was to examine the acute effects of protein ingestion on rates of myofibrillar and mitochondrial protein synthesis in association with selected cellular/molecular responses following a bout of consecutive resistance exercise and cycling. It was hypothesized that, compared to placebo, protein ingestion would enhance anabolic and metabolic signalling and subsequent protein synthesis during the early recovery period following exercise.

4.2 Methods

Subjects

Eight healthy male subjects [age 19.1 ± 1.4 yr, body mass 78.1 ± 15.6 kg, peak oxygen uptake ($V_{O_{2peak}}$) 46.7 ± 4.4 ml·kg^{-1}·min^{-1}, leg extension one repetition maximum (1-RM) 130 ± 14 kg; values are mean ± SD] who had been participating in regular concurrent resistance and endurance training (~3×/week; > 1 year) volunteered to participate in this study. The experimental procedures and possible risks associated with the study were explained to all subjects, who gave written informed consent before participation. The study was approved by the Human Research Ethics Committee of RMIT University.

Study Design

The study employed a randomised double-blind, cross-over design in which each subject completed two acute concurrent resistance and cycling exercise sessions with either post-exercise placebo (PLA) or protein (PRO) ingestion separated by a three week recovery period, during which time subjects maintained their habitual physical activity pattern.

Preliminary Testing

VO_{2peak}. Peak oxygen uptake was determined during an incremental test to volitional fatigue on a Lode cycle ergometer (Groningen, The Netherlands). The protocol has been described in detail previously (Hawley and Noakes 1992). In brief, subjects commenced cycling at a workload equivalent to 2 W/kg for 150 s. Thereafter, the workload was increased by 25 W every 150 s until volitional fatigue, defined as the inability to maintain a cadence >70 revolutions/min. Throughout the test subjects breathed through a mouthpiece attached to a metabolic cart (Parvomedics, USA) to determine oxygen consumption.

Maximal Strength. Quadriceps strength was determined during a series of single repetitions on a plate-loaded leg extension machine until the maximum load lifted was established (1 RM). Repetitions were separated by a 3-min recovery and were used to establish the maximum load/weight that could be moved through the full range of motion once, but not a second time. Exercise range of motion was 85° with leg extension endpoint set at -5° from full extension.

Diet/Exercise Control. Before an experimental trial (described subsequently), subjects were instructed to refrain from exercise training and vigorous physical activity, and alcohol and caffeine consumption for a minimum of 48 h. Subjects were provided with standardized prepacked meals that consisted of 3 g carbohydrate/kg body mass, 0.5 g protein/kg body mass, and 0.3 g fat/kg body mass consumed as the final caloric intake the evening before reporting for an experimental trial.

Experimental Testing Session

On the morning of an experimental trial, subjects reported to the laboratory after a ~10-h overnight fast. After resting in the supine position for ~15 min, catheters were inserted into the anticubital vein of each arm and a baseline blood sample (~3 mL) was taken (Figure 4.1). A primed constant intravenous infusion (prime: 2 $\mu mol \cdot kg^{-1}$; infusion: 0.05 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) of L-[*ring*-$^{13}C_6$] phenylalanine (Cambridge Isotopes Laboratories, USA) was then administered. Under local anaesthesia (2–3 mL of 1% Xylocaine) a resting biopsy was obtained 3 h after commencement of the tracer infusion from the vastus lateralis using a 5-mm Bergstrom needle modified with suction. Subjects then completed the exercise intervention (described

subsequently). Immediately following the cessation of exercise, subjects ingested 500 mL of either a placebo (PLA: water, artificial sweetener) or protein beverage (PRO: 25 g whey protein). The protein beverage was enriched to 5% L-[*ring*-$^{13}C_6$] phenylalanine to prevent dilution of the steady-state isotope enrichment implemented by the constant infusion. Subjects rested throughout a 240-min recovery period and additional muscle biopsies were taken 60 and 240 min post-exercise. Each muscle biopsy was taken from a separate site 2-3 cm distal from the right leg for the first trial and left leg for the second trial with all samples stored at -80°C until subsequent analysis. Blood samples were collected in EDTA tubes at regular intervals during the post-exercise recovery period.

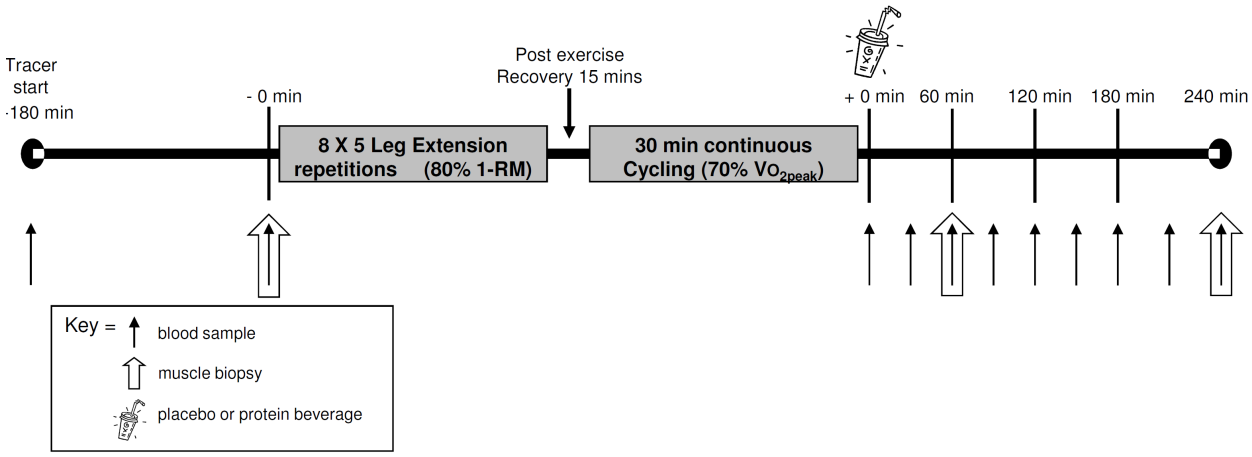

Figure 4.1 Schematic representation of the experimental trial. Subjects reported to the laboratory following an overnight fast and an after initial resting blood sample began a constant infusion of L-[*ring*-$^{13}C_6$] phenylalanine. 180 min after commencement of tracer infusion, a baseline muscle biopsy (*vastus lateralis*) was obtained, and subjects then completed a concurrent exercise session consisting of resistance (8 sets of 5 leg extensions at 80% 1-RM) and endurance (30 min cycling at 70% VO_{2peak}) exercise separated by 15 minutes. Immediately after exercise, subjects consumed a 500 mL bolus of protein (25 g whey) or placebo. Additional muscle biopsies were taken at 1 and 4 h post-exercise.

Resistance Exercise

After a standardised warm-up (2 ×5 repetitions at ~50% and ~60% 1 RM, respectively), subjects performed eight sets of five repetitions at ~ 80% 1 RM. Each set was separated by a 3 min recovery period during which time the subject remained seated on the leg extension machine. Contractions were performed at a set metronome cadence approximately equal to 30°/s and strong verbal encouragement was provided during each set. Subjects then rested for 15 min before beginning the cycling protocol.

Cycling Exercise

Subjects performed 30 min of continuous cycling at a power output that elicited ~70% of individual VO_{2peak}. Subjects were fan-cooled and allowed *ad libitum* access to water throughout the ride. Visual feedback for pedal frequency, power output, and elapsed time were provided to subjects.

Analytical Procedures

Blood Glucose and Plasma Insulin Concentration. Whole blood samples (5 mL) were immediately analysed for glucose concentration using an automated glucose analyser (YSI 2300, Yellow Springs, USA). Blood samples were then centrifuged at 1000 *g* at 4º C for 15 min, with aliquots of plasma frozen in liquid N_2 and stored at -80°C. Plasma insulin concentration was then measured using a radioimmunoassay kit according to the manufacturer's protocol (Linco Research, Inc., St Charles, MO, USA).

Plasma Amino Acids and Enrichment. Plasma amino acid concentrations were determined by high performance liquid chromatography (HPLC) from a modified protocol (Moore et al. 2009a). Briefly, 100 µL of plasma was mixed with 500 µL of ice cold 0.6 M PCA and neutralised with 250 µL of 1.25 M potassium bicarbonate (KHCO3). Samples were then subsequently derivatised for HPLC analysis. Plasma [*ring*-$^{13}C_6$] phenylalanine enrichments were determined as previously described (Glover et al. 2008b).

Mitochondrial and Myofibrillar Protein Synthesis

A piece frozen of wet muscle (~ 100 mg) was homogenised with a Dounce glass homogenizer on ice in an ice-cold homogenising buffer (1M Sucrose, 1M Tris/HCl, 1M KCl, 0.5M EDTA) supplemented with a protease inhibitor and phosphatase cocktail tablet (PhosSTOP, Roche Applied Science, Mannhein, Germany) per 10 ml of buffer. The homogenate was transferred to an eppendorf tube and centrifuged to pellet a fraction enriched with myofibrillar proteins and collagen that was stored at -80˚C for subsequent extraction of the myofibrillar fraction (described below). The supernatant was transferred to another eppendorf tube and centrifuged to pellet the mitochondrial enriched protein fraction. The supernatant was placed in a separate eppendorf and stored at -80˚C for Western Blot analysis (described below). The mitochondrial enriched pellet was then washed, lyophilized and amino acids were liberated by adding 1.5 mL of 6M HCL and heating to 110˚C overnight.

The myofibrillar pellet stored at -80˚C was washed twice with the homogenization buffer, centrifuged and supernatant was discarded. Myofibrillar proteins were solubilised in 0.3 M sodium hydroxide and precipitated with1 M perchloric acid. Amino acids were then liberated from the myofibrillar enriched precipitate by adding 2.0 ml of 6 M HCL and heating to 110˚C overnight.

Free amino acids from myofibrillar and mitochondrial enriched fractions were purified using cation-exchange chromatography (Dowex 50WX8-200 resin; Sigma-Aldrich Ltd) and converted to their N-acetyl-n-propyl ester derivatives for analysis by gas chromatography combustion-isotope ratio mass spectrometry (GC-C-IRMS: Hewlett Packard 6890; IRMS model Delta Plus XP, Thermo Finnagan, Waltham, MA, USA).

Intracellular amino acids (IC) were extracted from a separate piece of wet muscle (~20 mg) with ice-cold 0.6 M PCA. Muscle was homogenized and the free amino acids in the supernatant were purified by cation-exchange chromatography and converted to their heptafluorobutyric (HFB) derivatives before analysis by GC-MS (models 6890 GC and 5973 MS; Hewlett-Packard, Palo Alto, USA) as previously described (Moore et al. 2009a).

Calculations

The rate of mitochondrial and myofibrillar protein synthesis was calculated using the standard precursor–product method: FSR (%·h−1) = [(E2b − E1b) / (EIC x t)] × 100

Where E2b - E1b represents the change in the bound protein enrichment between two biopsy samples, EIC is the average enrichment of intracellular phenylalanine between the two biopsy samples and t is the time between two sequential biopsies.

Western Blots. The supernatant frozen at -80˚C from the previous mitochondrial enriched fraction extraction was used for determination of protein concentration using a BCA protein assay (Pierce, Rockford, IL, USA). The supernatant was subsequently resuspended in Laemelli sample buffer, separated by SDS-PAGE, transferred to polyvinylidine fluoride membranes and incubated with primary antibody (1:1,000) overnight at 4°C on a shaker. Membranes were incubated with secondary antibody (1:2,000), and proteins were detected via enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK; Pierce Biotechnology, Rockford, IL) and quantified by densitometry (Chemidoc, BioRad, Gladesville, Australia). All sample (40 µg) time points for each subject were run on the same gel. Polyclonal anti-phospho-Akt^{Ser473} (no.9271), -mTOR^{Ser2448} (no. 2971), -Glycogen Synthase (GS) ^{Ser641} (no.3891) -eEF2^{Thr56} (no. 2331), and monoclonal anti- AMPKα^{Thr172} (no. 2535) and p70S6K^{Thr389} (no.9234) were from Cell Signalling Technology (Danvers, USA). Data are expressed relative to α-tubublin (no. 3873, Cell Signalling Technology, Danvers, USA) in arbitrary units.

RNA Extraction and Quantification

Skeletal muscle tissue RNA extraction was performed on previously snap frozen samples with TRIzol according to the manufacturer's directions. Briefly, ~ 20 mg of skeletal muscle was homogenised in TRIzol and chloroform added to form an aqueous RNA phase. This RNA phase was then precipitated by mixing with isopropanol alcohol and the resulting pellet was washed and re-dissolved in 50 µl of RNase-free water. Extracted RNA was quantified using a QUANT-iT analyser kit (Invitrogen, Melbourne, Australia, Cat No Q32852) according to the manufactures directions. Quality of RNA was further determined on a NanoDrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA) by measuring absorbance at 260 nm and 280 nm

with a 260/280 ratio of ~ 1.88 recorded for all samples. The RNA samples were diluted as appropriate to equalise concentrations, and stored at -80˚C for subsequent reverse transcription.

Reverse Transcription and Real-Time PCR

First-strand complementary DNA (cDNA) synthesis was performed using commercially available TaqMan Reverse Transcription Reagents (Invitrogen, Melbourne, Australia) in a final reaction volume of 20 µL. All RNA and negative control samples were reverse transcribed to cDNA in a single run from the same reverse transcription master mix. Serial dilutions of a template RNA (AMBION; Cat No AM7982) was included to ensure efficiency of reverse transcription and for calculation of a standard curve for real-time quantitative polymerase chain reaction (RT-PCR). Quantification of mRNA (in duplicate) was performed on a 72-well Rotor-Gene 3000 Centrifugal Real-Time Cycler (Corbett Research, Mortlake, Australia). Taqman-FAM-labelled primer/probes for MuRF-1 (Cat No. Hs00261590), Atrogin (Cat No. Hs01041408), Myostatin (Cat No. Hs00976237), PGC-1α (Cat No. Hs01016719), Hexokinase (Cat No. Hs00175976) and VEGF (Cat No. Hs00900055) were used in a final reaction volume of 20 µL. PCR treatments were 2 min at 50 ºC for UNG activation, 10 min at 95 ºC then 40 cycles of 95 ºC for 15 s and 60 ºC for 60 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cat No Hs Hs99999905) was used as a housekeeping gene to normalize threshold cycle (CT) values. The relative amounts of mRNAs were calculated using the relative quantification (ΔΔCT) method (Livak and Schmittgen 2001).

Statistical Analysis

All data were analysed by two-way ANOVA (two factor: time × treatment) with repeated measures and Student-Newman-Keuls post hoc analysis. Statistical significance was established when $P < 0.05$ (SigmaStat for windows Version 3.11). All data are expressed as arbitrary unit's ± SD.

4.3 Results

Plasma Insulin, Amino Acids and Blood Glucose

There were significant effects for plasma insulin and total amino acid concentration with PRO but not PLA ($P < 0.001$; Figure 4.2A, B). Peak plasma insulin (~ 535%) and amino acid (~ 70%) concentrations occurred 40 min post-exercise ($P < 0.001$). The same effect was evident for BCAA concentration (~180%, $P < 0.001$; Figure 4.2C). Blood glucose was not different at any time in either treatment (data not shown).

Plasma tracer enrichments

Plasma L-[*ring* $^{13}C_6$] phenylalanine enrichment at rest, and 60, 120, 180 and 240 min post-exercise for PRO and PLA were 0.0688, 0.0557, 0.0679, 0.0673 and 0.0609, and 0.0617 0.0558, 0.0616, 0.0558, and 0.0606 tracer-to-tracee ratio: t·T-1, respectively. Linear regression analysis indicated that the slopes of the plasma enrichments were not significantly different from zero, showing isotopic plateau/steady-state.

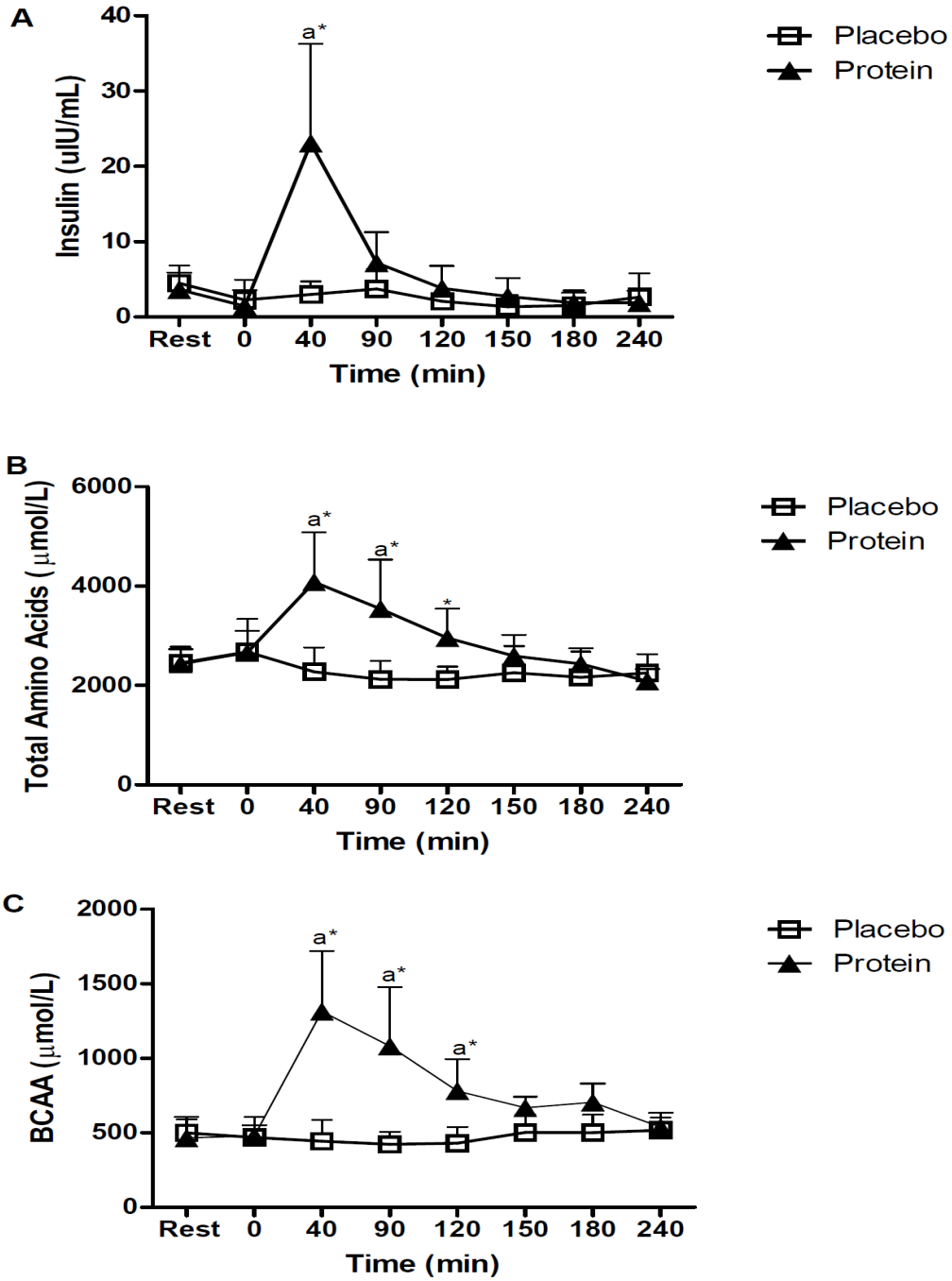

Figure 4.2 Plasma insulin (A), total plasma amino acid (B) and plasma branched chain amino acid (BCAA) concentrations (C) at rest and during 240 min recovery following a concurrent exercise of session resistance (8 sets of 5 leg extensions at 80% 1-RM) and endurance (30 min cycling at 70% VO_{2peak}) exercise and ingestion of either 500 mL placebo or protein beverage immediately post-exercise. Values are mean ± SD. Significantly different ($P < 0.05$) versus (a) rest; and (asterisk) between treatments (placebo vs. protein).

Akt-mTOR-p70S6K-eEF2. There were significant effects for Akt^{Ser473} phosphorylation for time and treatment ($P < 0.05$, Figure 4.3A). Akt^{Ser473} phosphorylation increased above rest with PRO (~ 175%; $P < 0.05$) but not PLA 1 h after exercise. This disparity in Akt^{Ser473} resulted in a significant difference between treatments at 1 h ($P < 0.05$). Phosphorylation in PRO then returned to resting levels 4 h following recovery from exercise ($P < 0.05$).

There were significant effects for time and treatment for mTOR^{Ser2448} phosphorylation ($P<0.05$, Figure 4.3B). mTOR phosphorylation increased after PRO (~ 400%, $P < 0.001$) and PLA (~ 100%, $P < 0.05$) ingestion at 1 h, and this increase was markedly higher with PRO (~ 300%, $P < 0.001$). mTOR^{Ser2448} phosphorylation remained elevated above rest 4 h post-exercise with PLA only (~ 130%, $P < 0.05$), resulting in a significant disparity between treatments ($P < 0.05$).

There were significant effects for p70S6K^{Thr389} phosphorylation for both time and treatment ($P < 0.05$, Figure 4.3C). p70S6K^{Thr389} phosphorylation increased above rest with PRO (~ 3000%; $P < 0.001$) but not PLA 1 h after exercise. This disparity in p70S6K^{Thr389} resulted in a significant difference between treatments at 1 h ($P < 0.05$). Phosphorylation of p70S6K after PRO returned to resting levels after 4 h of recovery from exercise ($P < 0.001$).

There were significant effects for eEF2^{Thr56} phosphorylation for time in both treatments ($P < 0.05$, Figure 4.3D). One hour post-exercise, phosphorylation of eEF2 decreased ~ 60% ($P < 0.05$) with PLA and ~ 75% ($P < 0.05$) with PRO and remained at this level for the duration of the recovery (4 h).

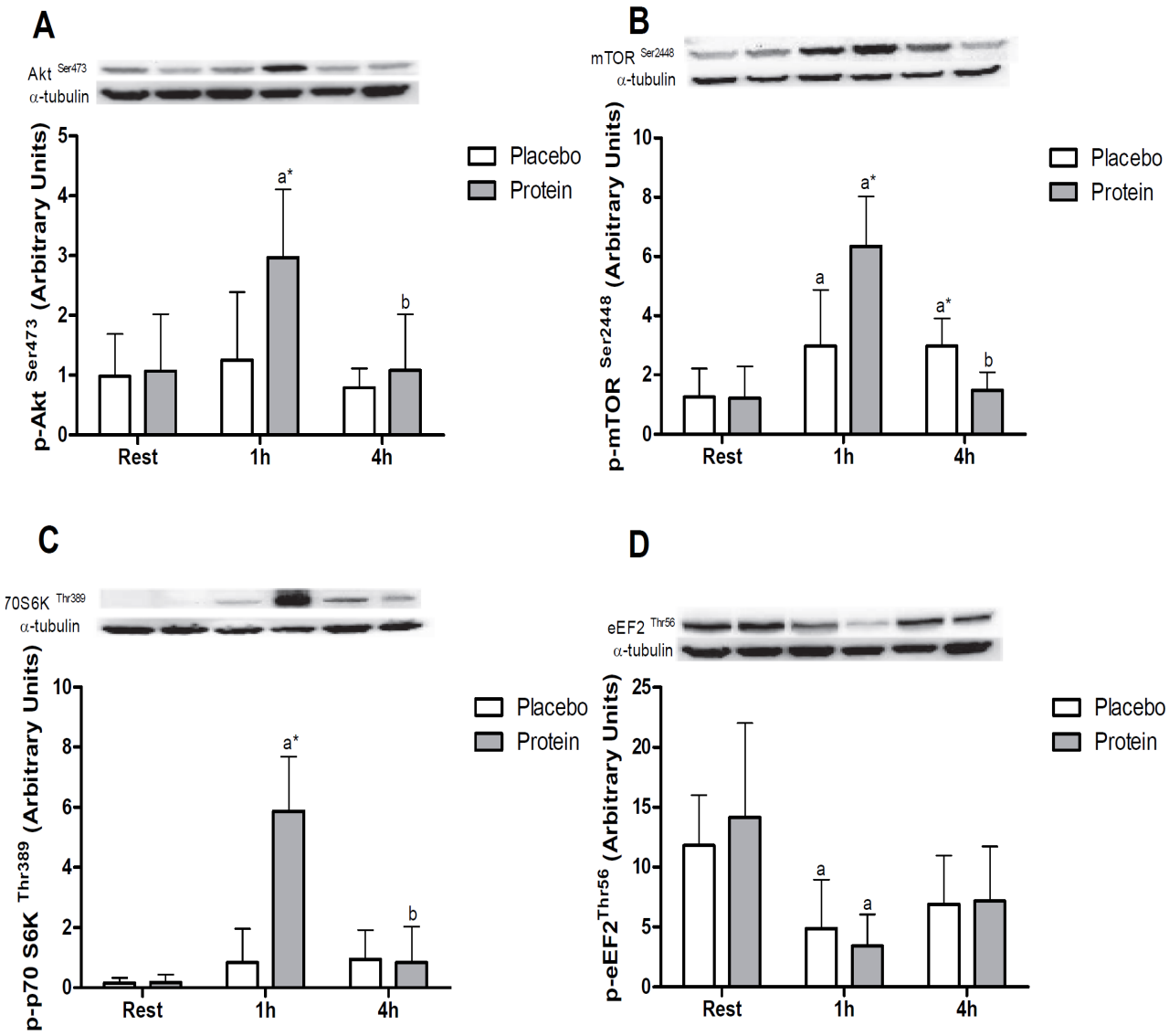

Figure 4.3 (A) Akt^{Ser473} (B) mammalian target of rapamycin (mTOR) ^{Ser2448} (C) p70S6K^{Thr389} and (D) eukaryotic elongation factor 2 (eEF2) ^{Thr56} phosphorylation in skeletal muscle at rest and during 4 h post-exercise recovery following a concurrent exercise session of resistance (8 sets of 5 leg extensions at 80% 1-RM) and endurance (30 min cycling at 70% VO_{2peak}) exercise and ingestion of either 500 mL placebo or protein beverage immediately post-exercise. Values are expressed relative to α-tubulin and presented in arbitrary units (mean ± SD, n = 8). Significantly different ($P < 0.05$) versus (a) rest, (b) 1 h and (asterisk) between treatments (placebo vs. protein) at equivalent time-point.

AMPK – GS. There were significant effects for both time and treatment for $AMPK^{Thr172}$ phosphorylation ($P < 0.05$, Figure 4.4A). $AMPK^{Thr172}$ phosphorylation decreased from 1 h to 4 h post-exercise after PRO only (~ 70 %, $P < 0.05$) and was higher in PLA, however post hoc analysis failed to show any differences for any individual time point.

There were significant effects for time for GS^{Ser64}、 phosphorylation ($P < 0.05$, Figure 4.4B). GS^{Ser641} phosphorylation was lower from rest at 1 h (~ 80%, $P < 0.05$) and 4 h (~ 70%, $P < 0.05$) post-exercise with PLA. GS phosphorylation similarly decreased at 1 h with PRO compared to rest (~ 90%, $P < 0.05$) but was not different at 4 h.

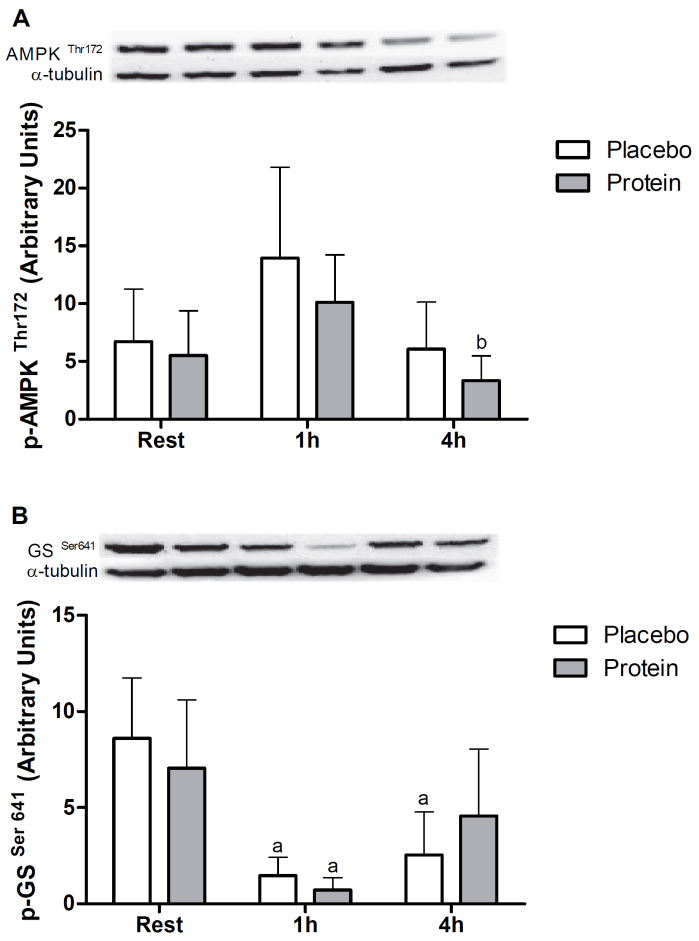

Figure 4.4 (A) 5' adenosine monophosphate-activated protein kinase (AMPK) ^{Thr172} and (B) Glycogen Synthase (GS) ^{Ser641} phosphorylation in skeletal muscle at rest and during 4 h post-exercise recovery following a concurrent exercise session of resistance (8 sets of 5 leg extensions at 80% 1-RM) and endurance (30 min cycling at 70% VO_{2peak}) exercise and ingestion of either 500 mL placebo or protein beverage immediately post-exercise. Values are expressed relative to α-tubulin and presented in arbitrary units (mean ± SD, n = 8). Significantly different ($P < 0.05$) versus (a) rest, (b) 1 h and (asterisk) between treatments (placebo vs. protein) at equivalent time-point.

mRNA expression

MuRF1-Atrogin-1-Myostatin

There were significant effects for time and treatment for MuRF1 mRNA abundance ($P < 0.05$, Figure 4.5A). MuRF1 increased significantly above resting levels at 1 h (~ 315% vs. ~ 230%, $P < 0.001$) and 4 h (~ 250% vs. ~ 140%, $P < 0.05$) post-exercise after both PLA and PRO, respectively. MuRF1 was higher in PLA compared to PRO at both post-exercise time points (1 h: 78%, 4 h: 105%, $P < 0.05$).

Atrogin-1 mRNA expression increased above rest only with PLA 1 h post-exercise (~ 50%, $P < 0.05$; Figure 4.5B). The disparity in Atrogin-1 mRNA at 1 h resulted in a significant difference between treatments ($P < 0.05$).

There was a significant effect of time for myostatin mRNA abundance ($P < 0.05$, Figure 4.5C). Myostatin decreased from rest at 1 h (~ 40% vs. ~ 55%, $P < 0.05$) and 4 h (~ 70% vs. ~ 80%, $P < 0.001$) after both PLA and PRO, respectively. Myostatin mRNA at 1 h was different from 4 h after PLA (~ 120%, $P < 0.05$).

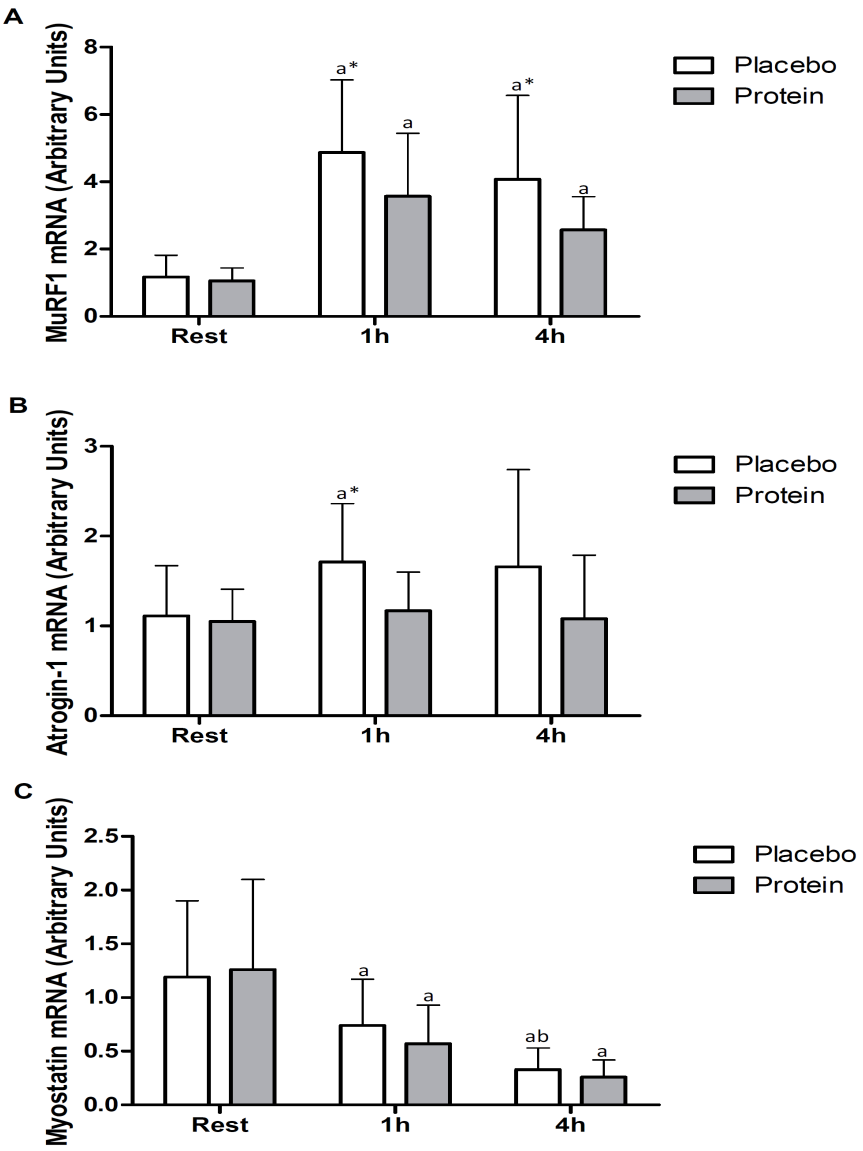

Figure 4.5 (A) Muscle ring finger 1 (MuRF1), (B) atrogin and (C) myostatin mRNA abundance at rest and during 4 h post-exercise recovery following a concurrent exercise session of resistance (8 sets of 5 leg extensions at 80% 1-RM) and endurance (30 min cycling at 70% VO_{2peak}) exercise and ingestion of either 500 mL placebo or protein beverage immediately post-exercise. Values are expressed relative to GAPDH and presented in arbitrary units (mean ± SD, n = 8). Significantly different ($P < 0.05$) versus (a) rest, (b) 1 h and (asterisk) between treatments (placebo vs. protein) at equivalent time-point.

PGC-1α-Hexokinase-VEGF

There were significant effects for PGC-1α mRNA abundance for time ($P < 0.05$, Figure 4.6A). PGC-1α expression increased above resting and 1 h levels following 4 h post-exercise recovery in PLA (~ 730%, $P < 0.001$) and PRO (~ 620%, $P < 0.001$).

There were signifiant effects for time and treatment for hexokinase mRNA expression ($P < 0.05$, Figure 4.6B). Hexokinase increased above rest at 4 h in PLA only (~ 120%, $P < 0.05$) whereas in PRO there were no changes. This disparity resulted in a significant difference between treatments at 4 h ($P < 0.05$).

VEGF mRNA expression increased above rest at both 1 h (~ 200%, $P < 0.001$) and 4 h (~ 210%, $P < 0.001$) with PLA (Figure 4.6C). Likewise, VEGF also increased with PRO at 1 h (~ 170%, $p < 0.05$) and 4 h (~ 180; $P < 0.05$). There were no differences between treatments at any post-exercise time point.

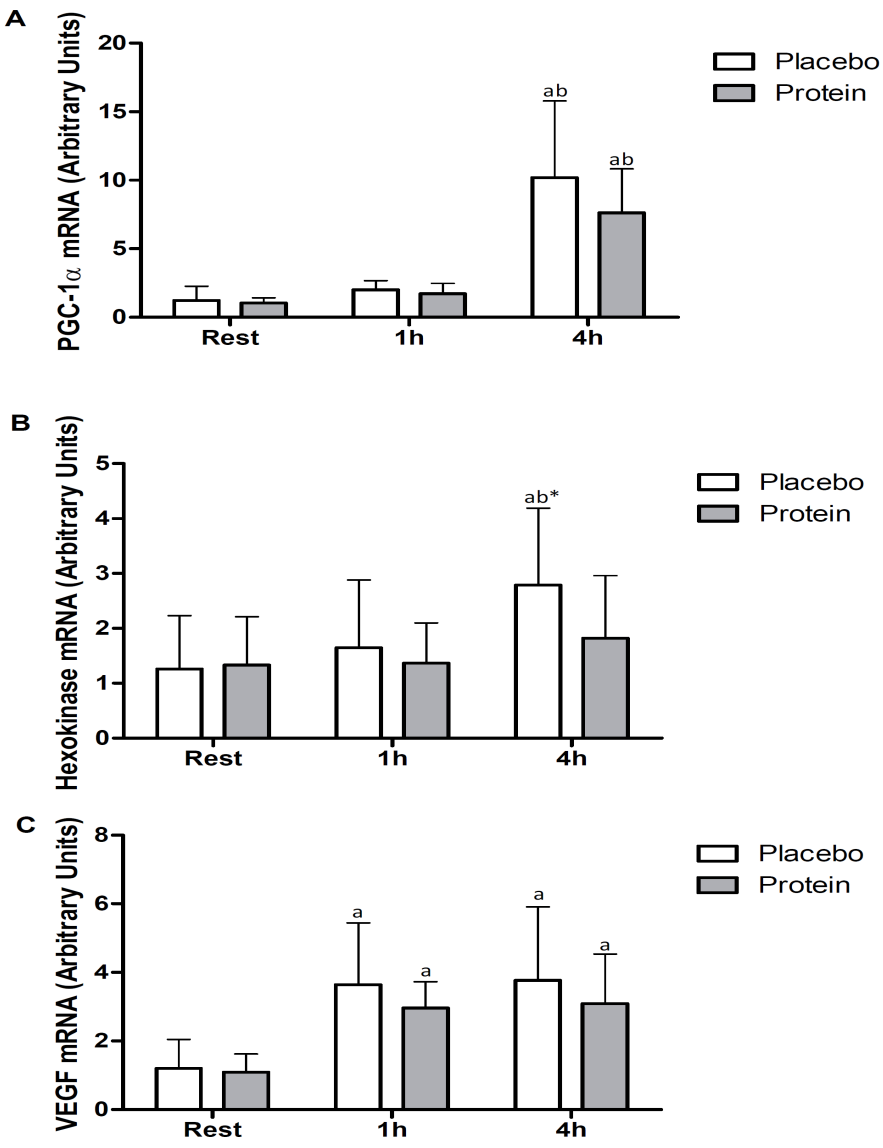

Figure 4.6 (A) Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), (B) hexokinase and (C) vascular endothelial growth factor mRNA abundance at rest and during 4 h post-exercise recovery following a concurrent exercise session of resistance (8 sets of 5 leg extensions at 80% 1-RM) and endurance (30 min cycling at 70% VO_{2peak}) exercise and ingestion of either 500 mL placebo or protein beverage immediately post-exercise. Values are expressed relative to GAPDH and presented in arbitrary units (mean ± SD, n = 8). Significantly different ($P < 0.05$) versus (a) rest, (b) 1 h and (asterisk) between treatments (placebo vs. protein) at equivalent time-point.

Muscle Protein Synthesis

Rates of myofibrillar protein synthesis increased above rest between 1 h and 4 h post-exercise after both PLA (~ 75%, P < 0.05) and PRO (~ 145%, P<0.001) (Figure 4.7A). This post-exercise increase in the rate of myofibrillar synthesis was greater with PRO compared to PLA (P < 0.05). Rates of mitochondrial protein synthesis (n = 6) were unchanged during the acute post-exercise period and there were no differences in post-exercise fractional synthesis rates between treatments (Figure 4.7B).

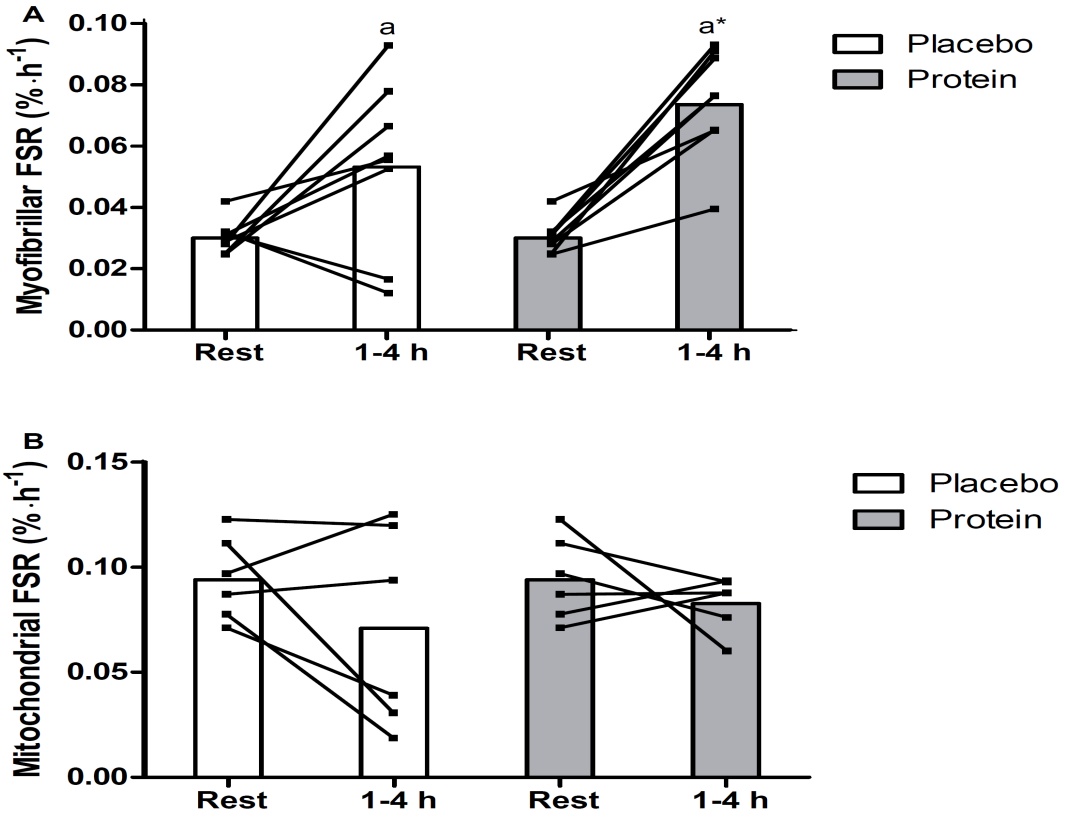

Figure 4.7 (A) Myofibrillar (n = 8) and (B) Mitochondrial (n = 6) protein fractional synthetic rates between 1 - 4 h recovery following a concurrent exercise session of resistance (8 sets of 5 leg extensions at 80% 1-RM) and endurance (30 min cycling at 70% VO_{2peak}) exercise and ingestion of either 500 mL placebo or protein (25 g whey protein) beverage immediately post-exercise. Values are expressed as %•h and presented as individual data with group mean. Significantly different (P < 0.05) versus (a) rest, and (asterisk) placebo vs. protein.

4.4 Discussion

Adaptations to concurrent resistance and endurance exercise may be 'compromised' when compared with training for either exercise mode alone (Coffey and Hawley 2007b, Hickson 1980, Wilson et al. 2012). Results from this work show that in moderately trained individuals the combined effects of resistance and endurance exercise resulted in elevated rates of myofibrillar but not mitochondrial protein synthesis. It was also reported for the first time that protein ingestion promotes insulin/insulin-like growth factor (IGF) pathway signalling and myofibrillar protein synthesis, but does not enhance mitochondrial protein synthesis rates during the early recovery period following consecutive resistance exercise and cycling. In addition, these findings provide new information to demonstrate that post-exercise protein ingestion attenuates mRNA expression of markers of muscle catabolism following a concurrent training session.

Athletes from a variety of sports undertake resistance and endurance training concurrently to enhance both anabolic/growth and metabolic/oxidative adaptations in skeletal muscle. As such, concurrent training presents a unique integration of divergent contractile activity. The primary novel finding of the present study was that a single bout of concurrent training promoted an adaptation response favouring muscle anabolism in moderately trained males, and post-exercise protein supplementation preferentially enhanced rates of myofibrillar but not mitochondrial protein synthesis (Figure 4.7). Donges and colleagues (Donges et al. 2012) have recently shown that a concurrent training bout was capable of upregulating translational signalling, and myofibrillar and mitochondrial protein synthesis in untrained, middle-aged subjects to a similar extent as resistance and endurance exercise bouts performed in isolation. The results of the present study provide support for this exercise-mediated effect on the myofibrillar fraction of skeletal muscle with placebo ingestion, but failed to elevate rates of mitochondrial protein synthesis in this cohort of subjects.

Enhanced rates of myofibrillar protein synthesis following resistance exercise with post-exercise protein ingestion are well established (Burd et al. 2009, Moore et al. 2009a). However, this is the first study to report increased rates of myofibrillar synthesis with protein supplementation compared to placebo during the acute post-exercise recovery period following concurrent resistance exercise and cycling. Therefore, these findings suggest that resistance exercise generates a sufficient adaptive signal to retain the capacity to stimulate myofibrillar protein

synthesis despite a subsequent bout of endurance exercise. Such an acute response would be expected to ultimately result in muscle hypertrophy with repeated bouts of resistance exercise in a chronic concurrent training program.

A similar selective increase in myofibrillar protein synthesis rate has previously been demonstrated in response to protein-carbohydrate co-ingestion following a high-intensity repeated sprint protocol (Coffey et al. 2011). Given the high load (0.75 $Nm \bullet kg^{-1}$) and subsequent mechanical force required to complete maximal sprint cycling repetitions, the overload stimulus in this previous study may be considered resistance-like exercise that might promote a modest hypertrophy response with protein ingestion (Coffey et al. 2011). However, Breen and co-workers have also recently reported increases in rates of myofibrillar, but not mitochondrial, protein fractional synthetic rates when carbohydrate-protein was co-ingested compared to carbohydrate feeding alone following 90 min of steady state cycling at ~75% VO_{2peak} (Breen et al. 2011b). While some increase in muscle mass in untrained/sedentary individuals likely occurs with contractile overload per se (Harber et al. 2012), endurance exercise does not induce substantial hypertrophy (Hickson 1980, Wilson et al. 2012) and Breen and colleagues postulate that a potential mechanism for the increase in myofibrillar protein synthesis following prolonged endurance exercise and protein ingestion was repair and remodelling of muscle fibres. In contrast, Donges and co-workers (Donges et al. 2012) have reported an endurance exercise bout combined with post-exercise protein ingestion failed to increase myofibrillar protein synthesis above rest compared with a resistance exercise and concurrent training bout. Whether the myofibrillar synthetic response observed in the present study is exclusively the result of the resistance exercise or some interaction with the endurance exercise remains unclear. Regardless, the enhanced protein synthesis with protein ingestion is undoubtedly beneficial for retaining/augmenting muscle mass and promoting adaptation with concurrent training.

Results from this study also show variable rates of mitochondrial protein synthesis that failed to increase following the concurrent training bout with either treatment (Figure 4.7B). Previous studies in untrained or sedentary subjects have shown an increase in mitochondrial protein synthesis regardless of the mode of exercise i.e. resistance, endurance or concurrent exercise bouts (Burd et al. 2012, Donges et al. 2012, Wilkinson et al. 2008). Consequently, it is suggested the training status of subjects in the present study may have required a greater overload stimulus

to generate an acute increase in mitochondrial protein synthesis. Indeed, Breen and co-workers (Breen et al. 2011b) determined the effect of protein ingestion on muscle protein synthesis in well-trained cyclists and also failed to observe any effect on mitochondrial FSR. Rowlands and co-workers reported an enhanced mitochondrial transcriptome associated with protein ingestion following endurance exercise, an effect that was only evident late (48 h) but not early (3 h) in the post-exercise period (Rowlands et al. 2011). Therefore, it cannot be ruled out that quantification of mitochondrial protein synthesis later in recovery (e.g. 24 h) may have revealed differences in the adaptation response to exercise and protein ingestion.

The enhanced myofibrillar protein synthesis was associated with increases in the phosphorylation status of signalling proteins that regulate translation initiation and elongation. A similar time course for Akt-mTOR-S6K phosphorylation was shown in Chapter 2 during the early recovery period following single bouts of resistance exercise and cycling. Others have also previously shown endurance and resistance exercise in isolation activate the insulin/IGF signalling pathway (Benziane et al. 2008, Moore et al. 2009a). Collectively, these findings indicate specific translational processes in skeletal muscle are not an important factor determining the specificity of training adaptation. More recently, a concurrent training bout has been shown to enhance Akt/mTOR-mediated signalling responses (Lundberg et al. 2012, Wang et al. 2011). The results of the present study extend these findings by demonstrating that protein ingestion can augment Akt-mTOR-S6K phosphorylation following concurrent training (Figure 4.3). Consequently, it is contended that Akt-mTOR-S6K signalling may be indicative of nutrient sensitivity and/or muscle overload but fails to discriminate between divergent contraction stimuli. Exercise also generated a decrease in phosphorylation (activation) of the peptide chain elongation factor eEF2 although there were no differences between treatments indicating it may be unresponsive to protein ingestion (Figure 4.3D). Thus, nutrient-mediated increases in muscle protein synthesis following exercise are likely due in part to enhanced translation initiation rather than elongation.

The AMPK has been implicated in repressing anabolic signalling and protein synthesis in skeletal muscle via inhibition of mTOR-mediated signalling to initiate translation (Dreyer et al. 2008a, Gwinn et al. 2008). However, post-exercise increases in $AMPK^{Thr172}$ phosphorylation in the present study were modest and were concomitant with increases in mTOR phosphorylation (Figure 4.4A). This may reflect an inability of concurrent exercise session used in this work to

significantly disrupt cell energy status to a level required to modulate AMPK signalling despite changes in glycogen metabolism with exercise (Figure 4.4B) (Coffey et al. 2009b). Nonetheless, previously work from this lab (Coffey et al. 2009b) and others (Benziane et al. 2008, Dreyer et al. 2008a, Mascher et al. 2011, Wang et al. 2011) have previously failed to observe an AMPK-associated inhibition of translation initiation signalling or protein synthesis during recovery from exercise in human studies and such a causal relationship has yet to be clearly established in vivo human muscle.

A novel finding of the present study was the attenuated mRNA responses of genes associated with muscle proteolysis and catabolism. MuRF1 and Atrogin-1 mRNA expression was elevated above rest following the concurrent training bout, however this increase was attenuated with protein ingestion (Figure 4.5A and B). Harber and colleagues (Harber et al. 2010) have previously shown a similar effect on MuRF1 mRNA abundance with ingestion of a protein/carbohydrate supplement following 60 min of cycling and Borgenvik and co-workers (Borgenvik et al. 2012) demonstrated an amino acid-enriched beverage decreased MuRF1 protein levels at rest and after a resistance exercise bout. Therefore, coordinated attenuation in MuRF1 and Atrogin-1 expression with provision of exogenous amino acids may have provided substrate for muscle remodelling/hypertrophy that might otherwise be achieved through muscle breakdown following exercise in the fasted state. There were no differences between treatments in myostatin mRNA expression during the acute recovery period (Figure 4.5C). Reduced myostatin expression has been demonstrated following an acute bout of endurance (Louis et al. 2007) and resistance (Louis et al. 2007) exercise, and it appears myostatin mRNA expression is responsive to contraction per se rather than a specificity of training response and/or nutrient availability. There were comparable increases in mRNA abundance of metabolic/mitochondrial proteins following the consecutive resistance and endurance exercise bouts but protein ingestion failed to induce any noteworthy increase in PGC-1α, hexokinase or VEGF mRNA levels (Figure 4.6). Accordingly, while concurrent training is capable of generating an adaptive mRNA profile supportive of mitochondrial, metabolic and angiogenic processes in skeletal muscle, this response is not enhanced by amino acid provision.

In conclusion, the results of the present study demonstrate that protein ingestion after consecutive resistance and endurance exercise selectively increased rates of myofibrillar, but not

mitochondrial, protein synthesis in the early (4 h) recovery period. Protein ingestion also attenuated post-exercise increases in genetic markers associated with muscle proteolysis. Given endurance exercise interferes in strength/hypertrophy adaptation responses with concurrent training, these findings suggest protein intake can be beneficial following successive resistance and endurance exercise by promoting myofibrillar protein synthesis and decreasing ubiquitin ligase expression. Accordingly, post-exercise protein ingestion may ameliorate the potential "interference effect" of endurance exercise on muscle hypertrophy, and represents an important nutritional strategy for concurrent training.

Chapter Five

Summary and Conclusion

The overall aim of the studies undertaken for this thesis was to investigate the molecular adaptations regulating the specificity of training adaptation in response to divergent modes of exercise in human skeletal muscle. Nutrient ingestion (Chapter 2 and 3) and concurrent training (Chapter 3) were also employed to elucidate their capacity to modulate the adaptation response.

The first study (Chapter 2) compared the exercise-induced responses of Akt-mediated and AMPK signalling during the acute post-exercise recovery period following resistance and endurance exercise. Contractile activity results in the acute activation of signalling pathways that contribute to adaptation responses in human skeletal muscle. However, resistance exercise is characterised by high-intensity, short-duration contractions compared to the prolonged, low-intensity contractions with endurance exercise. These differences in contractile overload are also associated with the development of divergent phenotypes. Therefore, the hypothesis of this study was that the differences in contraction mode would generate a disparity in acute post-exercise cell signalling to match adaptation processes promoted by each exercise stimuli. Specifically, prolonged, moderate-intensity contraction associated with endurance cycling would selectively initiate AMPK and Akt-mediated signalling specific for glucose transport (AS160) and glycogen resynthesis (GS) with small effects on translation signalling. In contrast, resistance exercise would have negligible effects on Akt-mediated substrate/metabolic signalling, but selectively promote phosphorylation of signalling proteins regulating translation initiation to mediate increases in protein synthesis. Contrary to this hypothesis, a similar time course for Akt-mTOR-p70S6K signalling was observed during the acute 60 min recovery period despite the divergent contractile activity between exercise modes. However, as expected, selective increases in phosphorylation responses for proteins promoting glucose transport (AS160) and glycogen resynthesis (GS) only occurred with endurance exercise. Collectively, results from this study indicate that peak phosphorylation for many of the proteins of the Akt signalling cascade in the acute recovery period occurs 30 - 60 min after both resistance and endurance exercise in the fasted state. This finding has several important implications that enhance the current understanding of the specificity of adaptation responses with resistance and endurance exercise in human skeletal muscle.

Firstly, in the context of *in vivo* human skeletal muscle there is no purported ‘AMPK-Akt’ switch following contrasting high-intensity, short-duration and prolonged, low-intensity contractile

stimuli, at least in the acute post-exercise recovery period. Therefore, acute post-exercise increases in Akt-mTOR-S6K signalling are indicative of muscle overload per se rather than a progression towards a hypertrophy phenotype. Thus, the similar time course observed with resistance and endurance exercise shows translational signalling responses in human skeletal muscle lacks sensitivity to divergent exercise stimuli and is not an important factor determining the specificity of training adaptation. However, these acute post-exercise signalling responses intuitively contribute to translation initiation for gene expression that, when repeated over time, generate the specific exercise-induced phenotype associated with chronic training. Specifically, the early translation signalling may be a rate limiting step in the synthesis of myofibrillar and mitochondrial proteins with resistance and endurance exercise, respectively. Nonetheless, AMPK, AS160, GSK-β and GS phosphorylation were selectively phosphorylated with endurance exercise. The greater glycogen depletion following endurance compared to resistance exercise may promote a specificity of adaptation with endurance exercise.

A novel finding from this study was also elucidating appropriate post-exercise time points for analysis of exercise-induced Akt-mediated cell signalling. Previous studies have sought to characterise contraction-induced changes in cell signalling in human skeletal muscle but differences in the timing of post-exercise biopsy sampling, among other factors, has contributed to the inconsistency in Akt-mediated signalling in skeletal muscle after exercise. The results from this study provide the first evidence that 30 - 60 min after exercise in the fasted state (endurance or resistance) is the most appropriate time for detecting changes in phosphorylation during the early recovery period.

In light of the similar translational signalling time courses when endurance and resistance exercise are performed in isolation, a logical progression was to examine how combining these two exercise modes would influence exercise-induced adaptation responses (Chapters 3 and 4). This multi-faceted proposition has relevance to many activities, particularly team sports, which require a combination of hypertrophy/strength and endurance/aerobic capacity. The second study (Chapter 3) determined the effects of resistance exercise undertaken with low muscle glycogen on markers of translation initiation and myofibrillar protein synthesis and the impact of protein-carbohydrate co-ingestion during recovery. The hypothesis was that low glycogen concentration

would suppress the muscle anabolic response to resistance exercise but nutrient provision in the early recovery period would restore muscle anabolism and promote hypertrophy.

This study provides novel data showing anabolic signalling and rates of myofibrilar protein synthesis are not altered during the acute recovery period following resistance exercise commenced with low muscle glycogen concentration. This was unexpected given muscle protein synthesis is an energy consuming process and a low glycogen state was expected to suppress activity of ATP-requiring processes at the expense of higher priority cellular activities such as glycogen resynthesis. Moreover, protein-carbohydrate ingestion was anticipated to 'rescue' any attenuation in the anabolic response to resistance exercise in a low glycogen state by providing exogenous substrate for glycogen resynthesis and muscle protein synthesis. Thus, the muscle protein synthesis machinery functions independently of alterations in muscle glycogen concentration and there is no additive nutrient-mediated anabolic adaptation when glycogen concentration is low.

The results from this study provide 'real world' information on the impact and interaction of glycogen status and training periodisation. Commencing resistance exercise with low or sub-optimal glycogen concentration is likely to be a common occurrence particularly for individuals undertaking concurrent training and/or multiple high-intensity exercise bouts in a day. This study provides the first evidence that anabolic adaptations to a high intensity, low volume resistance exercise bout are not compromised by suboptimal muscle glycogen availability. It is often debated when undertaking concurrent resistance and endurance exercise which exercise order should be prioritised (i.e.: which mode performed first) in a typical training session/day and what is sufficient recovery time between training sessions. This study shows the performance of endurance or other glycogen-depleting activity before resistance exercise does not attenuate rates of myofibrillar protein synthesis provided the required volume/intensity of resistance exercise can be completed, although an overnight recovery period (or equivalent time) may be necessary. In addition, despite no interaction between nutrient ingestion and low glycogen, the findings from this study still support the use of post-exercise ingestion of protein and carbohydrate to enhance myofibrillar protein synthesis and glycogen resynthesis. An intriguing consideration is whether differences in the response to resistance exercise with altered glycogen availability are manifest through changes in muscle protein breakdown. Higher atrogin-1 and myostatin mRNA

abundance in the low glycogen state was observed compared with normal glycogen levels after 4 h recovery in the nutrient group. Whether increases in markers of muscle protein breakdown following exercise reflect the adaptation response necessary for remodelling or repair of the muscles contractile protein, or is elevated due to inflammation/damage and results in net protein breakdown, is unknown.

The final study (Chapter 4) investigated the acute molecular response following a concurrent training bout and the effects of protein ingestion on rates of myofibrillar and mitochondrial protein synthesis. The hypothesis was the anabolic effects of protein ingestion would override any incompatibility and enhance metabolic and anabolic adaptation responses to concurrent training in the early recovery period. This study is the first to show higher rates of myofibrillar protein synthesis with protein compared to placebo ingestion following a concurrent training bout and that rates of mitochondrial protein synthesis were unchanged in the early recovery period.

The selective increase in myofibrillar protein synthesis likely relates to the large size (~ 60%) of this protein pool and may be the preferential site of disposal for free amino acids with protein ingestion. Equally, the lack of response in rates of mitochondrial protein synthesis following a concurrent training bout may be explained by the low volume of endurance exercise employed (30 min at ~ 70% $V{O_2}_{peak}$) and the training status of the subjects requiring a greater overload stimulus to increase mitochondrial protein synthesis. Greater amounts of protein (i.e.: > 20g) may also need to be ingested to generate an increase in rates of mitochondrial protein synthesis. It is also possible the quantification of mitochondrial protein synthesis during a longer recovery (i.e.: 24 - 48 h) may reveal differences in the adaptation response to exercise and protein ingestion.

The novel finding that rates of myofibrillar synthesis are increased with protein supplementation compared to placebo during the acute post-exercise recovery period following consecutive resistance exercise and cycling suggest the anabolic adaptations to resistance exercise are retained despite a subsequent bout of endurance exercise. Nonetheless, this finding has significant practical importance for individuals undertaking concurrent training that may have previously segregated resistance- and endurance-based training to avoid detrimental effects on hypertrophy and strength. Therefore, this study provides the first mechanistic evidence that post-exercise protein ingestion may have the capacity to ameliorate any potential ‘interference effect’ of endurance training on muscle hypertrophy. Accordingly, protein ingestion is an important

nutritional strategy during the early recovery period for increased muscle size and strength adaptations with concurrent training. Such information also has important implications for clinical populations seeking to simultaneously maintain/ increase muscle mass and enhance cardio-respiratory health.

This study also provided new information showing protein ingestion may be beneficial for hypertrophy following successive resistance and endurance exercise by decreasing ubiquitin ligase expression and subsequent rates of muscle protein breakdown. The reduction in ubiquitin ligase expression shows protein ingestion may reduce muscle protein breakdown with the potential to support maintenance of muscle mass with concurrent training. The increases in PGC-1α, hexokinase and VEGF mRNA abundance following concurrent exercise suggest the adaptive phenotype resulting from combined resistance and endurance exercise promotes metabolic gene expression. Interestingly, protein ingestion failed to substantially enhance the expression profile of PGC-1α and VEGF mRNA. Thus, in contrast to the expression profile of genes related to muscle catabolism, these metabolic genes appear to be insensitive to exogenous amino acid provision and are regulated exclusively through exercise-induced muscle contraction.

In summary, the studies undertaken for this thesis provide novel information regarding the effect of exercise mode, glycogen availability, nutrient supplementation and concurrent training on the molecular mechanisms involved in the specificity of training adaptation. Several questions remain that should be addressed by future work. For example, Akt-mediated translational signalling lacks sensitivity to divergent exercise stimuli, however the expression profile of other as yet uncharacterised signalling proteins may be an important factor determining the specificity of training adaptation (Chapter 2). It is unclear if alternate resistance exercise protocols incorporating different contraction volumes and intensities can generate a more pronounced effect of glycogen availability on the anabolic response in skeletal muscle (Chapter 3). In addition, future research should investigate if chronic concurrent training (~10 - 12 weeks) combined with protein ingestion attenuates the ‘interference’ by endurance training on hypertrophy and subsequent strength adaptations by comparing molecular and functional measures of adaptation responses (i.e.: correlation between changes in rates of muscle protein synthesis to changes in one repetition maximum or accrual of lean mass) to resistance exercise undertaken in isolation (Chapter 4). Similarly, defining the relative importance of the length of

recovery time (i.e.: 15 mins to 8 h) between exercise bouts during concurrent training will be a critical factor to inform training practice and in determining ‘interferences’ in adaptation (Chapter 4).

Resistance and endurance exercise assume opposite positions on a theoretical adaptation continuum by virtue of their divergent biochemical and morphological adaptations and resultant phenotype in skeletal muscle. Nonetheless, the exact molecular determinants underpinning this specificity in adaptation remain elusive and likely involve the contribution of numerous putative contraction-induced molecular responses including differences in muscle protein synthesis and breakdown, transcriptome expression, substrate metabolism, and muscle fibre type activation. The continued discovery of novel molecular targets and pathways regulating the specificity of training adaptation will ultimately assist in the development of innovative and personalised training strategies to improve athletic performance, and may also be extrapolated to clinical populations requiring effective treatment for skeletal muscle diseases.

Chapter Six

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Appendix I

Appendix II