Autophagy and Protein Turnover Responses to Exercise-Nutrient Interactions in Human Skeletal Muscle

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Statement of Authorship and Sources

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 program and any editorial work, paid or unpaid, carried out by a third party is acknowledged, and ethics procedures and guidelines have been followed. Unless otherwise stated, all work comprising of this thesis has been undertaken by the candidate. This candidature commenced at Royal Melbourne Institute of Technology (RMIT) University. The candidate subsequently transferred to the Australian Catholic University (ACU) in January of 2014 following the move of the Principal and Co-Supervisors.

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It is acknowledged that the work comprising this thesis was supervised by the following ACU staff members:

Principal Supervisor: Prof. John A Hawley (ACU, Fitzroy, VIC / RMIT University, Bundoora, VIC)

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Romans 8

^{1}*Therefore, there is now no condemnation for those who are in Christ Jesus, ^{2}because through Christ Jesus the law of the Spirit who gives life has set you free from the law of sin and death... ^{38}For I am convinced that neither death nor life, neither angels nor demons, neither the present nor the future, nor any powers, ^{39}neither height nor depth, nor anything else in all creation, will be able to separate us from the love of God that is in Christ Jesus our Lord.*

Table of Contents

Publications

Publications arising directly from this thesis

1. **Smiles WJ**, Areta JL, Coffey VG, Phillips SM, Moore DR, Stellingwerff T, Burke LM, Hawley JA, Camera DM. Modulation of autophagy signalling with resistance exercise and protein ingestion following short-term energy deficit. **Am J Physiol Regul Integr Comp Physiol. 2015 Sep; 309 (5).**

2. **Smiles WJ**, Parr EB, Coffey VG, Lacham-Kaplan O, Hawley JA, Camera DM. Protein co-ingestion with alcohol following strenuous exercise attenuates alcohol-induced intramyocellular apoptosis and inhibition of autophagy. **Am J Physiol Endocrinol Metab. 2016 Nov 1; 311 (5).**

3. **Smiles WJ**, Hawley JA, Camera DM. Effects of skeletal muscle energy availability on protein turnover responses to exercise. **J Exp Biol. 2016 Jan; 219 (Pt 2).**

Book chapter arising directly from this thesis

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1. **Smiles WJ**, Conceição MS, Telles GD, Chacon-Mikahil MP, Cavaglieri CR, Vechin FC, Libardi CA, Hawley JA, Camera DM. Acute low-intensity cycling with blood-flow

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1. **Smiles WJ** and Camera DM. The Guardian of the Genome p53 Regulates Exercise-Induced Mitochondrial Plasticity: Plausible Role for p53 in the Concurrent Training Interference Effect on Muscle Hypertrophy. **Sports Med.**

2. Camera, DM, Burniston, JG, Pogson, MA, **Smiles WJ**, Hawley JA. Dynamic Proteome Profiling of Individual Proteins in Human Skeletal Muscle. **FASEB.**

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Conference Presentations

1. **Smiles WJ**, Van Loon LJ, Hawley JA, Camera DM. The Effects of High-Fat Availability on Exercise- and Protein-Induced Muscle Protein Turnover. **Cell Symposia, Exercise Metabolism** – Gothenburg, 2017.

2. **Smiles WJ**, Parr EB, Coffey VG, Lacham-Kaplan O, Hawley JA, Camera DM. Protein co-ingestion with alcohol following strenuous exercise attenuates alcohol-induced intramyocellular apoptosis and inhibition of autophagy. **Exercise and Sport Science Australia, Research to Practice** – Melbourne, April 2016.

3. **Smiles WJ**, Areta JL, Coffey VG, Phillips SM, Moore DR, Stellingwerff T, Burke LM, Hawley JA, Camera DM. Modulation of autophagy signalling with resistance exercise and protein ingestion following short-term energy deficit. **Cell Symposia, Exercise Metabolism** – Amsterdam, 2015.

List of Figures

List of Tables

Abbreviations

4E-BP1	eukaryotic initiation factor 4E-binding protein-1
ACC	acetyl-CoA carboxylase
ADP	adenosine diphosphate
AIF	apoptosis-inducing factor
ALC	alcohol
AMPK	adenosine monophosphate-activated protein kinase
AS160	Akt substrate of 160 kDa
Atg	autophagy-related gene
ATP	adenosine triphosphate
ATPAF1	F_1-ATP synthase complex assembly factor 1
AUC	area under the curve
BAG3	Bcl-2-associated athanogene 3
Bax	Bcl-2-associated X protein
BCAA	branch chain amino acid
Bcl-2	B-cell lymphoma 2
BECN-1	beclin-1
BM	body mass
BNIP3	Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3
Ca^{2+}	calcium
CaMK	calcium/calmodulin-dependent protein kinase
CaN	calcineurin
CARM1	coactivator-associated arginine methyltransferase-1
CASA	chaperone-assisted selective autophagy
CHO	carbohydrate
CLEAR	Coordinated Lysosomal Expression and Regulation
CMA	chaperone-mediated autophagy
COX	cytochrome c oxidase
DAG	diacylglycerol
Deptor	Dep domain-containing protein interacting with mTOR
DGKζ	DAG kinase ζ
DRP1	dynamin-related protein 1
EB	energy balance
ECM	extracellular matrix
ED	energy deficit
eEF	eukaryotic elongation factor
EGR-1	early growth response-1
eIF	eukaryotic initiation factor
END	endurance exercise
ER	endoplasmic reticulum
ERK	extracellular signal-regulated protein kinase
ETC	electron transport chain
FA	fatty acid
FFA	free fatty acid
FFM	fat-free mass

FIP200	FAK-interacting protein of 200 kDa
FLNC-γ	filamin C-γ
FOXO	Forkhead box transcription factor
GABARAP	γ-aminobutyric-acid-type-A-receptor-associated protein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GDP	guanosine diphosphate
GLUT4	glucose transporter 4
GSH	reduced glutathione
GSSG	oxidised glutathione
GTP	guanosine triphosphate
H2B	histone 2B
HFD	high-fat diet
IL-6	interleukin-6
IMTG	intramuscular triglyceride
IRS1	insulin receptor substrate 1
LC3b	microtubule-associated protein 1 light chain 3b
LIR	LC3-interacting region
LKB1	liver kinase B1
LRS	leucyl-tRNA synthetase
MaFbx	muscle atrophy F-box
mLST8/GβL	mammalian lethal with SEC13 protein 8/G protein β-like-subunit protein
MPB	muscle protein breakdown
MPS	muscle protein synthesis
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial DNA
mTOR	mammalian target of rapamycin
MuRF-1	muscle-specific RING finger-1
NAD	nicotinamide adenine dinucleotide
NEDD4	neural precursor cell expressed, developmentally downregulated 4
NEFA	non-esterified (free) fatty acids
NRF-1	nuclear respiratory factor-1
OMM	outer mitochondrial membrane
OPA1	optic atrophy 1
p38 MAPK	p38 mitogen-activated protein kinase
p70S6K	ribosomal protein s6 p70 kinase
PA	phosphatidic acid
PARP1	poly (ADP-ribose) polymerase 1
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator-1α
P_i	inorganic phosphate
PI3K	phosphatidylinositol-3-kinase
PI3KC3	phosphatidylinositol 3-kinase protein complex
PI3P	phosphatidylinositol 3-phosphate
PIC	pre-initiation complex
PINK1	PTEN-induced putative kinase 1
PLA	placebo
PLD1	phospholipase D1

PLIN	perilipin
PPARγ	peroxisome proliferator-activated receptor-γ
PRAS40	proline-rich Akt substrate of 40 kDa
PRO	protein
PTEN	phosphatase and tensin homologue
PUMA	p53-upregulated modulator of apoptosis
Raptor	regulatory associated protein of mTOR
REX	resistance exercise
Rheb	Ras homologue enriched in brain
Rictor	rapamycin-insensitive companion of mTOR
RM	repetition maximum
ROS	reactive oxygen species
rpS6	ribosomal protein S6
RT-PCR	reverse transcription and real-time Polymerase Chain Reaction
SCO2	synthesis of cytochrome c oxidase 2
SD	standard deviation
SEM	standard error of the mean
Tfam	mitochondria transcription factor A
TFEB	transcription factor EB
TNFα	tumour necrosis factor-α
TOP	terminal oligopyrimidine tract
tRNA	transfer ribonucleic acid
TSC	tuberous sclerosis complex
ULK1	Unc-51-like kinase 1
UPR	unfolded protein response
UPS	ubiquitin proteasomal system
v-ATPse	vacuolar H^+-ATPase
VDAC1	voltage-dependent anion channel 1
VEGF	vascular endothelial growth factor
$\dot{V}O_{2peak}$	peak oxygen uptake
Vps34	vacuolar protein sorting 34
W	watts
yr	year

Abstract

Skeletal muscle is a dynamic tissue comprising the largest protein reservoir of the human body with a rate of turnover of ~1-2% per day. Protein turnover is regulated by the coordination of intracellular systems regulating protein synthesis and breakdown that converge in a spatiotemporal manner on lysosomal organelles responsible for integrating a variety of contractile and nutritional stimuli. One such system, autophagy, which literally means to 'self-eat,' involves capturing of cellular material for deliver to, and disintegration by, the lysosome. The autophagic 'cargo' is subsequently recycled for use in synthetic reactions and thus maintenance of protein balance. As a dynamic system, autophagy responds to intracellular perturbations to homeostasis elicited by exercise and changes in nutrient availability in an attempt to restore energy balance. However, little is known regarding how autophagy is modulated following exercise in response to changes in nutrient availability. This thesis is comprised of three independent studies in which the effects of divergent forms of exercise-nutrient interactions are investigated in relation to autophagy-mediated protein turnover processes in skeletal muscle.

Study 1 assessed whether resistance-based exercise undertaken following a short-term period of dietary energy restriction activates autophagic cell signalling, and whether high-protein availability during recovery from exercise attenuates the autophagic response. This latter supposition was based on the anabolic properties of amino acids that may temporarily repress autophagy *in vitro*. In contrast to one of the original hypotheses, protein availability promoted the largest accumulation of proteins implicated in the induction of skeletal muscle autophagy and thus, turnover-remodelling, which is required to support the elevated synthetic demands imposed by resistance exercise contraction in a state of energy deficit.

Study 2 investigated the effects of alcohol intoxication during recovery from vigorous exercise on autophagy and whether concomitant protein availability could 'rescue' alcohol-exposed muscle tissue from the toxic effects of alcohol metabolism. It was hypothesised that the largest autophagic response (e.g., the accumulation of specific autophagy-related proteins in different subcellular compartments) would be seen when alcohol with carbohydrate, but not protein, was co-ingested, thereby promoting greater rates of protein degradation. However, the results from this study showed that alcohol availability consistently attenuated the abundance of numerous autophagy-related proteins that culminated in cell death responses. Protein availability, in part, through a compensatory induction of mitochondrial biogenesis, facilitated 'sparing' of the alcohol-exposed tissue from these deleterious effects of alcohol metabolism, thus revealing the intrinsic anti-apoptotic effects of exogenous protein. While excess alcohol consumption should be avoided following sport and/or exercise training, protein co-ingestion may relieve some of the intracellular damage and facilitate recovery-adaption.

Study 3 investigated the impact of acutely elevating systemic fatty acid availability and its impact on skeletal muscle protein turnover. Participants received either a lipid infusion with or without the addition of exercise, or a saline control, and following these infusions ingested a bolus amount of protein. It was hypothesised that lipid availability would attenuate markers of cellular anabolism (i.e., translation initiation) that would be ameliorated by exercise. Whereas the lipid infusion alone induced an elevated autophagic flux, combining the lipid infusion with exercise inhibited this activation of autophagy and, in response to protein ingestion, promoted the largest intracellular anabolic protein translational response. In addition, exercise performed during the lipid infusion resulted in a novel mitochondria-specific autophagic response independent of canonical routes of autophagic degradation. Therefore, the anabolic sensitivity of

skeletal muscle to protein ingestion, despite high-circulating free fatty acids, was 'rescued' by strenuous exercise performed during this infusion and was associated with the disposal of mitochondrial organelles presumably damaged by lipid availability. Combining strenuous exercise with high-protein availability in the context of excess circulating lipids is a powerful stimulus for promoting muscle protein turnover-remodelling.

Taken collectively, the results from this thesis demonstrate that nutrient availability alters the responsiveness of skeletal muscle protein turnover, in particular autophagy, to exercise stimuli. The optimal nutrient 'pairing' with exercise in regards to optimising muscle quality and quantity for athletes and non-athletes alike, in the context of dietary energy restriction or excess alcohol and fat availability, is the consumption of high-quality sources of protein.

Chapter One

1 Introduction and Overview

Skeletal muscle is a dynamic tissue accounting for approximately 40% of body mass (BM) and is the body's largest protein reservoir, with a turnover rate of ~1-2% each day (Wagenmakers 1998). Aside from an obligatory role in locomotion, skeletal muscle is the major site of glucose disposal accounting for up to 80% of post-prandial glucose load and therefore, is integral to whole-body metabolic homeostasis (Hawley & Lessard 2008). Skeletal muscle displays remarkable plasticity and can alter its morphological and metabolic characteristics in response to external environmental stimuli such as habitual levels of contractile activity and prevailing substrate availability (Coffey & Hawley 2007; Hawley et al. 2014; Egan & Zierath 2013; Fluck & Hoppeler 2003). Exercise perturbs intracellular homeostasis that may cause transient fatigue with the potential to damage working muscle tissue. However, application of appropriate recovery strategies and reinforcement by subsequent exercise bouts induces skeletal muscle adaption by elevating the steady-state abundance of specific intracellular proteins and organelles that respond specifically to the imposed demands. Such a postulate is consistent with the hypotheses of the Canadian endocrinologist Hans Selye. More than 70 years ago, Seyle promulgated the mammalian general adaptation syndrome, suggesting that a given stressor triggers an alarm reaction (activating stress-sensing enzymes), followed by an adaptive remodelling period (biosynthesis of cellular constituents) and ultimately resistance against the imposed demands (augmented homeostasis and exercise capacity) (Selye 1946).

Manipulation of nutrient availability modulates exercise adaptation responses (Hawley et al. 2011), conferring a significant effect on chronic training-induced changes in muscle phenotype (Cermak et al. 2012). Exercise-nutrient interactions are sufficient to alter the activation status of

cellular signalling cascades that transduce a given perturbation to the nucleus, activating gene transcription programmes and specific mRNA translational responses. For example, several studies have shown that altering carbohydrate availability either before or after endurance-based exercise amplifies signalling cascades that improve the ability to oxidise carbohydrate- and fat-based fuels (Lane et al. 2015; Psilander et al. 2013; Camera et al. 2015; Starkie et al. 2001). Similarly, prior resistance-based exercise markedly sensitises the working muscle to the anabolic (i.e., protein-synthesising) properties of amino acids contained within ingested proteins (Burd et al. 2011; Moore et al. 2009).

Adaptation to exercise involves intricate maintenance of skeletal muscle protein homeostasis ("proteostasis") that incorporates crosstalk between biological pathways regulating protein synthesis, breakdown, trafficking and folding. Exercise training essentially fine-tunes these processes as part of the adapting cellular environment (Coffey & Hawley 2007; Hawley et al. 2014). Autophagy, derived from the Greek to "self-eat", is a rate-limiting pathway for maintaining proteostasis and involves the lysosome-dependent degradation of cellular components such as protein macromolecules and nutrient substrate. Autophagy is constitutively active, but engaged in a dynamic manner by energy stress stimuli such as fasting and/or amino acid deprivation (Mizushima & Klionsky 2007). As such, autophagy has emerged as a crucial regulator of exercise response-adaptation processes (Vainshtein & Hood 2016). However, in contrast to the large body of literature demonstrating the sensitivity of exercise-induced muscle protein synthesis (MPS) to specific nutrients (Hawley et al. 2011), little is known regarding how exercise-nutrient interactions alters processes associated with autophagy activation (i.e., rates degradative of flux), especially in human skeletal muscle.

While training-induced protein accumulation depends on the type, frequency, intensity and/or volume of a given contractile stimulus, the rate at which these proteins are synthesised and degraded is a direct function of their half-life. Hence, proteins with shorter half-lives (e.g., contractile) are generally turned over more rapidly than proteins with longer (e.g., mitochondrial) half-lives. Because autophagy is responsible for cellular "recycling", reduced constituents of its degradation can be subsequently utilised in anabolic processes. Thus, it is conceivable that any exercise-stimulated MPS response is dependent, in part, on autophagy-mediated recycling of longer-lived proteins, particularly those damaged by contraction-provoked mechanical and/or oxidative stresses (Phillips et al. 1997). For example, selective autophagic degradation of the actin crosslinking protein, filamin C-γ, that mechanically undergoes conformational changes during contraction (Rognoni et al. 2012), is complemented by simultaneous synthesis processes to replenish the degraded protein (Ulbricht et al. 2013a; Ulbricht et al. 2015; Kathage et al. 2017). In addition, MPS is an energy-consuming process (discussed subsequently) and thus autophagy likely partitions requisite energy substrate to sustain prolonged (>12 h) increases in MPS following acute exercise (Phillips et al. 1997; MacKenzie et al. 2009).

Divergent exercise stimuli promote the development of distinct phenotypic characteristics. Specifically, high-force resistance-based exercise promotes the synthesis of contractile myofibrillar proteins leading to hypertrophy and improvements in strength (force-generating) capacity (Coffey & Hawley 2007). In contrast, endurance-based exercise stimulates mitochondrial volume-density (biogenesis) to enhance the oxidation of carbohydrate and fat-based fuels (Egan & Zierath 2013; Coffey & Hawley 2007; Camera et al. 2016). It is therefore not surprising that equally divergent forms of autophagic pathways have been discovered in skeletal muscle that can localise to skeletal muscle sites of force transmission and/or

mitochondria (Ulbricht et al. 2013a; Ulbricht et al. 2013b; Kim et al. 2007). Moreover, the terminal point of autophagic breakdown, the lysosome, is a carrier of glycosidase, protease and lipase enzymes that thrive in the intraluminal acidic environment of the lysosome for selective catabolism of carbohydrates, proteins and fat, respectively (Singh & Cuervo 2011). Hence, autophagy is intimately positioned at the crossroads of cellular metabolism and protein turnover and represents a central biological hub integrating contractile and nutritional cues.

The work for this thesis comprises three independent studies that have examined the role of divergent exercise and nutritional stimuli on modulating autophagy and protein turnover responses in human skeletal muscle.

Study 1: The effects of resistance-based exercise on intracellular autophagy signalling after short-term energy restriction (Chapter 4).

Study 2: The effects of post-exercise alcohol ingestion on autophagy and apoptotic cell signalling with altered protein availability (Chapter 5).

Study 3: The effects of high-fat availability on exercise- and protein feeding-induced anabolic and autophagy signalling (Chapter 6).

Chapter Two

2 Literature Review

This chapter has been adapted from the following published reviews:

1. Camera DM and **Smiles WJ**. Autophagy, exercise and lifestyle modification. In: *Autophagy and Cardiometabolic Diseases: From Mechanisms to Molecules to Medicine* (Invited Review). Elsevier; Accepted for publication.

2. Camera DM, **Smiles WJ**, Hawley JA. Exercise-induced skeletal muscle signalling pathways and human athletic performance. *Free Radic Biol Med*. 2016 Sep; 98: 131-43.

3. **Smiles WJ**, Hawley JA, Camera DM. Effects of skeletal muscle energy availability on protein turnover responses to exercise. *J Exp Biol*. 2016 Jan; 219 (Pt 2): 214-25.

Introduction

Muscle protein turnover is regulated by several biological systems that coordinate protein synthesis and breakdown. Regulation of protein balance is largely an intrinsic process with several intracellular systems synergistically impacting synthesis, breakdown and folding of proteins. These intracellular systems, which include, but are not limited to, the protein translational machinery, the ubiquitin proteasome system (UPS) and autophagy are all inextricably linked to energy availability and can thus mediate exercise-induced adaptation responses. The purpose of this literature review is 1) to provide an overview of the biological regulation of protein turnover, 2) how these systems regulate exercise training adaptation, 3) the interactive effects of nutrient availability, and 4) highlight gaps in the literature and areas for future investigation.

2.1 Muscle Protein Synthesis

The initial step in intracellular protein synthesis is the transcription of specific genetic sequences of amino acids encoded in DNA into messenger RNA (mRNA). Amino acids containing this message are translated into proteins via sequential reactions of translation initiation, elongation and termination of the peptide chain. In terms of the cellular energy requirements of this process, the elongation step consumes the majority of energy required for protein synthesis, utilising four high-energy phosphates per peptide bond (Merrick 1992). Protein synthesis of skeletal muscle tissue is comprised predominantly of the myofibrillar protein pool. Myofibrils are highly organised cylindrical structures consisting of specialised contractile units termed sarcomeres. Incorporation of nascent myofilament proteins actin and myosin into sarcomeres requires concomitant synthesis of several giant molecular scaffolds including, but not limited to, titin,

nebulin and obscurin (Kontrogianni-Konstantopoulos et al. 2009). Therefore, given that the ATP cost of protein synthesis is largely dictated by peptide chain elongation, proteins with longer peptide chains would impose greater corresponding synthesis-energy demands, thereby accounting for the high-energy demands required to maintain skeletal muscle mass (Pasiakos et al. 2010).

The molecular events surrounding protein translation have been characterised extensively at the preceding steps of translation initiation, which involves formation of two regulatory complexes regulated by eukaryotic initiation factors (eIFs). In brief, eIF2·GTP·Met-tRNAi ternary complexes recruit small 40S ribosomal subunits to form the 43S preinitiation complex (PIC), while eIF4F complexes are involved in binding to mRNA and assembling with the PIC, where the mRNA is scanned by ribosomes to locate the translation start site (recognition of an AUG start codon) (Kimball & Jefferson 2010; Kapp & Lorsch 2004). Formation of a translation-competent 80S ribosome (required for peptide elongation) requires eIF2-bound GTP hydrolysis and its detachment from the ribosome. For subsequent rounds of translation, the GTP on eIF2 is recycled by eIF2B (Wang & Proud 2008). Indeed, these processes are related to the energy state of the cell and regulated by several upstream kinases sensitive to energy availability, in particular the mechanistic (or mammalian) target of rapamycin complex 1 (mTORC1)/AMP-dependent protein kinase (AMPK) signalling pathways.

mTORC1/AMPK Signalling

mTOR is a serine/threonine protein kinase that nucleates two functionally distinct multi-protein complexes (mTORC1 and mTORC2) with contrasting sensitivities to the immunosuppressive

drug rapamycin. mTORC1 is the principal regulator of protein translation and is highly rapamycin-sensitive. mTORC1 contains the regulatory subunit of mTOR, Raptor, as well as a positive regulator of mTOR kinase activity mLST8/GβL (mammalian lethal with SEC13 protein 8/G protein β-like-subunit protein). Two negative regulators exist within mTORC1, including Deptor (Dep domain-containing protein interacting with mTOR) and PRAS40 (proline-rich Akt substrate of 40 kDa) (Goodman 2014). mTORC2, which is primarily responsible for remodelling of the actin cytoskeleton and facilitating growth factor signals (e.g., insulin-stimulated phosphorylation of Akt at the Ser^{473} residue), is largely resistant to rapamycin treatment and replaces Raptor in its complex with the rapamycin-insensitive companion of mTOR (Rictor) (Laplante & Sabatini 2012; Zoncu et al. 2011). Two well-characterised downstream substrates of mTORC1, ribosomal protein s6 (rps6) p70 kinase (p70S6K) and eIF4E-binding protein 1 (4E-BP1) orchestrate numerous signals arising from elevated mTORC1 activity. p70S6K has a number of substrates contributing to varying steps of protein translation and has been implicated in the transcriptional regulation of ribosome biogenesis, thereby augmenting translational capacity (Chauvin et al. 2014). 4E-BP1 phosphorylation results in the formation of an eIF4F complex that preferentially promotes translation of mRNAs encoding for translation factors and ribosomal proteins that contain a terminal oligopyrimidine (TOP) tract in their 5′-untranslated region (Thoreen et al. 2012).

Activation of mTORC1 is linked to numerous energetic and hormonal signals (e.g., growth factors, amino acids and mechanical stimuli) that converge on the lysosome (Figure 2.1). mTORC1 signalling is activated by Ras-homologue enriched in brain (Rheb), a small GTPase that when GTP-bound has direct affinity for mTOR and stimulates its kinase activity (Inoki et al. 2003a). Tuberin, or TSC2, is a protein that cycles on and off the lysosome and is a negative

regulator of Rheb and thus mTORC1 (Inoki et al. 2003a; Inoki et al. 2003b). Growth factors, such as the insulin-induced Akt signalling pathway (illustrated schematically in Figure 2.1), trigger lysosomal dissociation of TSC2 and mTORC1 activation (Menon et al. 2014; Li et al. 2002). In contrast to the action of growth factors, amino acids, through a proposed "inside-out" mechanism, accumulate inside the lysosomal lumen and control tethering of mTORC1 to the lysosomal surface by Rag GTPases and the v-ATPase-Ragulator complex (Sancak et al. 2008; Sancak et al. 2010). Particular emphasis has been placed on the anabolic properties of the branched-chain amino acid (BCAA) leucine, which, owing to its ability to independently activate mTORC1, is regarded as a nutrient trigger for stimulating protein synthesis (Wolfson et al. 2016). Another mTORC1 activator, the glycerophosholipid phosphatidic acid (PA), binds directly to mTOR and has been shown to enhance mTORC1 signalling in response to amino acid availability (Yoon et al. 2016) and high-force resistance-like contraction (You et al. 2012; You et al. 2014). The latter exercise stimulus can also promote TSC2 lysosomal dissociation that, like amino acid-induced activation of Rheb/mTORC1, is Akt-independent (Jacobs et al. 2013; Jacobs et al. 2017). Thus, the optimal stimulus for activating mTORC1 is high-force contractile activity combined with the ingestion of BCAA-enriched protein sources (Figure 2.1).

Perturbations to metabolic homeostasis activates the intracellular "metabolic fuel gauge" AMPK, which upregulates catabolic ATP-synthesising processes and attenuates anabolic ATP-consuming processes (Hardie et al. 2012). AMPK exists in heterotrimeric complexes consisting of catalytic α-subunits (α1, α2) and two regulatory subunits (β1, β2 and γ1, γ2, γ3) (Hardie et al. 2012). Energy stress that elevates the AMP/ADP: ATP ratio upregulates AMPK activity by phosphorylation of Thr^{172} in its catalytic α-subunit activation loop from upstream kinases such as liver kinase B1 (LKB1) (Suter et al. 2006). In addition, high-rates of glycogenolysis upregulate

AMPK activity via its β-subunits that bind endogenous carbohydrate in the basal state (McBride et al. 2009). AMPK propagates signal transduction to repress protein synthesis by activating Sestrin-2, the leucine-sensitive negative regulator of mTORC1, or by phosphorylating TSC2 and Raptor, thereby upregulating energy-harvesting systems to restore homeostasis (Inoki et al. 2006; Inoki et al. 2003b; Gwinn et al. 2008; Browne et al. 2004; Zhang et al. 2014). Of note is that in response to energy stress (e.g., glucose deprivation), AMPK is also activated by the v-ATPase-Ragulator complex that recruits an alternate complex comprised of the scaffold protein AXIN and LKB1, in turn activating AMPK on the lysosomal surface (Zhang et al. 2014). The regulation of protein and energy balance is therefore an intrinsic process with several intracellular systems synergistically responding to substrate availability and regulating rates of protein turnover.
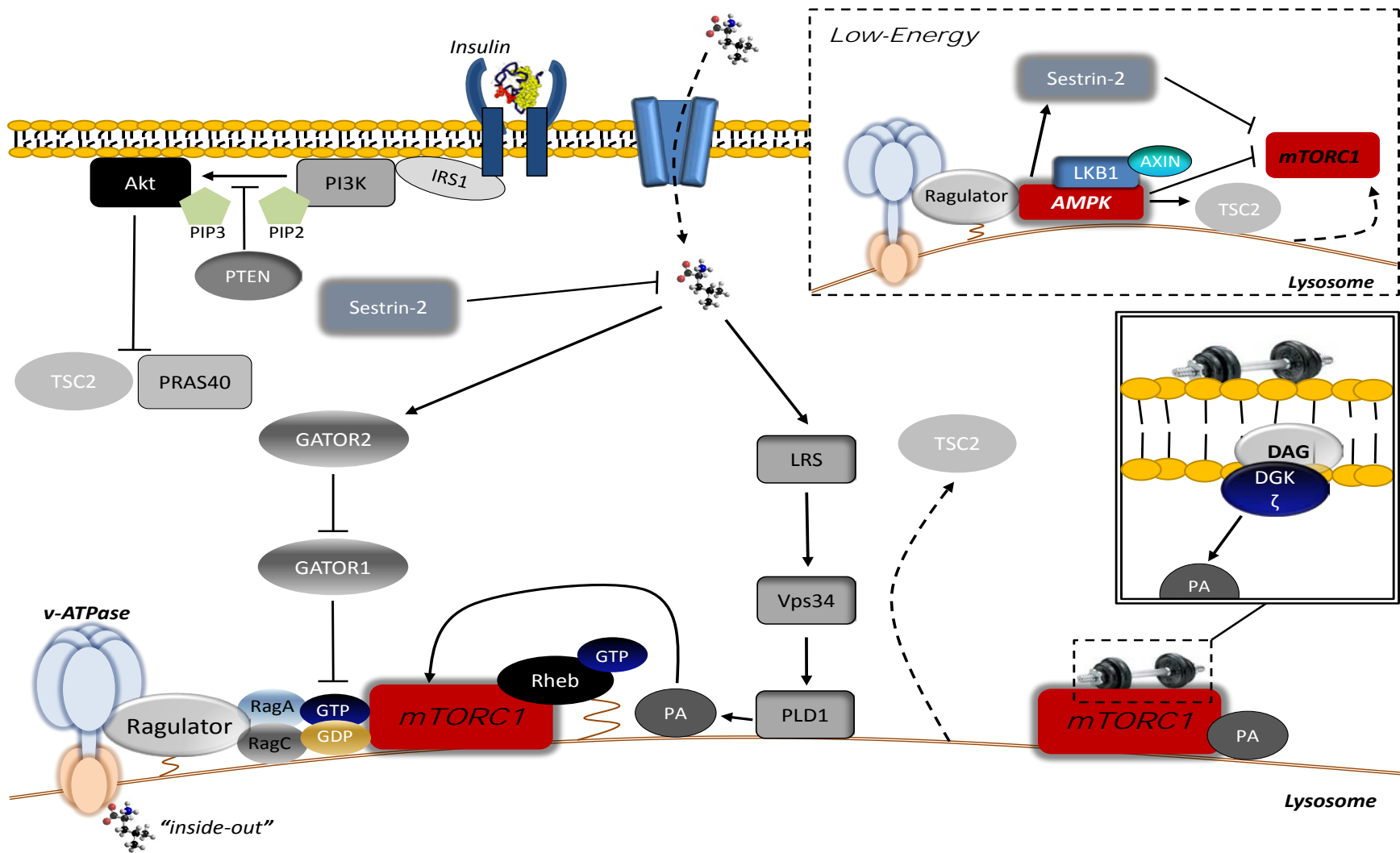
Figure 2.1. Schematic overview depicting mTORC1 activation at the lysosome with insulin, amino acids and resistance-based exercise

Figure 2.1. Continued

Growth factors such as insulin activate mTORC1 through Akt-mediated repression of negative regulators of mTORC1 (TSC2 and PRAS40). Amino acids on the other hand, through an "inside-out" mechanism of intralysosomal accumulation, recruit mTORC1 to the lysosomal surface via Rag GTPases (e.g., RagA and RagC dimers), where mTORC1 can be activated by Rheb. Alternate pathways of amino acid-stimulated mTORC1 signalling involve activation of the GATOR2 complex (a regulator of Rag activity), and/or of leucyl-tRNA synthetase (LRS). Activated LRS promotes phospholipase D-catalysed synthesis of phosphatidic acid (PA), another binding partner, and enhancer of, mTORC1. Also depicted is how resistance exercise contraction, by promoting the local production of PA at the plasma membrane by diacylglycerol (DAG) kinase-ζ (DGKζ) isoforms, or TSC2 removal from the lysosome, induces mTORC1 activation. Finally, the consequences of low-energy availability toward mTORC1 signalling have been depicted, involving an AMPK/Sestrin-2-mediated repression of mTORC1. Notably, Sestrin-2 has been found to sense endogenous levels of the amino acid leucine and has become recognised as an mTORC1 inhibitor (Wolfson et al. 2016).

2.2 Muscle Protein Breakdown

The predominant intracellular systems regulating muscle protein breakdown (MPB) are proteasome- and lysosome-dependent. Because lysosome-dependent MPB is governed largely by autophagy, it will be discussed separately (Section 2.4). Proteasome-dependent proteolysis or the UPS involves ubiquitination of target substrates and their flux through the 26S proteasome. In brief, transfer of a ubiquitin chain onto a given substrate involves E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases, of which in skeletal muscle, proteins encoding the 'atrogenes' muscle atrophy F-box (MAFbx or atrogin-1) and muscle-specific RING finger-1 (MuRF-1) have been the most well-characterised (Bodine & Baehr 2014). Expression of atrogenes is regulated by the Forkhead box transcription factors 1 and 3a (FOXO1, FOXO3a), whose nuclear import can be enhanced and inhibited by AMPK and Akt phosphorylation, respectively (Stitt et al. 2004; Nakashima & Yakabe 2007). Activation of FOXOs by AMPK induces MPB (Nakashima & Yakabe 2007), presumably owing to the specific targeting of myofibrillar proteins and translation factors by atrogenes for proteasomal degradation (Bodine & Baehr 2014). Akt-mediated repression of atrogene transcription on the other hand is contingent upon pro-growth scenarios, such as elevated systemic growth factors and/or chronic mechanical overload of skeletal muscle (Leger et al. 2006; Stitt et al. 2004). Neural precursor cell expressed, developmentally downregulated 4 (NEDD4) is a another skeletal muscle-enriched ubiquitin ligase that becomes elevated under pro-atrophy conditions (Koncarevic et al. 2007), but may also regulate the activities of alternate degradative systems (i.e., autophagy) by tagging their molecular machinery for destruction through the proteasome (Platta et al. 2012). Thus, substantial crosstalk exists between intracellular pathways controlling MPB.

2.3 Exercise-Induced Muscle Protein Turnover

Protein Turnover Responses to Resistance Exercise (REX)

Mechanical loading that underpins resistance-based exercise (REX) contraction promotes hypertrophy, an increase in myofibre cross-sectional area (Adams & Bamman 2012), by stimulating MPS and net myocellular protein accretion of individual muscle fibres (Phillips 2009). REX elevates rates of MPS above basal levels for 24-48 h (Phillips et al. 1997; Chesley et al. 1992; MacDougall et al. 1995; Tang et al. 2008) along with a relatively small rise (~31%) in MPB (Phillips et al. 1997). Thus, any training-induced hypertrophic response results in the accretion of contractile myofibrillar protein. However, in order to supply the energy demands of protein synthesis, adaptations to resistance training may involve rapid degradation of proteins with shorter half-lives by the proteasome via muscle-specific ubiquitin ligases. This probably accounts for 'ubiquitin-mediated protein degradation' being one of the most over-represented pathways in a phosphoproteomic analysis of acute responses to REX (Potts et al. 2017), coupled by the increased expression of atrogenes during early phases of recovery after REX (Raue et al. 2007; West et al. 2012; Mascher et al. 2008). To promote a net positive protein balance, chronic training ultimately attenuates the expression of these degradative enzymes and/or their transcription factors (Stefanetti et al. 2015; Mascher et al. 2008). In addition, the provision of high-quality protein following REX reduces the rate of transcription and/or protein content of ubiquitin ligases (Areta et al. 2013; Borgenvik et al. 2012). This latter observation likely stems from higher amino acid availability mitigating the need for abrupt degradation of endogenous protein and liberation of its amino acid constituents.

Homeostatic Response-Adaptations to Resistance Exercise (REX)

REX-induced increases in MPS are largely attributable to upregulation of protein translation initiation and control by mTORC1 signalling (Goodman 2014), as evidenced by the diminished MPS response to REX in humans when rapamycin, an mTORC1 inhibitor, is administered before an exercise bout (Drummond et al. 2009; Gundermann et al. 2014). The precise upstream, mechanical tension-sensing kinase(s) that stimulate mTORC1 activity following REX remain elusive, although the lysosome has emerged as a key site implicated in mitigating mTORC1 translational responses to resistance-like exercise stimuli (Jacobs et al. 2014). Work from Troy Hornberger's laboratory (Jacobs et al. 2013; Jacobs et al. 2017) has demonstrated that high-force contractions in mice skeletal muscle induced phosphorylation of TSC2, triggering its lysosomal dissociation to promote mTORC1 activation. This response has been shown to act independent of upstream growth factor signals that activate mTORC1 signalling via Akt (Hornberger et al. 2004; Hamilton et al. 2010; Miyazaki et al. 2011; O'Neil et al. 2009; Hornberger & Chien 2006; Jacobs et al. 2017). Hence, the kinase responsible for targeting TSC2 and inducing its lysosomal displacement remains unknown, although components of transmembrane adhesive structures such as integrins and Z-disc proteins with intrinsic kinase activity (e.g., titin and obscurin) have emerged as candidate molecules involved in this process (Gan et al. 2006; Crossland et al. 2013; Potts et al. 2017). Mechanically-induced mTOR activation also increases intramyocellular concentrations of PA in rodent models where it is synthesised at membrane sites from diacylglycerol (Hornberger et al. 2006; O'Neil et al. 2009; You et al. 2014). Because PA becomes incorporated into the lysosomal lipid membrane (Jacobs et al. 2014), this organelle is evidently a key intracellular hub coordinating protein turnover responses to physiological stimuli such as resistance-based contraction.

Effects of Energy Availability

As MPS is energetically expensive, it is not surprising that periods of energy deficit (ED) attenuate both resting fasted (Pasiakos et al. 2010; Areta et al. 2014; Murphy et al. 2015) and feeding-induced MPS (Pasiakos et al. 2013; Murphy et al. 2015). REX undertaken subsequent to a 5 day, moderate (~1000 kcal·day^{-1}) ED which lowered basal MPS rates by 27% compared to resting energy balance (EB), failed to elevate MPS above the rates originally observed at rest in EB (Areta et al. 2014). Post-exercise whey protein provision in graded amounts of either 15 or 30 g augmented MPS and prolonged mTOR phosphorylation in a dose-responsive manner (Areta et al. 2014), demonstrating that the ED-induced repression of MPS is relieved with high exogenous amino acid availability. AMPK, which localises to the lysosome for activation following energy stress stimuli (Zhang et al. 2014), stimulates TSC2 activity under similar metabolic conditions (Inoki et al. 2003b). Although the inhibitory function of AMPK toward MPS following acute REX is equivocal (Dreyer et al. 2006), these findings suggest that in response to mechanical stimuli and downstream mTORC1 signalling, the prevailing anabolic response (involving a complete repression of TSC2) is optimised with sufficient intracellular energy availability and at least 30 g of protein ingested post-exercise.

Protein Turnover Responses to Endurance Exercise (END)

Endurance exercise (END) enhances whole-body maximum oxygen uptake ($\dot{V}O_{2max}$) and muscle oxygen extraction via increased capillarisation and blood flow to the working muscle. Seminal work by John Holloszy (1967) demonstrated that chronic endurance training elevated mitochondrial enzyme content, enhancing ATP synthesis through oxidative phosphorylation. Subsequent findings in humans demonstrated that short-term (3 wk) endurance training was sufficient to elevate concentrations of intramuscular mitochondrial

enzymes and enhance endurance capacity (Henriksson & Reitman 1977). Indeed, mitochondrial biogenesis is the hallmark adaptation to endurance training and accounts for the enhanced ability of skeletal muscle to oxidise both fat-based fuels (Holloszy 2011), and to the large increases in submaximal endurance capacity observed after endurance training (Holloszy 1967), at least when exercise is undertaken at the same absolute intensity pre- and post-training. Protein synthetic responses to END are in large part confined to the mitochondrial protein pool, although the latter constitutes a relatively small fraction of the skeletal muscle proteome (~10%). Endurance-based contractions preferentially increase mitochondrial versus myofibrillar protein synthesis in humans (Wilkinson et al. 2008; Donges et al. 2012). However, despite the capacity for protein ingestion to enhance MPS in response to REX (Moore et al. 2009; Burd et al. 2011), Breen et al. (2011) demonstrated that a protein and carbohydrate beverage after END augmented acute rates of myofibrillar, and not mitochondrial, protein synthesis. Due to the longer half-lives (~7 days) of several mitochondrial proteins (e.g., cytochrome *c*) (Booth & Holloszy 1977), peak rates of mitochondrial protein synthesis rates are likely stimulated at a later time point (>12 h) following END, which is in contrast to peak myofibrillar protein synthetic responses rapidly occurring within 5 h of REX recovery (Moore et al. 2009). Indeed, in response to fatiguing low-intensity resistance-based exercise [~30% 1 repetition maximum (RM) performed to muscular failure], mitochondrial protein synthesis peaked 24 h post exercise (Burd et al. 2012). Aside from variability in half-lives of myofibrillar versus mitochondrial proteins, it is also conceivable that the myofibrillar pool is more susceptible to mechanical damage, necessitating a faster post-exercise turnover response.

Homeostatic Response-Adaptations to Endurance Exercise (END)

END perturbs the intracellular milieu by accelerating substrate turnover and redox reactions [i.e., raising AMP/ADP, inorganic phosphate (P_i) and NAD^+ concentrations], triggering respiratory bursts of reactive oxygen species (ROS) and inducing repetitive Ca^{2+} oscillations that sustain locomotion via actomyosin cross-bridge cycling (Hood 2001; Egan & Zierath 2013; Coffey & Hawley 2007). Proximal signalling kinases sensitive to END-induced homeostatic perturbations include (but are not limited to) AMPK, p38 mitogen-activated protein kinase (p38 MAPK) and Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) that regulate downstream transcriptional responses to the stresses imposed (Akimoto et al. 2005; Irrcher et al. 2008; Jager et al. 2007; Puigserver et al. 2001; Jones et al. 2005; She et al. 2000; Rose & Hargreaves 2003; Wu et al. 2002).

The peroxisome proliferator-activated receptor-γ (*PPARγ*) coactivator-1α (PGC-1α) is considered the "master regulator" of mitochondrial biogenesis and is activated by the aforementioned protein kinases (Holloszy 2011). Another protein, p53, which is also activated by AMPK and p38 MAPK, has been identified as putative regulator of mitochondrial biogenesis (Saleem et al. 2013). Both p53 and PGC-1α are capable of translocating to mitochondrial DNA (mtDNA) and interacting with Tfam (mitochondrial transcription factor A) in response to exercise where they coordinate synthesis of the mitochondrial genome encoding for subunits of the respiratory chain (Saleem & Hood 2013; Safdar et al. 2011). Because meaningful changes in PGC-1α protein levels are not apparent until ~24 h after a single bout of END (Baar et al. 2002; Perry et al. 2010), immediate responses to endurance-based exercise are indicative of its activation, stabilisation and nuclear import (Little et al. 2010a; Little et al. 2011). PGC-1α also regulates its own transcription, probably accounting for rapid elevations in *PGC-1α* mRNA transcripts after END in humans (Edgett et al. 2013; Bartlett et al. 2012; Gibala et al. 2009; Perry et al. 2010;

Lane et al. 2015; Psilander et al. 2010). PGC-1α docks on and coactivates various nuclear transcription factors that upregulate genes encoding for proteins involved in respiration, substrate oxidation and antioxidant function (Scarpulla 2011).

The incorporation of nascent mitochondrial proteins into existing and/or new organelles is energy-consuming and inhibited by oxidative stress (Takahashi & Hood 1996). In human skeletal muscle, a functional change in mitochondrial content corresponds to completion of at least three strenuous END bouts (Perry et al. 2010), which may partially explain the delay in rates of mitochondrial protein synthesis observed during END recovery (conveyed hypothetically in Figure 2.2). In this regard, it can be postulated that maximal mitochondrial biogenesis is only achieved once energy equilibrium is restored.
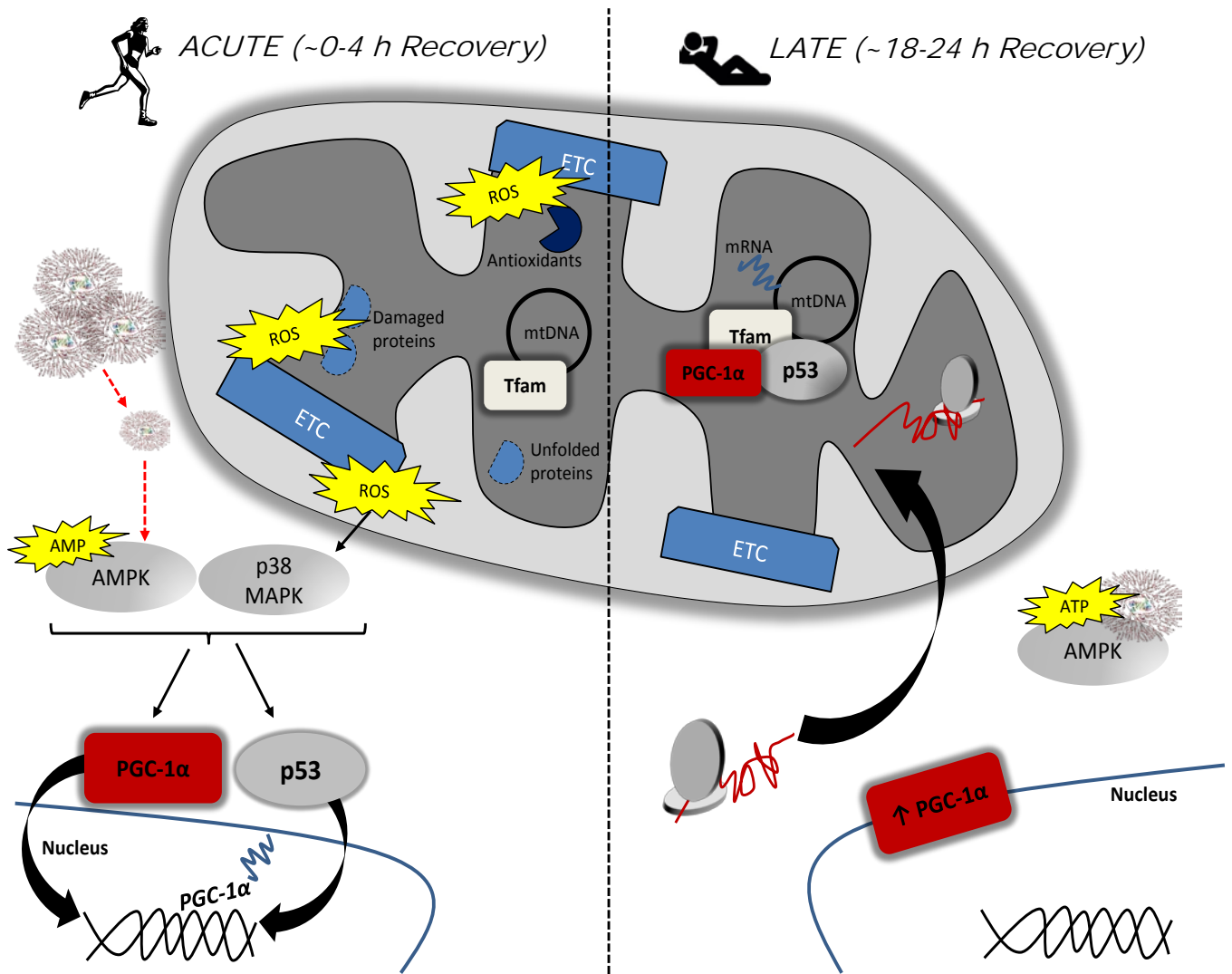
Figure 2.2. Hypothetical depiction of early (left; ~0-4 h) versus late (right; ~18-24 h) mitochondrial adaptive responses to endurance exercise.

During initial recovery, contraction-induced disturbances to mitochondrial protein homeostasis caused by reactive species production (ROS) from the electron transport chain (ETC) and diminished ATP (as well as glycogen) availability stimulates the kinase activity of AMPK and p38 MAPK, respectively, that stabilise and promote nuclear translocation of p53 and PGC-1α, critical regulators of mitochondrial protein transcription. Mitochondrial protein synthesis as well as PGC-1α content peaks ~24 h post-exercise, during which time mtDNA may also be transcribed upon restoration of energy and oxidative homeostasis (exemplified by AMPK rendered inactive by ATP and/or glycogen binding).

2.4 Autophagy

Autophagy is constitutively active and required for maintaining intracellular homeostasis via the turnover of an array of cellular components (Mizushima & Klionsky 2007). Autophagy commences with *de novo* formation of a double-membrane structure termed the phagophore that envelops a portion of cytoplasm in a selective or non-selective manner, the latter referred to as macroautophagy (henceforth autophagy). Closure of the phagophore generates autophagic vesicles termed autophagosomes that sequester cellular constituents for delivery to, and disintegration by, lysosomal hydrolytic enzymes. Reduced products of lysosomal degradation such as amino acid building blocks are utilised as precursors for synthetic reactions (Rabinowitz & White 2010). Alternate, yet highly selective forms of autophagic processes that involve deliberate targeting and degradation of specific organelles (e.g., mitochondria) and nutrients (e.g., lipid droplets) exist. In addition, direct lysosomal uptake of cellular material, bypassing autophagosome biogenesis, is executed by chaperone-mediated autophagy (CMA) and microautophagy. CMA recognises and transports substrates by cytosolic chaperones directly into the lysosomal lumen (Dice 2007), whereas microautophagy involves invagination of lysosomal membranes for instantaneous consumption of cytoplasmic material (Li et al. 2012).

Autophagosome Biogenesis

Autophagosome biogenesis is controlled by four sub-groups of autophagy-related gene (Atg) proteins described as the core molecular machinery of autophagy (Xie & Klionsky 2007). This core is comprised of the Unc-51-like kinase 1 (ULK1) and class III phosphatidylinositol 3-kinase (PI3KC3) protein complexes, as well as two ubiquitin-like conjugation systems. The molecular components of these complexes and their interactions with other systems are depicted in Figure 2.3. Activity of the mammalian ULK1 complex is regulated by AMPK and

mTORC1 phosphorylation of the ULK1 protein kinase at distinct residues that either activate, or inhibit the complex, respectively (Kim et al. 2011; Lee et al. 2010; Hosokawa et al. 2009; Sanchez et al. 2012). A low cellular energy state inhibits mTORC1 signalling, permitting AMPK-ULK1 interactions and downstream phosphorylation of ULK1's binding partners (Figure 2.3). This, in turn, stabilises the complex and associates it with other Atgs at phagophore assembly sites (Ganley et al. 2009; Jung et al. 2009). PI3KC3 contains, but is not limited to, a catalytic subunit vacuolar protein sorting 34 (Vps34) and the tumour suppressor protein Beclin-1 (He & Levine 2010). Of note is that ULK1 can activate the lipid kinase activity of Vps34 [triggering synthesis of phosphatidylinositol 3-phosphate (PI3P)] by directly phosphorylating Beclin-1 (Russell et al. 2013), while AMPK has also been shown to phosphorylate Beclin-1 in response to energy restriction (Kim et al. 2013). Posttranslational regulation of Beclin-1 and its dissociation from inhibitory B-cell lymphoma 2 (Bcl-2) binding has been shown to be a 'gatekeeper' for energy stress-induced autophagy in skeletal muscle (He et al. 2012). The resultant synthesis of PI3P at the phagophore assembly site recruits molecular machinery to further drive autophagosome biosynthesis (Axe et al. 2008; Obara & Ohsumi 2011).
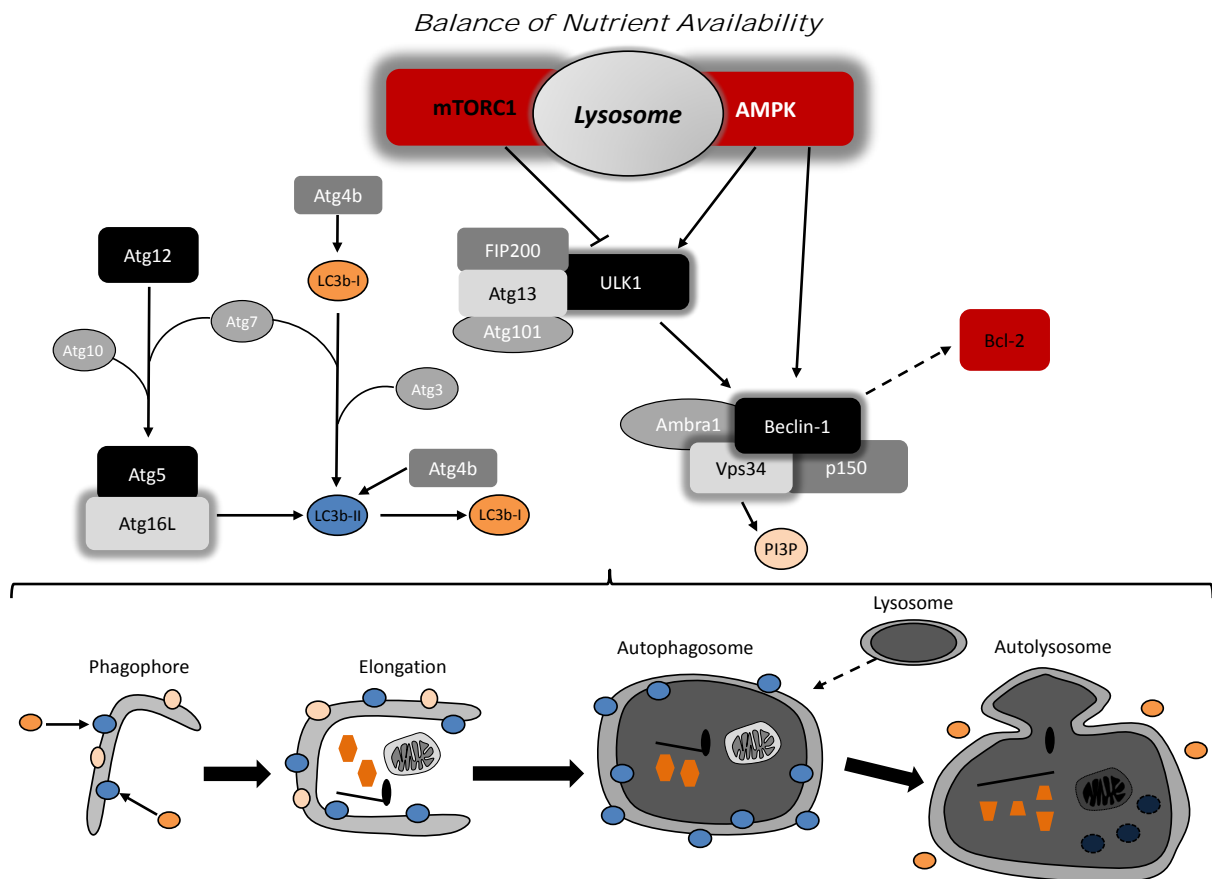
Figure 2.3. Schematic overview depicting cellular regulation of autophagy

Autophagy induction is governed largely by nutrient sensing mechanisms converging on the lysosome that are transduced to the core molecular machinery of autophagy by mTORC1 and AMPK. From left to right: formation of Atg16L involves conjugation of Atg12 to Atg5 via the E1- and E2-like activities of Atg7 and Atg10, respectively. LC3b is proteolytically cleaved by Atg4b, producing LC3b-I that is lipid-conjugated to form LC3b-II via the E1- and E2-like activities Atg7 and Atg3, respectively. Activities of ULK1 and the PI3P-synthesising PI3KC3 complexes are influenced by upstream signals that regulate mTORC1/AMPK signalling. Collectively, these events guide nucleation, expansion and ultimately closure of the autophagosomal double-membrane for the sequestration and degradation of cellular constituents by lysosomal hydrolases.

With regards to the ubiquitin-like conjugation systems, the enzymatic conjugation of Atg12 to Atg5 (cAtg12) results in the formation of a large (~800 kDa) multimeric complex called Atg16L. This complex then functions as an E3-like ligase for microtubule-associated protein 1 light chain 3b (LC3b) (Hanada et al. 2007; Fujita et al. 2008), the integral protein of the other, ubiquitin-like conjugation pathway. LC3b is proteolytically cleaved to form LC3b-I that becomes lipid-conjugated to LC3b-II isoforms that localise to either side of the autophagosomal membrane from the point of phagophore development to maturation into an

autophagosome. Note that upon autophagosomal fusion with the lysosome, outer membrane LC3b-II is deconjugated back to LC3b-I (Figure 2.3). p62 is an autophagy substrate-bridging protein that ultimately undergoes degradation alongside the autophagic cargo upon its sequestration by the autophagosome (Pankiv et al. 2007). As such, an increase in LC3b-II and concomitant decrease in p62 is a posited readout of autophagosome biogenesis (Mizushima & Yoshimori 2007; Bjorkoy et al. 2009). Changes in LC3b-II and p62 are commonly utilised to denote fluctuations in autophagic flux in response to strenuous exercise and altered energy availability, and will be discussed, along with several other putative exercise-energy-sensitive markers of autophagy in Section 2.7.

Transcriptional Regulation of Autophagy

Several transcription factors regulating autophagy are sensitive to changes in cellular bioenergetics (Pietrocola et al. 2013). These molecules include, but are not limited to, the FOXO transcription factors, transcription factor EB (TFEB) and p53 (Figure 2.4).

FOXOs

FOXO1, and in particular FOXO3a, are principal transcriptional regulators of muscle-specific ubiquitin ligases, but have also been shown to coordinate global skeletal muscle protein degradation by transactivating several *Atgs* in response to atrophy-inducing stimuli (Mammucari et al. 2007; Milan et al. 2015; Zhao et al. 2007). Although atrophying skeletal muscle cells elevate both lysosome- and proteasome-mediated proteolysis via FOXO3a (Zhao et al. 2007), this response likely represents a cell-survival mechanism to maintain a threshold level of cellular ATP. In rodent myotubes, forcing AMPK activation by the AMP analogue AICAR (mimicking a reduction in cellular nucleotides) induces AMPK-mediated FOXO3a

phosphorylation, its nuclear translocation and *Atg* expression (Sanchez et al. 2012). In that study, AICAR treatment concomitantly elevated ubiquitin ligase abundance and attenuated protein translation initiation (Sanchez et al. 2012). Therefore, AMPK transduces sensing of energy disturbances to activate autophagy at the transcriptional (e.g., via FOXO3a) and posttranslational level and amalgamates this autophagic response with alternate degradative pathways to create a net negative protein balance (Figure 2.4).

TFEB

TFEB is a basic helix-loop-helix leucine-zipper transcription factor discovered as a master regulator of the Coordinated Lysosomal Expression and Regulation (CLEAR) gene network (Palmieri et al. 2011; Sardiello et al. 2009). Recent evidence demonstrates that under basal conditions where nutrient availability is not limiting, TFEB localises to the lysosomal surface and is inactivated by mTORC1 phosphorylation (Settembre et al. 2012; Medina et al. 2015; Settembre et al. 2011). In contrast, phospho-activation by extracellular signal-regulated kinase 2 (ERK2) or dephosphorylation by Ca^{2+}-sensitive calcineurin triggers TFEB detachment from the lysosome and its subsequent nuclear import (Settembre et al. 2011; Medina et al. 2015). Amino acid availability is likely to regulate TFEB activity, since Rag GTPase overexpression experiments are sufficient to induce TFEB lysosomal retention and inhibition by mTORC1 (Settembre et al. 2012; Martina & Puertollano 2013). Recent novel findings have also uncovered a role for AMPK in the regulation of TFEB-mediated gene transcription. Specifically, AMPK activates FOXO3a that in turn represses the degradation of CARM1 (coactivator-associated arginine methyltransferase-1), a histone methyltransferase that coactivates TFEB (Shin et al. 2016). Because AMPK is activated by a reduction in cellular energy and Ca^{2+}, 'signals' commonly elicited by exercise (Coffey & Hawley 2007), it

would be relevant to determine whether TFEB is shuttled into the nucleus under similar physiological conditions (Figure 2.4).

p53

p53 regulates autophagy by negatively and positively affecting mTORC1 and AMPK signalling, respectively (Feng et al. 2005; Feng et al. 2007; Buckbinder et al. 1995; Stambolic et al. 2001; Ellisen et al. 2002; Budanov & Karin 2008). AMPK phosphorylation of p53 on Ser^{15} permits cell-survival following glucose deprivation (Jones et al. 2005), suggesting that AMPK may positively affect its own transcription to accelerate autophagic flux in energy-restricted cells. However, in contrast to its role as a transcription factor, cytoplasmic p53 has an inhibitory effect toward autophagy induction by inhibiting the accumulation of autophagic vesicles by an undetermined mechanism (Tasdemir et al. 2008). Akt is responsible for attenuating p53 activity by promoting its proteasomal removal (Ogawara et al. 2002). Whether this mechanism is implicated in the inhibition of autophagy, by cytoplasmic p53, is unknown. Figure 2.4 illustrates how all of these transcriptional loops overlap at some point in terms of sharing a common upstream activator and/or repressor. These pathways operate with a high degree of crosstalk, feedback regulation, as well as transient activation by both contractile activity and the prevailing cellular environment (i.e., energy availability), which will be discussed further (Section 2.7).
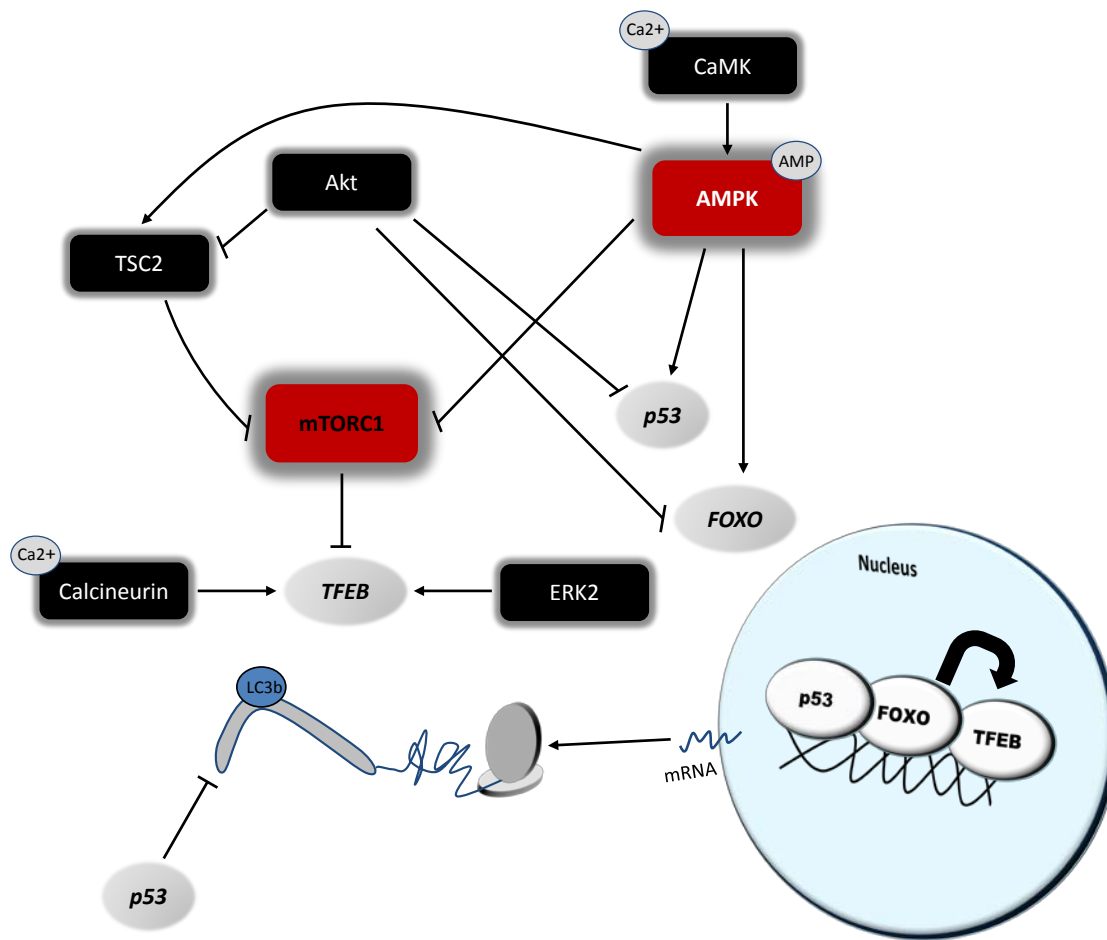
Figure 2.4. Simplified schematic of the transcriptional regulation of autophagy

Transcriptional activators of *Atgs* and genes encoding their regulatory proteins include, but are not limited to, the Forkhead box transcription factors FOXO1 and FOXO3a, transcription factor EB (TFEB) and p53. Of note is that there is considerable overlap between upstream regulators of these transcription factors and that p53 can promote (intranuclearly) and inhibit (cytosolically) autophagy induction.

2.5 Mitophagy and Mitochondrial Quality Control

Mitophagy is a selective form of autophagy that segregates defective and/or superfluous mitochondria for degradation (Kim et al. 2007). Mitochondrial oxidative phosphorylation supplies ~90% of cellular ATP, but not without consequential leakage of respiratory chain electrons that generate ROS (Powers et al. 2010). Although ROS emissions are an inevitable byproduct of cellular respiration, inefficient quenching of free radicals by endogenous

antioxidants can lower mitochondrial membrane potential and signal for mitophagy (Qiao et al. 2015). A failure of mitophagy to arrest high levels of ROS provokes the cytoplasmic release of apoptogenic factors that under basal conditions reside in the mitochondrial inter-membrane space (Daugas et al. 2000a). Prevailing apoptotic cascades trigger large-scale DNA fragmentation and chromatin condensation, hallmarks of apoptosis. Therefore, mitophagy is positioned at the interface of cell-fate responses. Mitochondrial quality control is also governed by pathways regulating mitochondrial membrane fission and fusion. Fission (or scission) of mitochondria is regulated by dynamin-related protein 1 (DRP1), a GTPase that assembles into multimeric spiral-shaped structures that constrict to sever mitochondrial membranes (Smirnova et al. 2001). This process generates two daughter organelles with uneven membrane potential, in which the depolarised unit is digested via mitophagy and the healthy unit undergoes fusion (Twig et al. 2008). Mitochondrial fusion of outer and inner membranes is regulated by the Mitofusins 1/2 and optic atrophy 1 (OPA1), respectively. Fusion permits the exchange of mitochondrial material that may facilitate survival of damaged mitochondria, thus sparing these organelles from mitophagy (Youle & van der Bliek 2012). Taken collectively, the dynamic nature of the mitochondrial network necessitates strict quality control at several levels with considerable crosstalk to preserve cellular viability. One such pathway involves regulation of mitochondrial turnover at the transcriptional level by TFEB/PGC-1α, and by the mitophagic machinery at the organelle level, PINK1 and Parkin and the mitophagy receptor BNIP3.

TFEB/PGC-1α

Transcriptional pathways of mitochondrial turnover are regulated by a PGC-1α/TFEB autoregulatory loop, whereby PGC-1α can coactivate TFEB (Scott et al. 2014; Tsunemi et al. 2012) and TFEB can in turn upregulate *PGC-1α* expression (Settembre et al. 2013). As such,

TFEB has emerged as a putative regulator of mitochondrial biogenesis (Settembre et al. 2013; Mansueto et al. 2017), which is coupled by its ability to modulate biogenesis of autophagic vesicles that capture and disintegrate long-lived mitochondrial organelles. Moreover, overexpression of *PGC-1α* has been shown to augment skeletal muscle autophagic vesicles and markers of mitophagy (Takikita et al. 2010; Lira et al. 2013), presumably via coactivation of TFEB and transactivation of *Atgs*. Hence, mitochondrial turnover is regulated by tight transcriptional coupling of molecules serving overlapping roles in mitochondrial breakdown and biogenesis.

PINK1/Parkin

PTEN-induced putative kinase 1 (PINK1) and Parkin are effectors of mitophagy. Under basal conditions, PINK1 is continually imported into the mitochondria and proteolytically cleaved (Jin et al. 2010). However, mitochondrial damage (e.g., membrane depolarisation) results in PINK1's redistribution to the outer mitochondrial membrane (OMM) where it recruits and activates Parkin, an ubiquitin ligase that specifically targets mitochondrial substrates for recognition by p62 and delivery to autophagosomes (Kazlauskaite et al. 2014; Kim et al. 2008; Narendra et al. 2010; Kane et al. 2014; Geisler et al. 2010). For example, Parkin ubiquitination of Mitofusins-1/2 disables mitochondrial fusion that would otherwise compensate for mitochondrial damage by merging dysfunctional organelles with healthy counterparts (Tanaka et al. 2010; Glauser et al. 2011). Miro, a scaffold protein that connects a motor complex to the mitochondria and facilitates mitochondrial motility, is phosphorylated by PINK1 and targeted by Parkin for proteasomal removal (Wang et al. 2011). Rapid proteasomal disposal of Miro quarantines mitochondria to inhibit fusion and present the idle organelles to autophagosomes (Wang et al. 2011). Numerous OMM proteins have been

identified as substrates of Parkin and the physiological manifestations of PINK1/Parkin-mediated mitophagy are not yet fully understood (Sarraf et al. 2013).

BNIP3

Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) has emerged as a putative regulator of mitophagy independent of canonical PINK1/Parkin signalling (Tracy et al. 2007; Quinsay et al. 2010). BNIP3 contains an LC3-interacting region (LIR) motif and phosphorylation of BNIP3 at Ser17 and 24 flanking this region facilitates recruitment of autophagosomes to damaged mitochondria (Hanna et al. 2012; Zhu et al. 2013). Thus, BNIP3 is a mitochondrial receptor for mitophagy and is accompanied by several other LIR motif-containing proteins such as FUNDC1, a downstream substrate of AMPK/ULK1 signalling (Wu et al. 2014; Tian et al. 2015), and the homologous BNIP3-like or Nix (Novak et al. 2010). Crosstalk between mitochondrial biosynthetic and degradative pathways under physiological conditions and the role of membrane receptors to preserve mitochondrial network integrity is a fertile area of future research.

2.6 Skeletal Muscle Autophagy

Masiero and colleagues (2009) have previously demonstrated that muscle-specific *Atg7* knockout mice experienced marked deterioration of skeletal muscle mass. Skeletal muscle of these mice was characterised by defective mitochondrial organelles and aberrant sarcoplasmic reticulum stress leading to reduced protein translation (Masiero et al. 2009). Similar phenotypes have been observed with alternate autophagy knockout models in skeletal muscle (Raben et al. 2008; Moresi et al. 2012; Bujak et al. 2015), and defective autophagy is known to underpin the inevitable deterioration of skeletal muscle tissue with ageing

(sarcopenia) (Zhou et al. 2017; White et al. 2016) and various skeletal myopathies in humans (Levine & Kroemer 2008). Reduced products of autophagic degradation are utilised to recycle cellular components to prevent unnecessary aggregation of aged organelles that otherwise instigate cellular ageing and disrupt whole-body metabolism. Thus, autophagy has emerged as a critical biological system regulating beneficial metabolic adaptations to strenuous exercise (discussed subsequently) and maintenance of muscle mass.

2.7 Exercise-Induced Autophagy and Protein Turnover

Resistance Exercise (REX)

As previously discussed, high-force REX concomitantly stimulates MPS and MPB. These REX-induced increases in MPB may be explained by the actions of muscle-specific ubiquitin ligases that increase in response to resistance contraction and are responsible for tagging myofibrillar proteins for proteasomal removal (Taillandier et al. 2003). However, because autophagy is required for maintaining the intracellular amino acid balance (Yu & Long 2015), some of the amino acids utilised in synthetic reactions following REX are likely partitioned via autophagic degradation. This supposition is based on the fact that MPB responses to acute REX persist for 12 h into recovery paralleling elevated MPS (Phillips et al. 1997), while peak proteasomal activities subside well before maximal protein anabolism is reached (Baehr et al. 2014). In cultured myotubes, amino acids culminating from autophagic degradation stimulate mTORC1 signalling (Yu & Long 2015). Mackenzie and colleagues (2009) previously demonstrated that high-force skeletal muscle mechanical overload of fasted rodents elevated intramuscular leucine content that accumulated before increased activity of the bifunctional protein Vps34 (Table 2.1). In addition to forming the pro-autophagy PI3KC3 complexes as described in Section 2.4, Vps34 is also an intracellular amino acid sensor and

effector of protein translation initiation (Yoon et al. 2011; Byfield et al. 2005). Thus, increased intramuscular leucine availability following high-force mechanical (resistance-like) stimuli may be a result of autophagy-mediated proteolysis.

REX is often accompanied by the ingestion of rapidly digestible protein sources such as whey and casein, due to the interactive stimulation of MPS to promote an even more positive net protein balance compared to just REX alone (Phillips 2011; Phillips & Van Loon 2011). Whether this prevailing plasma aminoacidemia attenuates autophagy-mediated amino acid cellular partitioning is unknown. While protein or amino acid mixtures ingested following REX can attenuate markers of proteasomal breakdown (Borgenvik et al. 2012; Areta et al. 2013), the nutritional effects of ingested protein toward autophagy activation are less clear and conceivably repress autophagy induction given its sensitivity to cellular bidirectional amino acid transport (Nicklin et al. 2009). LC3b-II has been shown to decrease below basal levels after REX in humans independent of amino acid availability (Table 2.1) (Glynn et al. 2010; Fry et al. 2013). The reduction in LC3b-II following REX could suggest that autophagy is repressed during early recovery from REX. This latter observation is noteworthy given that AMPKα_2 activity increases after a single bout of strenuous REX in humans and precedes the contraction-induced increase in MPS (Dreyer et al. 2006). Therefore, resistance-based contraction may mitigate alternate forms of autophagy activation, such as through AMPK phosphorylation of pro-autophagy Vps34 complexes and regulation of intracellular amino acid levels (Kim et al. 2013). Figure 2.5 presents a scenario in which this pro-autophagic activity of Vps34, specifically by increasing free cellular amino acid concentrations, subsequently stimulates the translational (mTORC1-dependent) machinery to upregulate MPS.

Table 2.1. Summary of acute autophagic responses to resistance-based exercise in human and rodent skeletal muscle

Reference	Subject Characteristics	Protocol	Major Findings in Skeletal Muscle in Response to Exercise
Fry et al. (2013)	Young and old humans	Leg extension; 8 sets x 10 repetitions @ 70% 1-RM	Reduced LC3b-II/ I ratio in both groups
Glynn et al. (2010)	Young, untrained humans	Leg extension; 10 sets x 10 repetitions @ 70% 1-RM plus amino acid ingestion with and without carbohydrate co-ingestion	Reduction in LC3b-II independent of nutritional condition
Ulbricht et al. (2015)	Young, untrained humans	High-intensity eccentric contractions	Increase in CASA (chaperone-assisted selective autophagy)-mediated degradation of the myofibrillar protein FLNC-γ
MacKenzie et al. (2009)	Adult Wistar rats	High-frequency stimulation of sciatic nerve	Increase in Vps34

Another form of autophagic stimuli sensitive to REX contraction is chaperone-assisted selective autophagy (CASA), which involves p62-facilitated delivery of ubiquitinated proteins to autophagosomes. Ulbricht et al. (2015) reported increased REX-induced colocalisation of the CASA co-chaperone Bcl-2-associated athanogene 3 (BAG3) with filamin C-γ (FLNC-γ), an anchoring and crosslinking protein for actin filaments that unfolds and is targeted for degradation by p62 in response to mechanical stress (Ulbricht et al. 2013a; Ulbricht et al. 2013b). Indeed, the molecular machinery regulating CASA localises to sarcomeric Z-discs, focal points of skeletal muscle force transmission. CASA is therefore ideally poised to respond to mechanically-invoked perturbations to sites of force transmission. As illustrated in Figure 2.5, these myocellular structures are also in close proximity to the site of PA synthesis (from DAG by DGKζ enzymes), the mechanically sensitive direct activator of mTORC1 (You et al. 2014). In addition, because lysosomes do not substantially contribute to degradation of myofibrillar proteins, for which the bulk responsibility is assumed by proteasomal degradation (Lowell et al. 1986), autophagic removal of structural FLNC-γ would render actomyosin complexes more susceptible to proteasomal degradation (Figure 2.5) (Solomon & Goldberg 1996). In response to four weeks of resistance training the steady-state abundance of CASA proteins are increased (Ulbricht et al. 2015), contrasting the otherwise negligible changes to markers of proteasomal breakdown after similar training protocols (Stefanetti et al. 2015). These findings suggest that in the trained state, CASA may 'reinforce' the contractile apparatus to compensate for a reduction in proteasome-dependent proteolysis. Taken collectively, high-force mechanical work necessitates synergistic turnover-remodelling of the architectural and locomotor-related components of skeletal muscle that contributes to increased cross-sectional area and enhanced force-generating capacity of the working tissue.
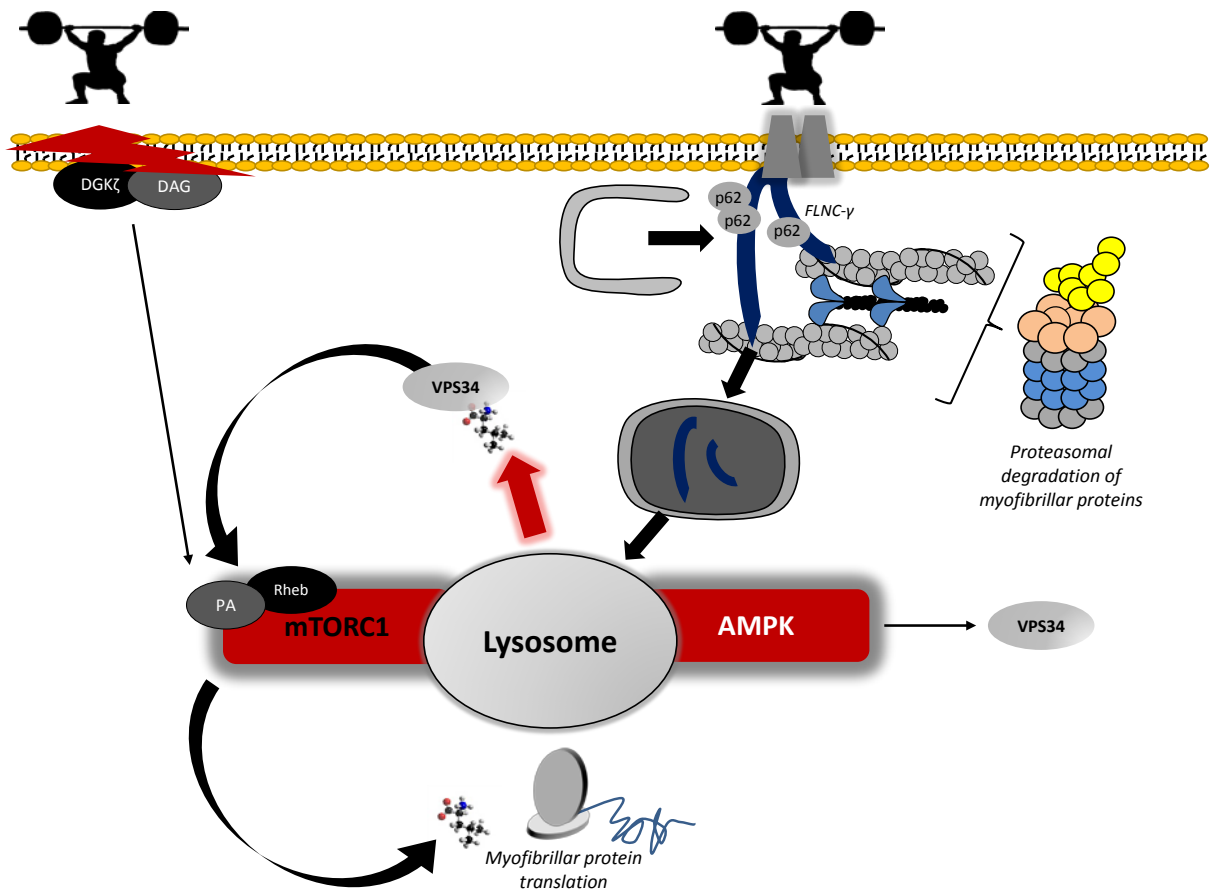
Figure 2.5. Schematic overview of resistance exercise-induced regulation of autophagy

High-force mechanical stimuli elicits sarcolemmal perturbations that are sensed by CASA (chaperone-assisted selective autophagy) that displaces, via p62-mediated autophagic degradation, components of the myofibrillar apparatus, including the actin crosslinking protein FLNC-γ leading to destabilisation of actomyosin filaments, thus providing readily-degradable substrates for the proteasome. Products of proteasome- and lysosome-mediated proteolysis, amino acids, can subsequently be detected by Vps34 activity that increases following acute REX contraction and serves overlapping roles in protein synthesis and autophagy. Amino acids that accumulate in the lysosomal lumen recruit mTORC1 to this site where it is brought into close proximity to its mechanically sensitive direct activators, PA (synthesised by DGKζ) and Rheb.

Endurance Exercise (END)

Autophagy is sensitive to endurance exercise stimuli in both rodents (Jamart et al. 2013; He et al. 2012; Grumati et al. 2011; Lira et al. 2013; Liu et al. 2015; Salminen & Vihko 1984; Vainshtein et al. 2015b; Pagano et al. 2014) and humans (Jamart et al. 2012a; Jamart et al. 2012b; Schwalm et al. 2015; Moller et al. 2015). However, differences in the exercise-induced regulation of autophagy between these species have been observed and the precise

mechanisms explaining the discrepancies are unknown (Table 2.2). Schwalm and colleagues (2015) showed in humans that increases in in AMPK-targeted ULK1[Ser317] phosphorylation following END were generally indistinguishable in fed versus fasted states, which is in contrast to findings from rodents whose skeletal muscle is more sensitive to autophagy activation when the exercise bout is undertaken fasted (Jamart et al. 2013). The same group also found that the LC3b-II/I ratio, a purported readout of autophagosome biogenesis, was attenuated after exercise compared to pre-exercise levels in whole cell lysates (Schwalm et al. 2015) and mitochondrial fractions (Schwalm et al. 2017), which is similar to other studies in humans reporting that LC3b-II abundance declines following acute END (Fritzen et al. 2016; Moller et al. 2015), but in contrast to previous rodent work (Table 2.2) (Jamart et al. 2012b). Because autophagosome biogenesis is an energy-consuming process (Plomp et al. 1987; Schellens et al. 1988), a reduction in LC3b-II content in the initial post-exercise recovery period (i.e., 0-8 h) may signify prioritisation of alternate cellular processes, such as restoration of energy and redox balance. A notable exception, however, is the large increase in LC3b-II protein content that occurred immediately after an ultramarathon (Jamart et al. 2012b), suggesting that prolonged contractile stress is required to increase autophagosome content during the exercise bout itself. Nevertheless, the discord between the proximal, AMPK-mediated activation of ULK1 and the distal reduction in LC3b lipidation in humans is unclear, and may be reconciled by AMPK's known role as an energy sensor and inhibitor of energy-consuming processes. This inhibition by AMPK may also extend to restricting the abrupt biogenesis of autophagosomes shortly after exercise when cellular homeostasis is already disrupted. A more immediate (and less energy-demanding) response to contractile stress could be the transcriptional regulation of autophagy. Lysosomal Ca^{+2} release caused by running exercise in rodent skeletal muscle triggers TFEB nuclear translocation (Medina et al. 2015), where it serves a dual role, alongside PGC-1α, in regulating autophagosome/lysosome

and mitochondrial biogenesis (Figure 2.6) (Scott et al. 2014; Mansueto et al. 2017; Halling et al. 2016). Hence, the TFEB/PGC-1α axis may represent one of the initial cellular autophagic responses to END-induced perturbations to metabolic homeostasis.

Table 2.2. Summary of acute autophagic responses to endurance-based exercise in human and rodent skeletal muscle

Reference	Subject Characteristics	Protocol	Major Findings in Skeletal Muscle in Response to Exercise
Human			
Jamart et al. (2012a)	Middle-aged, endurance-trained	Ultramarathon	General increase in autophagy gene expression (e.g., transcripts of Atg4b, Atg12, LC3b)
Jamart et al. (2012b)	Middle-aged, endurance-trained	Ultramarathon	Evidence for autophagosome formation based on elevated LC3b-II, as well as increases in AMPK phosphorylation
Schwalm et al. (2015)	Young, endurance-trained	2 h cycling, 55 or 70% $\dot{V}O2_{peak}$ in fed or fasted state	Reduced LC3b-II/I ratio, but largest increase in AMPK/ULK1 signalling following high-intensity exercise with otherwise negligible effects of prior fasting
Schwalm et al. (2017)	Young, endurance-trained	2 h cycling, 70% $\dot{V}O2_{peak}$ in fed or fasted state	Reductions in the LC3b-II/I ratio in mitochondrial fractions, but elevated mitochondrial BNIP3 and phosphorylated DRP1
Tachtsis et al. (2016)	Young, untrained	60 min cycling at 70% $\dot{V}O2_{peak}$	Reduced mitochondrial Atg5 and no change in LC3b-II content
Fritzen et al. (2016)	Young, active	60 min, single-leg knee-extensor exercise	Reduced LC3b-II/I ratio and no change in ULK1 Ser555 (AMPK-targeted) phosphorylation
Moller et al. (2015)	Young, active	60 min cycling, 50% $\dot{V}O2_{peak}$	Reduced LC3b-II/I ratio, but an increase in ULK1 Ser555 (AMPK-targeted) phosphorylation
Rodent			
Salminen & Vihko (1984)	NMRI mice	9 h treadmill running	Evidence of autophagic vesicles engulfing partly degraded mitochondria and cytoplasmic material 3 days post-exercise

Reference	Subject Characteristics	Protocol	Major Findings in Skeletal Muscle in Response to Exercise
Jamart et al. (2013)	C57BL6 mice	90 min treadmill running in fed or fasted state	Fasted running caused larger (relative to fed) increases in the LC3b-II/I ratio, conjugated Atg12 and *Atg* gene expression, as well as reductions in Akt signalling
Pagano et al. (2014)	C57BL/6 mice	Treadmill running to exhaustion	AMPK/ULK1 phosphorylation and evidence for elevated autophagic flux
Fritzen et al. (2016)	Wistar rats	4 h swimming	Elevated LC3b-II/I ratio
Kim et al. (2012)	ICR mice	50 min treadmill running	Reduction in autophagy protein levels (e.g., LC3b-II, Beclin-1, cAtg12 etc.), but an increase in MuRF1
He et al. (2012)	Wild-type and *Bcl-2^{AAA}* mice	Acute treadmill running to exhaustion	Increases in the number of autophagosomes associating with glucose uptake that was abolished by the mutant genotype
Lira et al. (2013)	Wild-type, *Atg6^{+/−}* and *MCK-PGC-1α* mice	Acute treadmill running bout to exhaustion	Oxidative wild-type muscles demonstrate a larger autophagic flux alongside mitochondrial biogenesis; effects induced by *PGC-1α* overexpression and abrogated in *Atg6^{+/−}* mice subjected to exercise
Medina et al. (2015)	CD1 mice	Acute treadmill running bout to exhaustion	Lysosomal calcium release and TFEB lysosome-to-nuclear translocation elicited by calcineurin dephosphorylation
Halling et al. (2016)	*PGC-1α* transgenic mice	1 h treadmill running	Augmented LC3b-II/I ratio and reduction in p62 in transgenic mice

The physiological significance of endurance-based exercise-induced autophagic activity in skeletal muscle remains unclear. Nevertheless, some insight can be gained from previous work taking advantage of mutant Bcl-2^{AAA} mice that are incapable of starvation- and exercise-induced autophagy due to repression of Beclin-1 activation (He et al. 2012). These rodents display impaired post-exercise AMPK phosphorylation, GLUT4 membrane translocation and glucose uptake associating with decrements in endurance performance (He et al. 2012), which may be attributable to the association between intramuscular autophagosome/lysosome content and glycogen storage (Takikita et al. 2010). In another study, endurance-based exercise stimulating local hypoxia was shown to facilitate autophagosome biogenesis and lysosomal digestion of ROS-emitting mitochondria (Qiao et al. 2015). However, by blunting the mitophagic-dependent disposal of mitochondria, the exercised rodents had deteriorated endurance performance arising from defective respiratory chain function (Qiao et al. 2015). Such defects are likely due to the fact that mitophagy precedes mitochondrial biogenesis and is required for differentiation of myotubes (Sin et al. 2015). Ultimately, recycled energy liberated by autophagy/mitophagy is likely acquired by the translational machinery for mitochondrial protein synthesis (Figure 2.6), as evidenced by findings demonstrating that autophagy is required for the endurance training-induced accrual of mitochondrial proteins (Lira et al. 2013; Ju et al. 2016). Thus, an increase in mitochondrial volume-density is met by corresponding increases in the autophagic machinery to compensate for a larger mitochondrial network in the endurance-trained state.

The endurance training-induced 'oxidative' phenotype is accompanied by an increase in intramuscular triglyceride (IMTG) content and elevated glycogen stores (Bergström & Hultman 1966; Cartee et al. 1989). This oxidative phenotype facilitates a larger prior loading of nutrient substrate to supply the bioenergetic demands of END (Martin et al. 1993; Hurley et al. 1986; Lithell et al. 1979). Lipophagy represents the capturing of intracellular lipid

droplets by autophagosomes and subsequent lysosomal degradation and liberation of fatty acids (FAs) provided to mitochondrial β-oxidation (Singh et al. 2009). Lipophagy can also selectively degrade lipid-coating Perilipins (Kaushik & Cuervo 2015), thereby exposing lipid droplets to cytosolic lipases that are contraction-sensitive and catalyse the release of FAs for mitochondrial β-oxidation (Lithell et al. 1979; Schmitt et al. 2003). Although no studies have directly assessed lipophagic flux and resultant FA oxidation rates following END, it is plausible that significant mitochondria-to-lysosome communication modulates the phenotypic and substrate handling characteristics of endurance-trained skeletal muscle (Figure 2.6).
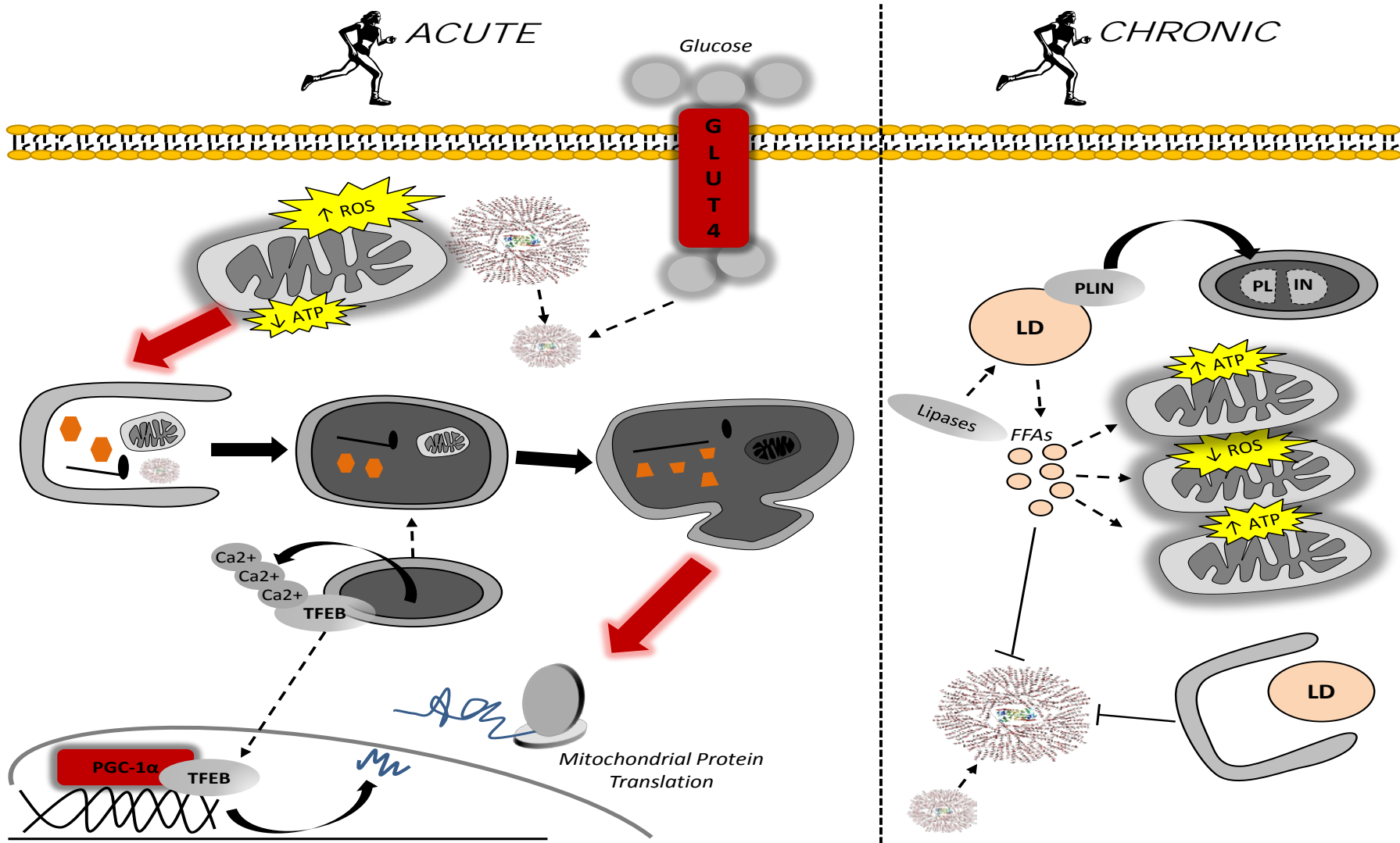
Figure 2.6. Schematic overview depicting the regulation of autophagy with acute and chronic endurance-based training

Figure 2.6. Continued

Acute endurance exercise provokes several metabolic perturbations to cellular homeostasis that stimulate an increased, compensatory autophagic flux. Autophagy is required for glucose uptake and removal of ROS-emitting mitochondria following acute END to mitigate contraction-induced disturbances to metabolic homeostasis. Lysosomal hydrolase-mediated degradation of autophagic "cargo" is returned to the cytoplasm as reduced constituents, for example amino acids that serve as precursors for mitochondrial protein synthesis. With chronic training and the establishment of the "endurance phenotype", lipid-specific autophagy (lipophagy) may supply intracellular FAs for mitochondrial β-oxidation by either engulfing lipid droplets (LD) whole, or selectively disposing of lipid-coating Perilipins (PLIN), rendering the latter amenable to cytosolic lipase-mediated lipolysis; these effects collectively relieve rates of glycogenolysis and resultant lactate accumulation, hallmark features of the endurance-trained state. Such undulations in nutrient and organelle breakdown and synthesis are appropriately controlled by an autoregulatory signalling-transcriptional loop of PGC-1α/TFEB.

2.8 Energy Availability and Autophagy and Protein Turnover

While the impact of exercise in stimulating skeletal muscle autophagy is well known, its modulation by specific nutrients, or lack thereof, is less well understood. Because this thesis comprises three studies investigating divergent forms of exercise-nutrient interactions in human skeletal muscle, the following sections will address the independent effects of these nutritional interventions on autophagy/protein turnover. Indeed, such interventions affect intracellular homeostasis and protein turnover responses and consist of low-energy availability, as well as potentially 'toxic' nutrients, high-alcohol and -fat.

Energy Deficit (ED)

Skeletal muscle autophagy supplies hepatic gluconeogenic substrates to maintain glycaemia during fasting (Bujak et al. 2015). Therefore, an elevated basal autophagic flux would be anticipated when dietary energy availability is restricted. Previous studies showed that chronic (six months) ED of ~500-1,000 kcal/day in animals (Wohlgemuth et al. 2010) and humans (Wohlgemuth et al. 2011) elevated markers of skeletal muscle autophagy. However, similar periods of prolonged ED also attenuate whole-body proteolysis and net protein turnover (Campbell et al. 2009; Stein et al. 1991). These observations suggest that autophagy is upregulated upon reaching a threshold level and/or duration of energy restriction to facilitate a new steady-state protein balance to prevent further tissue catabolism. In support of this postulate, during the initial phase of nutrient withdrawal *in vitro*, there are elevated rates of autophagosome-lysosome fusion (Yu et al. 2010). However, persistent nutrient limitation results in an eventual termination of autophagy, triggering mTORC1 reactivation and replenishment of the cellular complement of lysosomes (i.e., those that were previously fused with autophagosomes) (Yu et al. 2010). This process is likely regulated by the accumulation of amino acids inside the lysosomal lumen arising from autophagy-mediated proteolysis (Yu

& Long 2015). Thus, in response to prolonged periods of energy restriction, autophagy creates a new protein homeostasis to preserve skeletal muscle mass.

Shorter periods (5-10 days) of similar levels of energy restriction attenuate MPS and concomitantly elevate MPB in humans (Carbone et al. 2014; Areta et al. 2014; Pasiakos et al. 2010), which would facilitate a net negative protein balance and induce muscle atrophy. Increased protein breakdown responses to these periods of ED are hypothesised to culminate from elevated proteasome-mediated proteolysis and/or calpain cleavage of contractile proteins (Carbone et al. 2012). To date, no studies have examined the activation of autophagy and its potential contribution to skeletal muscle protein breakdown during this initial phase of energy restriction. Exogenous protein availability may mitigate the ED-induced loss of skeletal muscle mass by repressing ubiquitin-mediated proteolysis and enhancing MPS (Carbone et al. 2013), but its effects toward autophagy are unknown.

Alcohol

Excess alcohol consumption is implicated in skeletal muscle myopathy (Lang et al. 2005), yet despite the role for autophagy in preserving skeletal muscle size and quality, little is known regarding how alcohol affects this degradative process. The majority of work investigating the effects of alcohol on skeletal muscle metabolism has focused on the regulation of protein synthesis (Steiner & Lang 2015b). In rodent models, large quantities of alcohol were found to dysregulate the stimulation of MPS by growth factors and amino acids, an effect largely attributable to attenuation of protein translation (Kumar et al. 2002; Lang et al. 2003). In addition, maximal mechanical overload-induced growth of skeletal muscle was impaired by high- (Steiner & Lang 2014, 2015a), but not moderate-alcohol availability (i.e., intoxicating versus non-intoxicating, respectively) in rodents (Steiner et al. 2015). In humans, a post-

exercise alcohol drinking protocol (~12 standard drinks) restricted the peak stimulation of myofibrillar protein synthesis above basal levels (Parr et al. 2014). Since alcohol metabolism is energetically expensive and results in the production of highly toxic intermediates (Lieber 2005), these findings could suggest that a threshold amount alcohol is required to perturb skeletal muscle metabolism and impair cellular remodelling responses to strenuous contractile activity. However, the role(s) for autophagy in mitigating alcohol metabolism in exercised human skeletal muscle is unknown.

Thapaliya and colleagues (2014) showed that skeletal muscle of alcoholic cirrhosis patients compared to age-matched controls have reduced proteasomal activity, but elevated markers of autophagy commensurate with muscle atrophy. The pronounced skeletal muscle atrophy in this population group may arise from compensatory removal of damaged organelles and protein aggregates by autophagy to prevent apoptosis. Indeed, alcohol is preferentially metabolised in the mitochondria and its catabolism provokes ROS production and oxidative damage to cellular compartments (Lieber 2005), cues for autophagy induction. However, persistent ROS invoked by alcohol activates cell death pathways by triggering the opening of the mitochondrial permeability transition pore and inducing mitochondrial expulsion of apoptogenic factors [e.g., cytochrome *c* and apoptosis inducing factor (AIF)] that otherwise have basal roles in cellular respiration (Daugas et al. 2000a; Kluck et al. 1997). Although the liver serves as a first-pass of alcohol metabolism, the dense mitochondrial population of skeletal muscle places this tissue in a precarious position to handle the oxidative demands of alcohol metabolism. Thus, autophagy is likely to confer protection against the potentially DNA-damaging and apoptotic effects of skeletal muscle exposed to alcohol, but not without consequential tissue atrophy (Thapaliya et al. 2014). A failure of autophagy to arrest the intracellular threat of alcohol toxicity may activate harmful DNA damage-response pathways previously characterised in alcohol-induced liver injury (Ishii et

al. 2003). Taken altogether, excess alcohol can activate autophagy, and whether these effects are exacerbated or ameliorated by the 'stresses' associated with exercise is unknown.

Lipid

Genetic and dietary models of obesity downregulate autophagy in liver and pancreatic tissue, triggering endoplasmic reticulum (ER) stress, apoptotic signalling, and insulin resistance (Yang et al. 2010; Quan et al. 2012; Jung et al. 2008). However, the effects of lipid overload on autophagy in skeletal muscle are unknown. Mechanistically, high-fat availability alters membrane lipid composition and hampers the fusion of autophagic vesicles (Koga et al. 2010). In skeletal muscle, autophagy-deficient rodents have greater sensitivity than wild-type controls to the metabolic abnormalities (e.g., impaired glucose tolerance) elicited by a high-fat diet (HFD) (He et al. 2012). This observation is probably due to the impaired glucose uptake responses to contractile activity in autophagy-deficient rodents (He et al. 2012), suggesting that exercise-activated autophagy is an obligatory biological barrier against the deleterious effects of surplus dietary lipid. Of note is that post-exercise increases in adiponectin, an adipose tissue-secreted hormone that regulates blood glucose and insulin sensitivity (Diez & Iglesias 2003), is reduced in autophagy-mutant mice (He et al. 2012). Taken collectively, improvements in metabolic homeostasis (e.g., insulin sensitivity, glucose tolerance) promoted by exercise are dependent on intramuscular autophagy.

Recent findings have shown that the maximal mechanically-invoked growth of skeletal muscle is impaired in rodents fed a chronic HFD, which was associated with high-lipid availability blunting the activation of mTORC1 signalling (Sitnick et al. 2009). In addition, the muscle protein synthetic response to a bolus (21 g) administration of amino acids was shown to be impaired by 7 h of an intravenous (10%) lipid emulsion in young humans

(Stephens et al. 2015). In that study, an inability of amino acids to stimulate mTORC1-mediated 4E-BP1 phosphorylation, independent of changes in insulin/Akt signalling, accounted for the lipid-induced anabolic resistance (Stephens et al. 2015). Collectively, these findings suggest that excess fat availability may diminish the sensitivity of skeletal muscle to physiological 'anabolic' stimuli by specifically disrupting mTORC1 activation. Another possible effect of high-fat availability on cellular anabolism is the activation of the ER unfolded protein response (UPR). Rodents subjected to high-fat feeding or treating skeletal myotubes with saturated FAs, such as palmitate, activates the UPR which enhances phosphorylation of eIF2α at Ser[51], a posttranslational modification that attenuates 'general' protein translation to restore the folding capacity of the ER (Deldicque et al. 2011; Deldicque et al. 2010). This UPR response can also signal to autophagy, facilitating concomitant removal of misfolded proteins that consequently accumulate in the ER lumen under stress (B'Chir et al. 2013). Moreover, the ER is a major site of triglyceride formation (Wolins et al. 2006) and elevating the lipid saturation content of ER membranes induces activation of the UPR (Volmer et al. 2013); hence, lipid-induced reductions in MPS may serve as a homeostatic countermeasure against excess FAs infiltrating the ER. Whether an acute or chronic lipid challenge in skeletal muscle stimulates autophagy to mitigate removal of excess ER-accumulated and/or –synthesised triglyceride is unknown. Moreover, the potentially beneficial metabolic effect of strenuous exercise on these protein turnover responses to lipid availability is similarly, unresolved.

Chapter Three

3 Methodology and Design

In accordance with ACU guidelines, the methods used for each individual study are subsequently described fully. The methodology sections outlined in chapters four and five, contain methods presented in a format based on the specific requirements of the journals the respective studies have been published in.

3.1 Study 1: Modulation of Autophagy Signalling with Resistance Exercise and Protein Ingestion Following Short-Term Energy Deficit

Subjects

Skeletal muscle biopsy samples used for analysis in this study were obtained from human subjects that were recruited from a previous study (Areta et al. 2014). Details regarding methodology discussed herein pertain only to the analytical procedures undertaken on the samples analysed. Complete information regarding subject characteristics, ethical compliance, physiological testing, the experimental design (including dietary interventions) and experimental trials are outlined in detail in Chapter Four.

Analytical Procedures

Western Blotting

Approximately 40 mg of skeletal muscle was homogenised in a 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 4ºC and the supernatant was collected for Western blot analysis. After determination of protein concentration using a BCA protein assay (Pierce, Rockford, USA), lysate was resuspended in Laemmli sample buffer, separated by SDS-PAGE and transferred to polyvinylidine fluoride (PVDF) membranes, blocked with 5% non-fat milk, washed with 10 mM Tris·HCl, 100 mM NaCl and 0.02% Tween 20 and incubated with a primary antibody (1:1,000) overnight at 4°C on a shaker. Membranes were incubated the next day with a secondary antibody (1:2,000) and proteins were detected via enhanced chemiluminescence (Amersham Biosciences,

Buckinghamshire, UK; Pierce Biotechnology, Rockford, USA) and quantified by densitometry (Chemidoc, BioRad, Gladesville, Australia). All sample (40 µg protein) time points for each subject were run singularly on the same gel. For all proteins analysed, volume density of each target band was normalised to the total protein loaded into each lane using Stain-free technology (Gurtler et al. 2013).
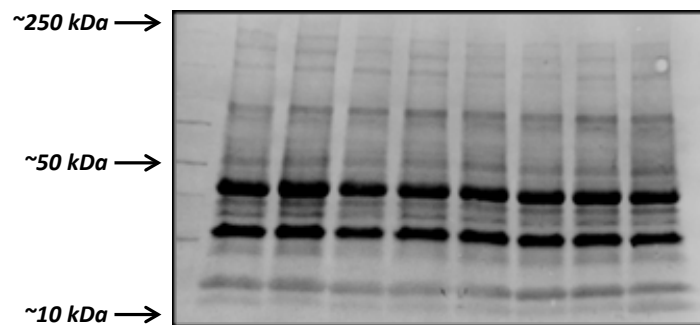
Figure 3.1. Representative of a Stain-Free image generated that is used for immunoblot protein quantification

RNA Extraction and Quantification

Skeletal muscle tissue RNA extraction was performed using a TRIzol-based kit according to the manufacturer's protocol (Invitrogen, Melbourne, Australia, Cat. No. 12183–018A). Briefly, ~20 mg of skeletal muscle tissue was removed from RNAlater-ICE solution and homogenised in TRIzol. After elution through a spin cartridge, extracted RNA was quantified using a QUANT-iT analyser kit (Invitrogen, Melbourne, Australia Cat. No. Q32852) according to the manufacturer's protocol.

Reverse Transcription and Real-Time PCR

Reverse transcription and real-time Polymerase Chain Reaction (RT-PCR) was performed as previously described (Camera et al. 2012). Specifically, first-strand complementary DNA (cDNA) synthesis was performed using commercially available TaqMan Reverse Transcription Reagents (Invitrogen) prepared in a final reaction volume of 20 μL. All RNA samples, including controls were reverse transcribed to cDNA in a single run from the same reverse transcription master mix. Serial dilutions of template RNA were added to ensure efficiency of reverse transcription to calculate a standard curve used for real-time RT-PCR. Quantification of mRNA (in duplicate) was performed using a Rotor-Gene 3000 Centrifugal Real-Time Cycler (Corbett Research, Mortlake, Australia). Taqman-FAM-labelled primer/probes were used. GAPDH (Cat. No. Hs 99999905) has been validated as an exercise housekeeping gene (Jemiolo & Trappe 2004) and was used to normalise threshold cycle (CT) values. GAPDH values were stably expressed between conditions (data not shown). The relative amount of mRNA was calculated using the relative quantification ($\Delta\Delta$CT) method (Livak & Schmittgen 2001).

Statistical Analysis

Data were analysed using two-way repeated measures analysis of variance (ANOVA) with Student-Newman-Keuls post hoc analysis (gender $\times$ time) (SigmaStat for Windows; Version 3.10). As there were no significant differences between sexes for cell signalling and gene expression responses, data were combined for further analysis using a one-way repeated measures ANOVA (two-tailed) with Student-Newman-Keuls post hoc testing (treatment only). Data were log-transformed when tests for normality and/or equal variance failed and statistical inferences were made based on these data. Statistical significance was set to

P<0.05 and all data are presented as mean ± standard deviation (SD) and expressed as arbitrary units.

3.2 Study 2: Protein Co-ingestion with Alcohol Following Strenuous Exercise Attenuates Alcohol-Induced Intramyocellular Apoptosis and Inhibition of Autophagy

Subjects

As for study 1, skeletal muscle biopsy samples analysed in study 2 were also obtained from human subjects recruited from a previous study (Parr et al. 2014). All details regarding methodology discussed subsequently relate only to the analytical procedures undertaken on samples obtained. Further information concerning the subject characteristics, the experimental design and trials can be found in Chapter Five.

Analytical Procedures

Skeletal Muscle Sample Preparation

For generation of whole muscle lysates, ~40 mg of skeletal muscle was homogenised as described in Section 3.1. Samples were spun at 16,000 g for 30 min at 4 ºC and the supernatant was collected for Western blot analysis. Nuclear and cytoplasmic extracts were prepared using an NE-PER fractionation kit according to the manufacturer's instructions (Thermo Scientific, Rockford, IL). In brief, ~30 mg of skeletal muscle was homogenised on ice, by hand, using a glass Dounce homogeniser in CER-I (cytoplasmic extraction reagent-I) buffer supplemented with protease and phosphatase inhibitors. Homogenised samples were vortexed and incubated on ice for 10 min, after which the CER-II buffer was added. CER-II-containing samples were vortexed and subsequently spun at 16,000 g for 5 min at 4 ºC with the supernatants containing cytoplasmic proteins removed and the remaining nuclei-containing pellet resuspended in nuclear extraction reagent buffer (supplemented with inhibitors). Nuclear lysates were subsequently incubated on ice and vortexed for 15 s every

10 min for a total of 40 min, with a final 10 min centrifugation (16,000 *g* at 4 ℃) for collection of nuclear supernatants.

Western Blotting

After determination of protein concentration using a BCA protein assay (Pierce, Rockford, USA), all lysates (50 μg and 10 μg of protein for whole muscle and fractionated lysates, respectively) were resuspended in Laemmli sample buffer and loaded into 4–20% Mini-PROTEAN TGX Stain-Free™ Gels (Bio-Rad, California, USA). All immunoblotting procedures for fractionated and whole cell lysates were then performed without any deviation from the protocol that has been described in Section 3.1. Purity of the cytoplasmic and nuclear fractions was determined by immunoblotting for the glycolytic enzyme GAPDH and the nuclear histone 2B, respectively.

RNA Extraction, Quantification, Reverse Transcription and Real-Time PCR

With the exception of quantification of mRNA being performed using a CFX96 Touch™ Real-Time PCR Detection System (Bio Rad, California, USA), this analysis was undertaken without any deviation from the protocol described in Section 3.1.

Cell Death ELISA

Detection of DNA damage and cell death (apoptosis) was performed using a cell death detection ELISA (Roche Diagnostics, Mannheim, Germany). The cell death ELISA quantitatively determines apoptotic DNA fragmentation by measuring the cytoplasmic

histone-associated mono- and oligonucleosomes. Briefly, even concentrations (120 µg) of nuclei-free cytoplasmic lysates were loaded as an antigen source to an anti-histone monoclonal antibody fixated to the walls of microplate modules. Lysates were loaded prior to the addition of an anti-DNA secondary antibody conjugated to peroxidase. The amount of peroxidase retained in the immunocomplex was determined photometrically by incubating with 2,2′-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) as a substrate on a plate shaker (300 rpm) for ~15 min at room temperature (20 °C). Absorbance was measured at a wavelength of 405 nm using a SpectraMax Paradigm plate reader (Molecular Devices, Sunnyvale, CA, USA). Samples were measured in duplicate on the same plate and absorbance values were normalised to µg of protein loaded in the assay per sample.

Reduced (GSH) to Oxidised (GSSG) Glutathione Ratio

Detection of oxidative stress was performed using a GSH/GSSG Ratio Detection Assay Kit (Abcam, Melbourne, Australia). Cytoplasmic lysates were first deproteinised by adding trichloroacetic acid (TCA) to the samples in a 1:10 dilution. Samples were then incubated on ice (15 min) and briefly centrifuged (5 min, 12,000 g at 4 °C) with the supernatant removed and neutralised of excess TCA using a neutralising solution (Abcam, Melbourne, Australia). The assay was performed using standards for reduced (GSH) and oxidised (GSSG) glutathione. Samples were incubated for ~45 min at room temperature (20 °C) in a Thiol Green Stock solution diluted in assay buffer (GAM) and a 25X GSSG Probe diluted in GAM solution for the detection of reduced and oxidised glutathione, respectively. Fluorescence was measured at an excitation/emission wavelength of 490/520 nm using a SpectraMax Paradigm plate reader (Molecular Devices, Sunnyvale, CA, USA). Samples were measured in duplicate

on the same plate and fluorescence values were normalised to μg of protein loaded in the assay per sample.

Statistical Analysis

Data were analysed using two-way repeated measures analysis of variance (ANOVA) with Student-Newman-Keuls post-hoc analysis (time $\times$ treatment) performed when an overall statistically significant difference in group means of a particular comparison was found (SigmaPlot for Windows; Version 12.5). Significance was set to $P<0.05$ and all data are presented as mean $\pm$ standard deviation (SD). When tests for normality and/or equal variance failed, data were log-transformed and statistical inferences were made based on these data.

3.3 Study 3: Exercise Sensitises Skeletal Muscle to the Intramyocellular Anabolic Effects of Protein Ingestion Following an Intralipid Infusion

Subjects

Twenty-nine sedentary, overweight, but otherwise apparently healthy male subjects volunteered to participate in this study. Subjects were excluded if they 1) regularly (>3 times per week) engaged in strenuous physical activity, 2) were smokers, or 3) taking medication(s) at the time of the study that contravened any of the outcome measurements. Subjects were advised of any possible risks associated with the study prior to providing written informed consent. The study was approved by the Human Research Ethics Committee of ACU (2016-53H) and was carried out in accordance with the standards set by the latest revision of the *Declaration of Helsinki*. The study was registered as an Australian/New Zealand Clinical Trial (ID: ACTRN12615000988550). One subject was excluded from analysis due to fasting blood glucose values on the morning of an experimental trial exceeding 6.0 mmol·L^{-1}. Therefore, twenty-eight subjects were included in the final analyses.

Experimental Design

The study employed a parallel-groups design in which subjects were allocated to a single experimental intervention where they received either an intravenous lipid infusion (lipid; $n = 9$), the same lipid infusion with the concomitant performance of exercise (exercise/lipid; $n = 10$), or a saline control infusion (control; $n = 9$). Group stratification was by fat-free mass (FFM), BMI and age.

Physiological Testing

At least 1 week prior to an experimental trial, subjects undertook assessments of body composition, peak oxygen uptake ($\dot{V}O_{2peak}$) and peak power output (PPO), and maximum strength of the quadriceps. Body composition was determined with whole-body dual-energy X-ray absorptiometry (DXA) analysis (GE Lunar iDXA Pro, enCORE software Version 16). $\dot{V}O_{2peak}$ was determined during an incremental test to volitional fatigue on a Lode cycle ergometer (Groningen, The Netherlands) using an automated breath-by-breath metabolic system (Parvo Medics, Utah, USA). Following a 5 min warm-up at 50-75 Watts (W), subjects commenced cycling at a workload of 1W/kg/BM for 150 s and the workload was increased incrementally by 25 W every 150 s until volitional fatigue. The test was terminated when at least two of the following criteria were met: 1) an inability to maintain a pedalling cadence over 70 rpms, 2) an RER >1.10, 3) an increase in HR within 10 bpm of the participant's age-predicted maximum, or 4) a rating of perceived exertion on the Borg scale >17. $\dot{V}O_{2peak}$ was recorded as the highest value obtained over any 30 s period. Maximum strength of the quadriceps was determined during a series of single repetitions on a seated, plate-loaded leg extension machine until the maximal load was established (1-RM), which was defined as an ability to displace the weight through the entire range of motion (i.e., complete extension of the knee) once, but not a second time.

Diet and Exercise Control

For the 48 h prior to an experimental trial, subjects were instructed to refrain from participating in any strenuous physical activity and to abstain from consuming any caffeine or alcohol. Subjects were provided with pre-packaged food and drinks to be consumed as the final, high-carbohydrate meal (3 g carbohydrate·kg^{-1} BM, 0.3 g fat·kg^{-1} BM, 0.5 g protein·kg^{-1} BM) the night before an experimental trial.

Experimental Trials

Subjects reported to the laboratory at ~0700 h on the morning of an experimental trial following a ~10-12 h overnight fast. After resting in the supine position for ~15 min, a Teflon catheter was inserted into the antecubital vein of each forearm and a baseline blood sample (~4 mL) was obtained. A 20% intravenous lipid (Baxter, NSW, Australia) infusion (intralipid; 20% soybean oil, 1.2% egg yolk phospholipids, 2.25% glycerin and water for infusion) was subsequently administered at a rate of 1.5 mL·min^{-1} for 5 h, or a saline (0.9%) control infusion at the identical rate for the same duration with blood samples taken every hour throughout the infusions. Heparin (Pfizer, NSW, Australia), an anticoagulant, was infused in combination with intralipid to catalyse the release of FFAs from the emulsion into the circulation. Following an initial bolus injection (200 U), heparin was infused at a continuous rate (600 U·h^{-1}) alongside intralipid for the 5 h period. Immediately after the commencement of intralipid, subjects in the exercise group undertook a concurrent exercise bout comprised of 30 min of cycling at ~60% of $\dot{V}O_{2peak}$ followed by resistance exercise (7 × 5 knee extension repetitions, 80% of 1 RM, with an additional 8th set at the same lifting intensity performed to volitional fatigue). Subjects in the non-exercising groups rested. After 2 h in all experimental groups, subjects received a primed, continuous infusion of L-[*ring*-$^{13}C_6$]-phenylalanine (prime: 2.1375 μmol·kg; continuous: 0.0475 μmol·kg·min^{-1}) and L-[3, 5-D$_2$]-tyrosine (prime: 0.824 μmol·kg; continuous: 0.018 μmol·kg·min^{-1}) that would continue for the remainder of the experimental trial day. Tracer infusions were administered for the determination of mixed-muscle and whole-body protein synthesis responses. Note that due to technical issues associated with measurement of plasma tracer enrichments, there has been an extended delay in obtaining these data. As such, tracer-derived estimates of protein synthesis are not included in this thesis but will be incorporated in the final manuscript arising from this study. Upon completion of the 5 h lipid or saline infusion, an initial muscle biopsy was

obtained from the *vastus lateralis* under local anaesthesia (1% lignocaine) using a 5 mm Bergstrom needle modified for suction. Immediately following the first muscle biopsy, subjects in each group ingested 500 mL of a whey protein beverage (30 g, WPI; Swisse, Victoria, Australia) and rested throughout a 4 h period, during which time blood samples were taken every hour. Additional muscle biopsies were obtained 2 and 4 h after protein ingestion (Figure 6.1). Biopsies were taken from separate incision sites (~2-3 cm apart), cleared of any visible adipose and/or connective tissue, snap-frozen in liquid nitrogen and stored at −80 ℃ for subsequent analysis. Blood samples were centrifuged at 4 ℃ (1,500 *g*, 10 min) and aliquots of plasma were stored at −80 ℃.

Analytical Procedures

Plasma Analyses

FFAs were analysed using a nonesterified fatty acid (NEFA) kit (Wako Pure Chemical Industries, Osaka, Japan). Blood glucose concentrations were determined using a YSI (model 2900) Biochemistry Analyser (Xylem, QLD, Australia). Plasma insulin, tumour necrosis factor α (TNF-α) and interleukin-6 (IL-6) were measured utilising commercially available and customised Milliplex human magnetic bead panels (Millipore, Massachusetts, USA) according to the manufacturer's instructions. Analytes were quantified in duplicate using the Magpix system using xPONENT 4.2 software. Two quality controls of designated ranges were run with each assay to ensure validity of the data generated.

Western Blotting

This analysis was performed without any deviation from the protocol described in Section 3.1.

Citrate Synthase Activity Assay

Citrate synthase activity was determined as previously described (Granata et al. 2016). In brief, samples were analysed in duplicate by loading 5 µL of whole muscle skeletal muscle lysate (prepared at a concentration of 2 mg·mL^{-1}) into each well of a 96-well microtiter plate containing a reagent cocktail of 25 µL of 1 mM DTNB [5,5′-Dithiobis(2-nitrobenzoic acid)], 40 µL of 3 mM Acetyl CoA and 165 µL of 100 mM Tris buffer (pH 8.3). Following the addition of 15 µL of Oxaloacetic acid (10 mM) to each well, absorbance was measured at 30 s intervals for 5 min at a wavelength of 412 nm using a SpectraMax Paradigm plate reader (Molecular Devices, Sunnyvale, CA). Citrate Synthase activity is expressed as µmol per minute, per gram of muscle protein.

Statistical Analysis

Data were analysed using two-way analysis of variance (ANOVA) with Student-Newman-Keuls post-hoc analysis (time $\times$ treatment) performed when an overall statistically significant difference in group means of a particular comparison was found (SigmaPlot for Windows; Version 12.5). Subject characteristics and plasma areas under the curve (AUC) were analysed using a one-factor ANOVA with the same post-hoc analysis. Significance was set to $P<0.05$ and all data are presented as mean ± SEM. When tests for normality and/or equal variance failed for Western blot densitometry values, data were log-transformed and statistical inferences were made based on these data.

Preface to Chapter Four

The following chapter examines the molecular activation/regulation of autophagy following a 5 day period of dietary energy restriction and in response to resistance-based exercise undertaken after this period, in which protein was ingested during recovery from the exercise bout. Chronic periods (e.g., 6 months) of energy restriction elevate markers of skeletal muscle autophagy (Wohlgemuth et al. 2011; Wohlgemuth et al. 2010), while endurance-based exercise is a potent stimulus for autophagy that may be augmented when the exercise bout is undertaken fasted (Schwalm et al. 2015; Jamart et al. 2013). Thus, although resistance exercise (REX) alone or in combination with the ingestion of protein/carbohydrate may attenuate post-exercise autophagic processes (Fry et al. 2013; Glynn et al. 2010), the synergistic effects of REX contraction in the face of low intracellular energy availability is an attractive physiological model to investigate these integrated effects on autophagy *in vivo* human skeletal muscle and will be addressed in the subsequent chapter.

Chapter Four

4 *Study 1*: Modulation of Autophagy Signalling with Resistance Exercise and Protein Ingestion Following Short-Term Energy Deficit

Adapted from:

4.1 Abstract

Autophagy contributes to remodelling of skeletal muscle and is sensitive to contractile activity and prevailing energy availability. Changes in targeted genes and proteins with roles in autophagy were investigated following 5 days of energy balance (EB), energy deficit (ED) and resistance exercise (REX) after ED. Muscle biopsies from 15 subjects (8 males, 7 females) were taken at rest following 5 days of EB [45 kcal·kg fat free mass $(FFM)^{-1}·day^{-1}$] and 5 days of ED (30 kcal·kg $FFM^{-1}·day^{-1}$). After ED, subjects completed a bout of REX and consumed either placebo (PLA) or 30 g whey protein (PRO) immediately post-exercise. Muscle biopsies were obtained at 1 and 4 h into recovery in each trial. Resting protein levels of autophagy-related gene protein 5 (Atg5) decreased after ED compared to EB (~23%, P<0.001) and remained below EB from 1-4 h post-exercise in PLA (~17%) and at 1 h in PRO (~18%, P<0.05). In addition, conjugated Atg5 (cAtg12) decreased below EB in PLA at 4 h (~20, P<0.05); however its values were increased above this time point in PRO at 4 h alongside increases in FOXO1 above EB (~22-26%, P<0.05). Notably, these changes were subsequent to increases in Unc-51 like kinase 1^{Ser757} phosphorylation (~60%) 1 h post-exercise in PRO. No significant changes in gene expression of selected autophagy markers were found, but EGR-1 increased above ED and EB in PLA (~417-864%) and PRO (~1417-2731%) trials 1 h post-exercise (P<0.001). Post-exercise protein availability, compared to placebo, can selectively promote autophagic responses to REX in ED.

4.2 Introduction

Maintenance and remodelling of skeletal muscle mass depends on the net balance between simultaneous processes of muscle protein synthesis (MPS) and muscle protein breakdown (MPB). Periods of energy deficit (ED) are associated with losses of lean body mass (Mettler et al. 2010) and it has been previously demonstrated that short-term ED through dietary restriction reduces post-absorptive rates of MPS compared to energy balance (EB) (Pasiakos et al. 2010; Areta et al. 2014). Intracellular pathways of protein degradation underlying this increased MPB response are not well characterised, although increased mRNA expression of ubiquitin proteasomal system (UPS) markers have been reported following short-term ED (Carbone et al. 2013; Carbone et al. 2014). However, this UPS response is repressed following protein consumption (Carbone et al. 2013), suggesting a possible muscle protein 'sparing' effect of protein ingestion when in ED.

The loss of muscle proteins resulting from ED (Mettler et al. 2010) may, in part, be attributed to enhanced activity of the autophagy-lysosomal pathway (henceforth termed autophagy); chronic (6 months) periods of ED increases mRNA and protein markers of autophagy (Wohlgemuth et al. 2010). Autophagy degrades, via lysosomal proteolysis, protein aggregates and damaged organelles that in turn are 'recycled' to yield energy for the maintenance of cellular homeostasis (Carroll et al. 2014). As detailed in Chapter 2, autophagy commences with the sequestration of a portion of cytoplasm by a double-membrane vesicle called the autophagosome, which delivers its contents for degradation upon fusion with the lysosome (Mizushima et al. 2011). Formation, expansion and lysosomal fusion of the autophagosome is coordinated by subgroups of autophagy-related gene (Atg) proteins, collectively referred to as the core molecular machinery of autophagy (Yang & Klionsky 2010).

Increases in protein levels and mRNA transcripts of Atgs observed following endurance exercise have been proposed to dispose of accumulative cell debris and alleviate the metabolic perturbations induced by strenuous contractile activity (Jamart et al. 2013; Jamart et al. 2012b; Lira et al. 2013; Schwalm et al. 2015; Moller et al. 2015). This autophagic response may be the result of attenuated insulin/Akt signalling and a diminished cellular energy charge that promotes autophagy through activation of upstream energy- and stress-responsive kinases. Autophagy conceivably initiates an adaptive response to contractile stimuli by restoring EB and facilitating an ultimate transition to intracellular anabolism through protein and nutrient resynthesis. In contrast to endurance exercise, several markers of autophagy decrease or remain unchanged following resistance exercise (REX) in humans (Glynn et al. 2010; Fry et al. 2013). This highlights ubiquitin-mediated proteolysis as the predominant degradation pathway responsible for the initial increases in MPB following REX (Phillips et al. 1997) and may indicate that augmenting the activation of autophagy is dependent on a substantial cellular energy perturbation. Whether the combined effects of short-term ED and REX activate muscle autophagy is unknown. Moreover, the effects of protein ingestion on autophagy signalling following ED and REX have not been determined. Amino acids have been shown to regulate autophagic flux in human HeLa and MCF7 cell lines (Nicklin et al. 2009), but these effects are largely unknown in human skeletal muscle. Accordingly, the aim of this study was to investigate targeted autophagy signalling responses at rest following 5 days ED and subsequently after REX and protein ingestion undertaken in a state of ED. It was hypothesised that ED would increase autophagy signalling and this response would be exacerbated by the metabolic stress induced by REX. An additional hypothesis was that increasing protein availability after REX would reduce the magnitude of autophagy signalling.

4.3 Materials and Methods

Subjects

Fifteen (8 males, 7 females) young, healthy, resistance-trained subjects [male: age 27 ± 5 yr, body mass (BM) 82.7 ± 6.6 kg, leg press 1-repetition maximum (1-RM) 300 ± 70 kg; female: age 28 ± 4 yr, BM 70.3 ± 7 kg, 1-RM 200 ± 28 kg; values are mean ± SD] were recruited in a previous study (Areta et al. 2014). Subjects were advised of any possible risks associated with the study prior to providing written informed consent. The study was approved by the Australian Institute of Sport Ethics Committee and conformed to the standards set by the latest revision of the Declaration of Helsinki.

Experimental Design

The study employed a within-subject design where each subject completed four experimental interventions for measures of resting energy balance (EB), resting energy deficit (ED), and then resistance exercise (REX) following ED with ingestion of protein or a non-nutrient placebo (PRO and PLA trials, respectively). The EB trial was always undertaken before ED trials to eliminate any possible metabolic disruptions elicited by a previous ED intervention (Figure 1). For ED trials, the protein/placebo ingestion following REX was randomised and counterbalanced.

Maximal Strength

Lower body strength was determined one week prior to experimental trials during a series of single repetitions on an inclined (45º) leg press (GLPH1100, Body-Solid, Forest Park IL) until the maximal load was established (1-RM).

Dietary Intervention

Subjects received individualised pre-packaged meals for 5 days prior to an experimental trial. Before the resting EB trial subjects received meals equivalent to an energy availability of 45 kcal·kg fat free mass $(FFM)^{-1}·day^{-1}$, where energy availability was defined as energy intake minus the energy cost of habitual exercise. For ED trials, diets consisted of an energy availability of 30 kcal·kg $FFM^{-1}·day^{-1}$. From days 1-3 of the dietary control period, subjects were permitted to exercise and the diet was adjusted to account for the energy expenditure of exercise sessions in order to maintain energy availability at a set level. In the 48 h prior to an experimental trial (days 4 and 5 of dietary control), subjects refrained from any strenuous physical activity. No alcohol was consumed during the 5 day dietary control period and subjects refrained from caffeine intake 24 h before each trial day. The 5 day ED period was based on previous studies showing that this timeframe is sufficient to cause perturbations in whole-body metabolic homeostasis (Loucks & Thuma 2003; Ihle & Loucks 2004). The protein, carbohydrate and fat content of the diets was 1.4-1.6, 4-4.5 and 1.5-2.5 g·kg body mass $(BM)^{-1}·day^{-1}$ for EB and 1.4-1.6, 3-3.5 and 0.5-1.5 g·kg $BM^{-1}·day^{-1}$ for ED, respectively (Areta et al. 2014). Experimental trials were separated by a 9 day washout period based on data demonstrating that the reduction in resting metabolic rate after a 20 day ED can be restored with 10 days of EB (Weinsier et al. 2000). During the washout period subjects resumed their habitual exercise and diet routine.

Experimental Trials

Following 5 days of dietary control, subjects reported to the laboratory between 0700 and 0800 h after a 10-12 h overnight fast. In both EB and ED trials, subjects rested for a 3 h period and a muscle biopsy was obtained from the *vastus lateralis* under local anaesthesia

(1% lidocaine) using a 5 mm Bergstrom needle modified for suction. For the ED trials, subjects completed a standardised warm up (2 x 5 repetitions at ~50 and ~60% 1-RM) on a leg-press machine before commencing the REX protocol comprising 6 sets of 8 repetitions at ~80% 1-RM (3 min recovery between sets). In a randomised and counterbalanced design, subjects ingested 500 mL of either placebo (PLA: water, vanilla flavour, artificial sweetener) or a protein beverage (PRO: 30 g, ISO8 vanilla WPI; 86.8 g protein, 1.5 g fat, 3.1 g carbohydrates per 100 g; Musashi, Australia) on separate occasions. Subjects would rest throughout a 240 min recovery period during which additional muscle biopsies were obtained at 60 and 240 min post-exercise (Figure 4.1). All muscle samples were stored at -80ºC for subsequent analysis.
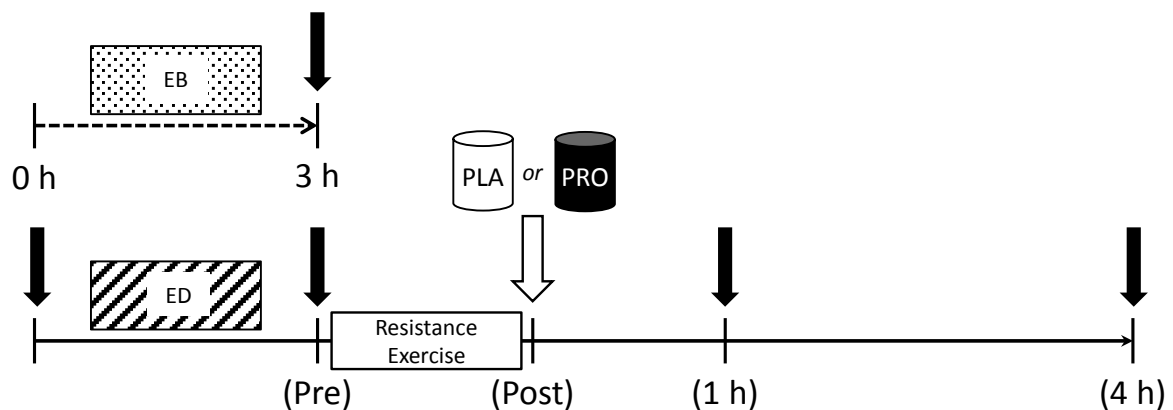
Figure 4.1. Overview of experimental design for Study 1

The resting energy balance (EB) trial was preceded by 5 days of a controlled diet providing 45 kcal·kg FFM-1·day-1. The energy deficit (ED) trials were preceded by 5 days of a controlled diet providing 30 kcal·kg FFM-1·day-1. Solid black arrow; muscle biopsy sample; PLA and PRO represent the respective placebo or 30 g whey protein drink (500 mL). Dashed time-line (EB trial) represent trials undertaken a single time by each subject. Times in parentheses are for ED trials involving exercise and beverage intake.

Analytical Procedures

Western Blotting

Approximately 40 mg of skeletal muscle was homogenised in a 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 4ºC and the supernatant was collected for Western blot analysis. After determination of protein concentration using a BCA protein assay (Pierce, Rockford, USA), lysate was resuspended in Laemmli sample buffer, separated by SDS-PAGE and transferred to polyvinylidine fluoride (PVDF) membranes, blocked with 5% non-fat milk, washed with 10 mM Tris·HCl, 100 mM NaCl and 0.02% Tween 20 and incubated with a primary antibody (1:1,000) overnight at 4°C on a shaker. Membranes were incubated the next day with a secondary antibody (1:2,000) and proteins were detected via enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK; Pierce Biotechnology, Rockford, USA) and quantified by densitometry (Chemidoc, BioRad, Gladesville, Australia). All sample (40 μg protein) time points for each subject were run singularly on the same gel. Polyclonal antibodies against phosphorylated p38 mitogen-activated protein kinase (p38 MAPK)$^{Thr180/Tyr182}$ (no. 4511), p53^{Ser15} (no. 9284), Unc-51-like kinase 1 (ULK1)^{Ser757} (no. 6888), Forkhead box O1 (FOXO1)^{Thr24} (no. 9464), FOXO3a^{Ser253} (no. 9466), eukaryotic initiation factor 2α (eIF2α)^{Ser51} (no. 9721), total autophagy-related gene protein 5 (Atg5) (no. 2630), Beclin-1 (no. 3738), microtubule-associated protein-1 light chain 3 beta (LC3b) (no. 2775), cAtg12 (no. 4180), p62 (no. 5114), ULK1 (no. 6439), p53 (no. 2527), p38 MAPK (no. 9212), FOXO1 (no. 9454), FOXO3a (no. 2497) and eIF2α (no. 9722) were all purchased from Cell Signalling Technology (Danvers, USA). The LC3b antibody detects bands for both the 14 kDa LC3b-I and 16 kDa LC3b-II isoforms. As consistent separation between bands for the

two isoforms was unattainable, only data for the more prominent LC3b-I band are presented. The presence of this specific band was verified by the use of an LC3 Control Extract (no. 11972) purchased from Cell Signalling Technology (Danvers, USA). For all proteins, volume density of each target band was normalised to the total protein loaded into each lane using Stain-Free technology (Gurtler et al. 2013).

RNA Extraction and Quantification

Skeletal muscle tissue RNA extraction was performed using a TRIzol-based kit according to the manufacturer's protocol (Invitrogen, Melbourne, Australia, Cat. No. 12183–018A). Briefly, ~20 mg of skeletal muscle tissue was removed from RNAlater-ICE solution and homogenised in TRIzol. After elution through a spin cartridge, extracted RNA was quantified using a QUANT-iT analyser kit (Invitrogen, Melbourne, Australia Cat. No. Q32852) according to the manufacturer's protocol.

Reverse Transcription and Real-Time PCR

Reverse transcription and real-time Polymerase Chain Reaction (RT-PCR) was performed as previously described (Camera et al. 2012). Quantification of mRNA (in duplicate) was performed using a Rotor-Gene 3000 Centrifugal Real-Time Cycler (Corbett Research, Mortlake, Australia). Taqman-FAM-labelled primer/probes for *LC3b* (Cat. No. Hs 00797944), γ-aminobutyric-acid-type-A-receptor-associated protein (*GABARAP*) (Cat. No. 00925899), *Beclin-1* (Cat. No. Hs 00186838), *Atg12* (Cat. No. Hs 01047860), *Atg4b* (Cat. No. Hs 00367088), Sirtuin-1 (*SIRT1*) (Cat. No. Hs 01009005), *FOXO1* (Cat. No. Hs 01054576), PPARγ-coactivator-1α (*PGC-1α*) (Cat. No. Hs 01016719), Bcl-2/adenovirus E1B

19 kDa-interacting protein-3 (*BNIP3*) (Cat. No. Hs 00969291), vascular endothelial growth factor (*VEGF*) (Cat No. Hs00900055) and early growth response-1 (*EGR-1*) (Cat. No. Hs 00152928) were used. *GAPDH* (Cat. No. Hs 99999905) has been validated as an exercise housekeeping gene (Jemiolo & Trappe 2004) and was used to normalise threshold cycle (CT) values. *GAPDH* values were stably expressed between conditions (data not shown). The relative amount of mRNA was calculated using the relative quantification ($\Delta\Delta$CT) method (Livak & Schmittgen 2001).

Statistical Analysis

Data were analysed using two-way repeated measures analysis of variance (ANOVA) with Student-Newman-Keuls post hoc analysis (gender $\times$ time) (SigmaStat for Windows; Version 3.10). As there were no significant differences between sexes for cell signalling and gene expression responses, data were combined for further analysis using a one-way repeated measures ANOVA (two-tailed) with Student-Newman-Keuls post hoc testing (treatment only). *EGR-1* and *VEGF* mRNA data were log-transformed. Statistical significance was set to $P<0.05$ and all data are presented as mean $\pm$ standard deviation (SD) and expressed as arbitrary units.

4.4 Results

Cell Signalling Proteins

p38 MAPK-p53-eIF2α

There were no significant changes in p38 MAPK$^{\text{Thr180/Tyr182}}$ phosphorylation at any time point (Figure 4.2A), however total p38 MAPK decreased from EB in PLA at 4 h (~23%, $P<0.05$)

and from 1-4 h post-exercise in PRO (~24-25%, $P<0.05$; Figure 4.2B). There were no significant changes in p53^{Ser15} phosphorylation (Figure 4.2C) or total p53 at any time point (Figure 4.2D). No significant changes in the phosphorylation state of eIF2α^{Ser51} (Figure 4.2E) or the abundance of its corresponding total at any time point were found (Figure 4.2F).
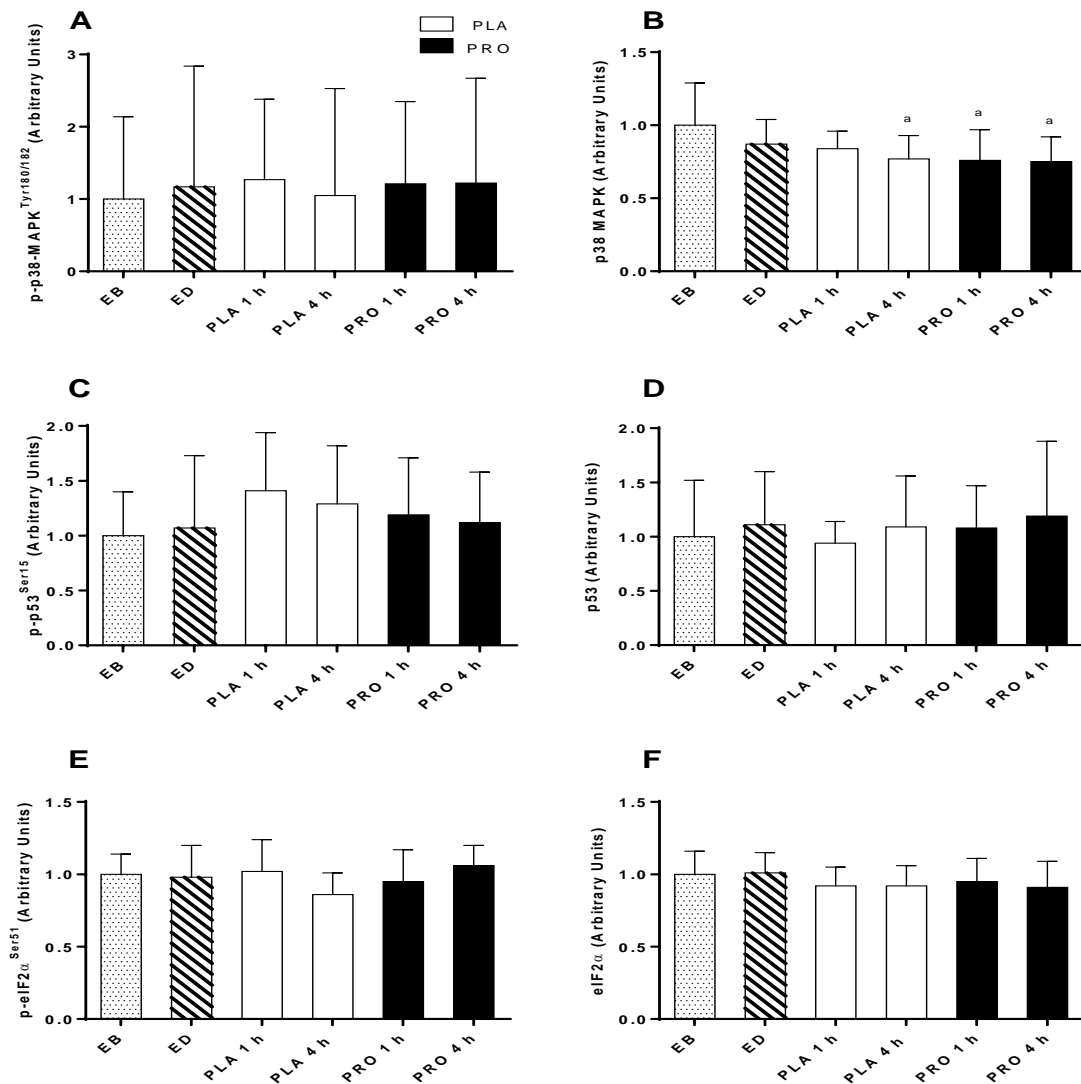
Figure 4.2. Phospho-p38 MAPK$^{Thr180/Tyr182}$ (A), total p38 MAPK (B), phospho-p53^{Ser15} (C), total p53 ($n = 9$; D), phospho-eIF2α^{Ser51} (E) and total eIF2α (F)

Analysed at rest following 5 days of EB, 5 days of ED and a bout of leg press (6 sets × 8 repetitions at 80% 1-RM) in ED with post-exercise ingestion of a placebo (PLA) or 30g of a whey protein drink (PRO). Values are expressed relative to total protein loaded determined by Stain-Free technology and presented in arbitrary units (mean ± SD). Significantly different ($P<0.05$) vs. (a) EB.

FOXO1-FOXO3a-LC3b-I

Although no significant differences in FOXO1^{Thr24} phosphorylation at any time point were observed (Figure 4.3A), there were main treatment effects for total levels of FOXO1 (Figure 4.3B). FOXO1 abundance increased in PRO at 4 h above ED and each PLA post-exercise time point (~20-22%, P<0.05). No significant differences in FOXO3a^{Ser253} phosphorylation (data not shown) or levels of its total were found despite a modest increase in total FOXO3a above resting EB and ED in PLA at 1 h post-exercise (Figure 4.3C). However, there were main treatment effects for total levels of LC3b-I (Figure 4.3D). Total LC3b-I decreased from EB and ED in PLA at 4 h (~21-26%, P<0.05) and below EB in PRO at 1 h (~19%, P=0.053).
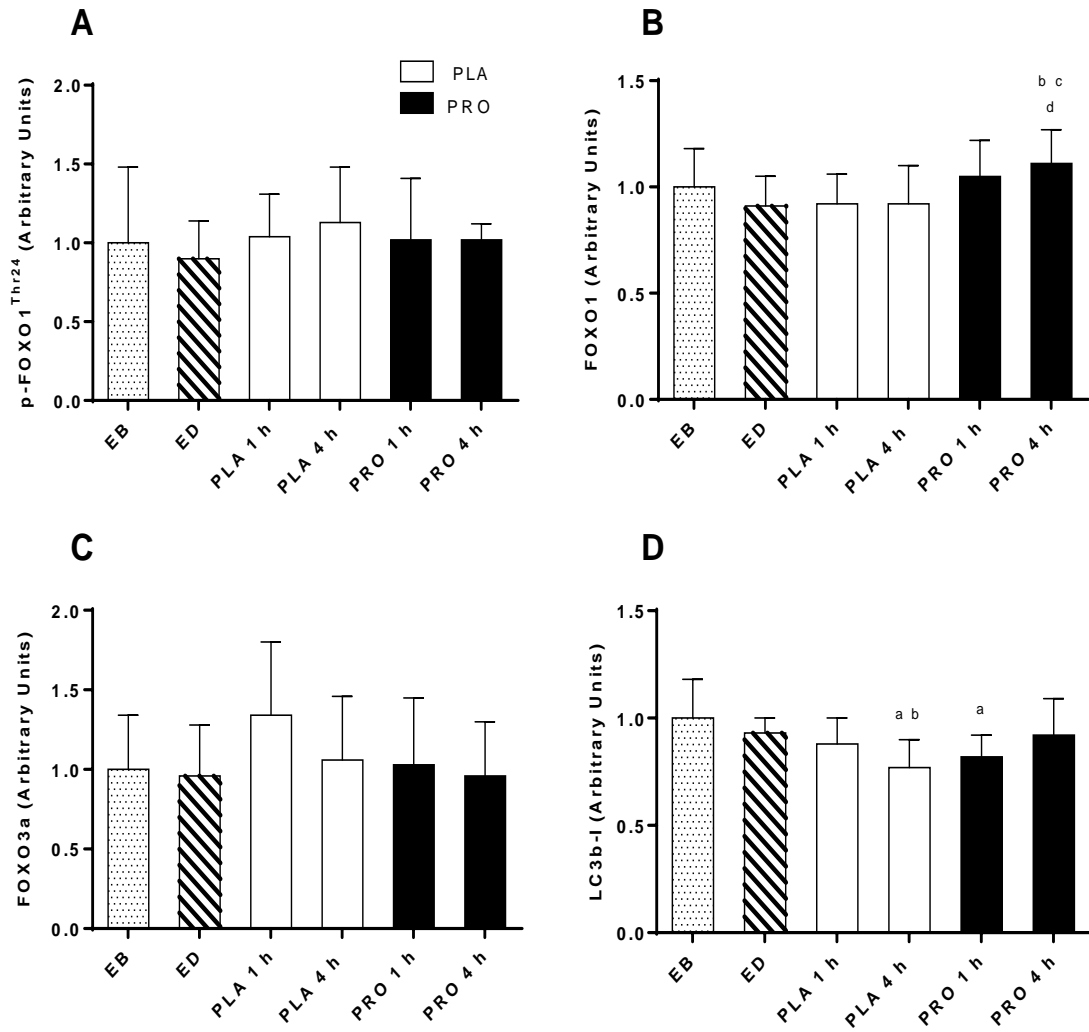
Figure 4.3. Phospho-FOXO1^{Thr24} ($n = 13$; A), total FOXO1 (B), FOXO3a ($n = 13$; C) and LC3b-I ($n = 12$; D)

Analysed at rest following 5 days of EB, 5 days of ED and a bout of leg press (6 sets × 8 repetitions at 80% 1-RM) in ED with post-exercise ingestion of a placebo (PLA) or 30g of a whey protein drink (PRO). Values are expressed relative to total protein loaded determined by Stain-Free technology and presented in arbitrary units (mean ± SD). Significantly different (P<0.05) vs. (b) ED, (c) PLA 1 h and (d) PLA 4 h.

ULK1-Atg5-cAtg12-Beclin-1-p62

There was a main effect of treatment for ULK1^{Ser757} phosphorylation (Figure 4.4A). ULK1 phosphorylation increased in PRO at 1 h post-exercise above resting EB and ED (~30-60%, P<0.05) and was higher than PRO at 4 h (~40%, P<0.05), along with a tendency toward increasing above PLA 4 h (~35%, P=0.07) at this time point. There was also tendency for total ULK1 to increase (~79%, P=0.089) above ED in PRO at 1 h after REX (Figure 4.4B). There were main treatment effects for total levels of Atg5 (Figure 4.4C). Atg5 decreased from EB to ED (~23%, P<0.001) and these levels remained below EB from 1-4 h post-exercise in PLA (~17%, P<0.05) and at 1 h in PRO (~18%, P<0.05). There were main treatment effects for total levels of cAtg12 protein (Figure 4.4D). cAtg12 decreased compared to EB in PLA 4 h (~20%, P<0.05), whereas it increased in PRO at 4 h above this time point (~26%, P<0.05) to similar values observed in EB. There were no significant differences in Beclin-1 (Figure 4.4E) or p62 (Figure 4.4F) protein expression. Beclin-1 tended to decrease from EB to ED but these changes did not reach significance (~14%, P=0.083).
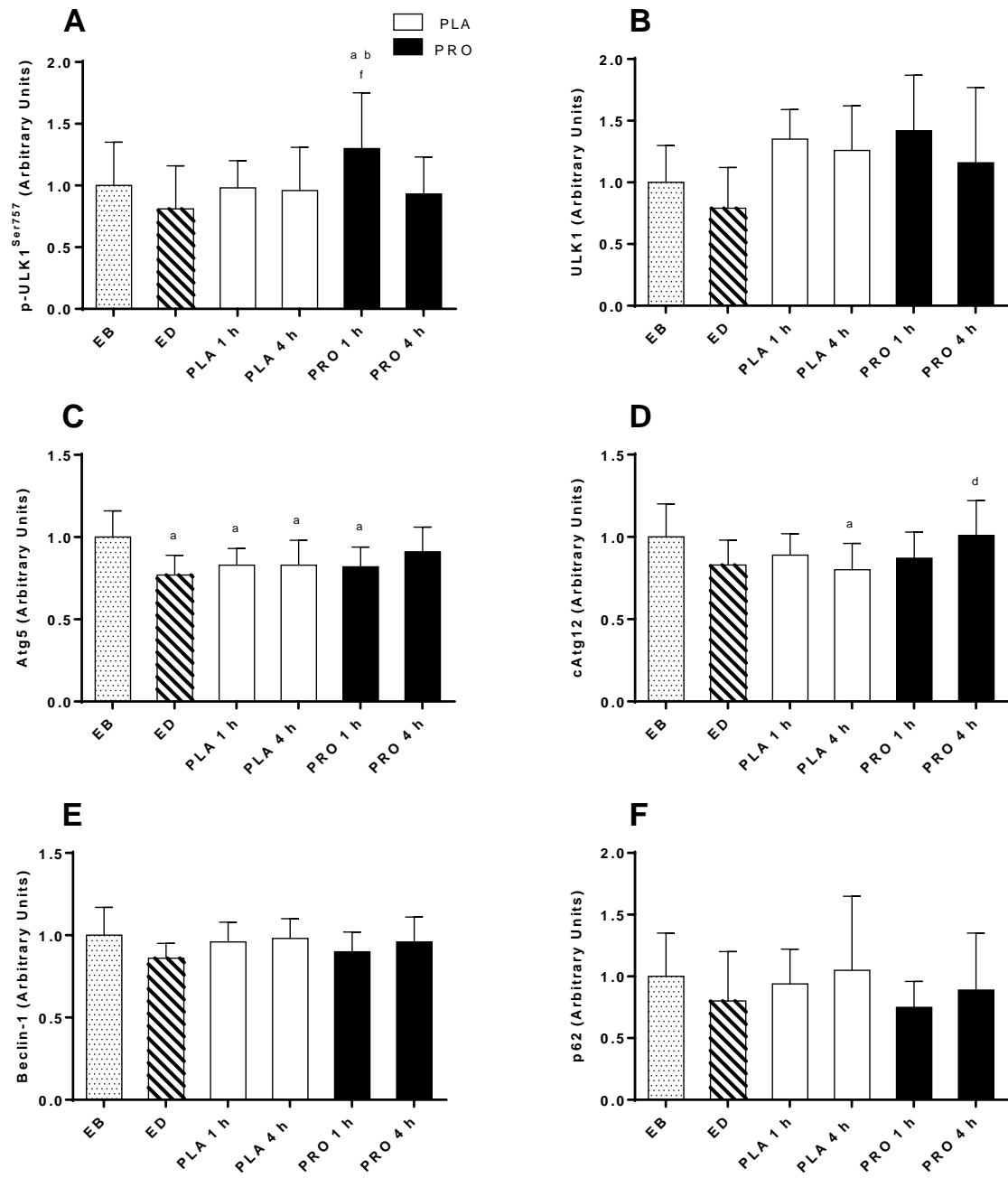
Figure 4.4. Phospho-ULK1^{Ser757} (A), total ULK1 (n = 8; B), Atg5 (C), cAtg12 (D), Beclin-1 (E) and p62 (F)

Analysed at rest following 5 days of EB, 5 days of ED and a bout of leg press (6 sets × 8 repetitions at 80% 1-RM) in ED with post-exercise ingestion of a placebo (PLA) or 30g of a whey protein drink (PRO).Values are expressed relative to total protein loaded determined by Stain-Free technology and presented in arbitrary units (mean ± SD). Significantly different (P<0.05) vs. (a) EB, (b) ED, (d) PLA 4 h and (f) PRO 4 h.

mRNA Expression

Atgs

There were no significant changes at any time in the mRNA expression of genes with roles in autophagy including *Beclin-1*, *Atg12*, *Atg4b*, *GABARAP*, *LC3b* and the mitophagy marker *BNIP3* (Figure 4.5).
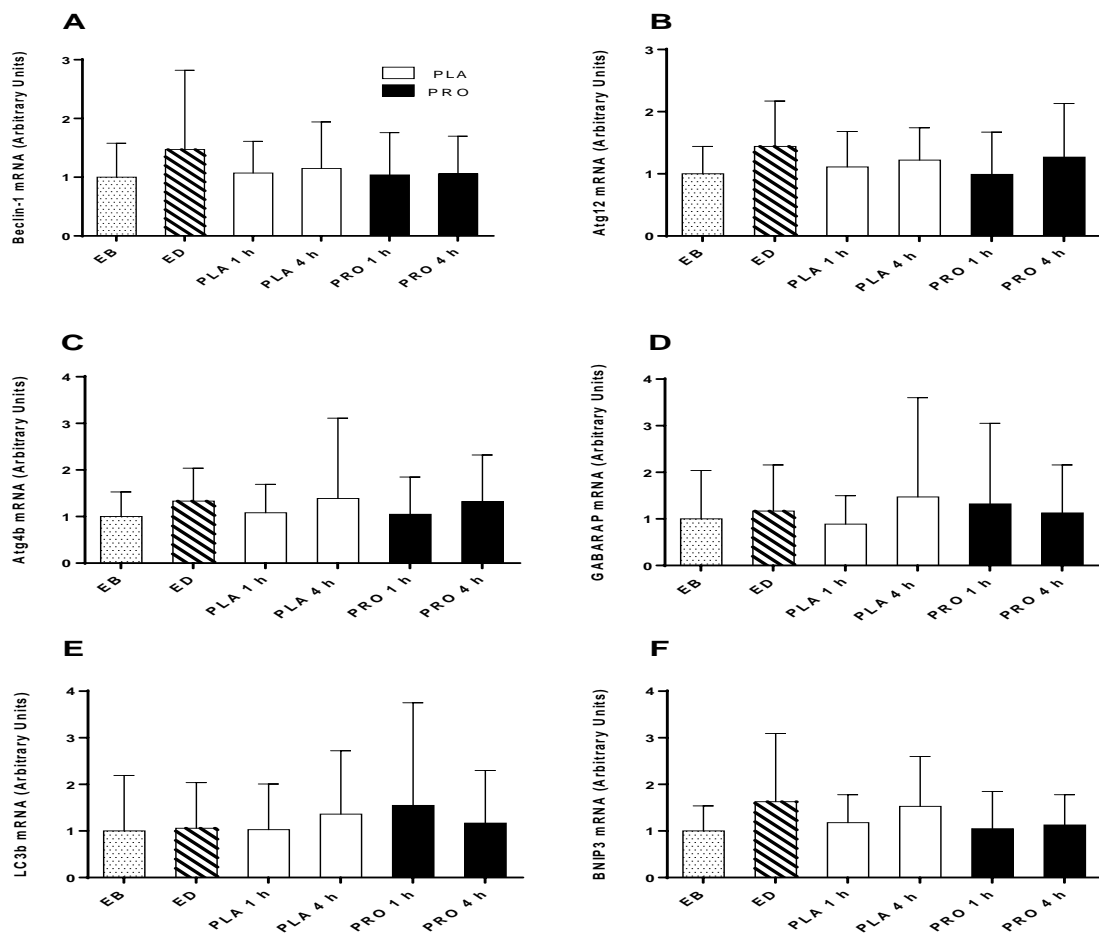
Figure 4.5. *Beclin-1* (A), *Atg12* (B), *Atg4b* (C), *GABARAP* (D), *LC3b* (E) and *BNIP3* (F) gene expression

Analysed at rest following 5 days of EB, 5 days of ED and a bout of leg press (6 sets $\times$ 8 repetitions at 80% 1-RM) in ED with post-exercise ingestion of a placebo (PLA) or 30g of a whey protein drink (PRO). Values are expressed relative to GAPDH and presented in arbitrary units (mean $\pm$ SD, $n = 12$).

SIRT1-FOXO1-PGC-1α-EGR-1-VEGF

There were no significant changes in *SIRT1*, *FOXO1* and *PGC-1α* mRNA expression at any time point (Figure 4.6A-C). There was a main effect of treatment for *EGR-1* mRNA expression after exercise (Figure 4.6D). *EGR-1* mRNA increased above EB (PLA, ~864%; PRO, ~2731%, $P<0.001$) and ED (PLA, ~417%; PRO, ~1417%, $P<0.001$) 1 h post-REX and was higher in PRO at 1 h post-REX than PLA at 4 h (~136%, $P=0.015$). *EGR-1* expression in PRO at 4 h was also elevated above EB and ED (~353-746%, $P<0.01$), whereas its expression in PLA at 4 h only increased significantly above EB (~1100%, $P<0.05$) with a trend toward increasing above ED (~543%, $P=0.083$). Lastly, *VEGF* mRNA increased significantly above both resting conditions in PLA at 4 h only (~47-51%, $P<0.05$; Figure 4.6E).
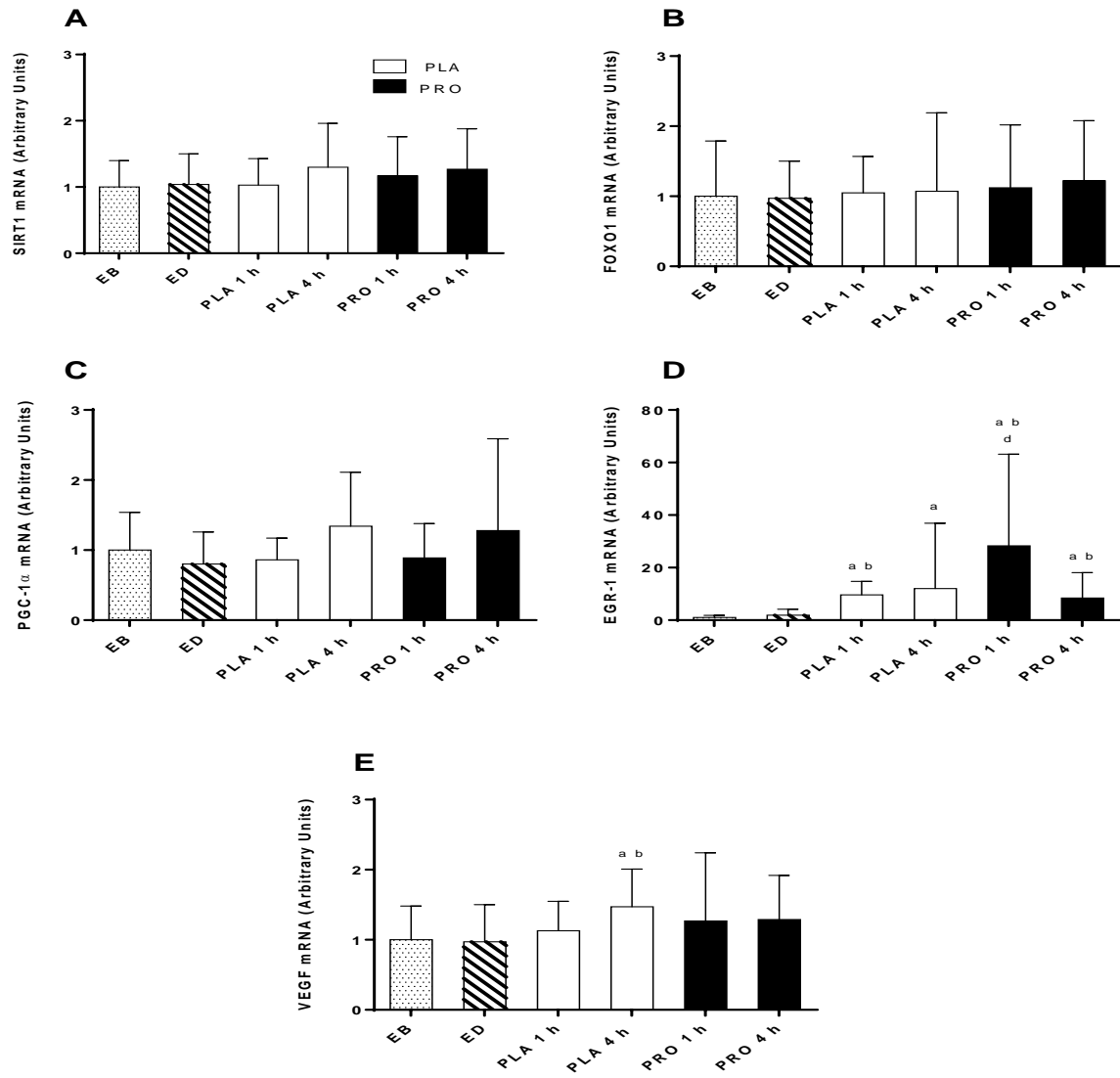
Figure 4.6. *SIRT1* (A), *FOXO1* (B), *PGC-1α* (C) *EGR-1* (D) and *VEGF* (E) gene expression

Analysed at rest following 5 days of EB, 5 days of ED and a bout of leg press (6 sets × 8 repetitions at 80% 1-RM) in ED with post-exercise ingestion of a placebo (PLA) or 30g of a whey protein drink (PRO). Values are expressed relative to GAPDH and presented in arbitrary units (mean ± SD, *n* = 12). Significantly different (P<0.05) vs. (a) EB, (b) ED and (d) PLA 4 h.
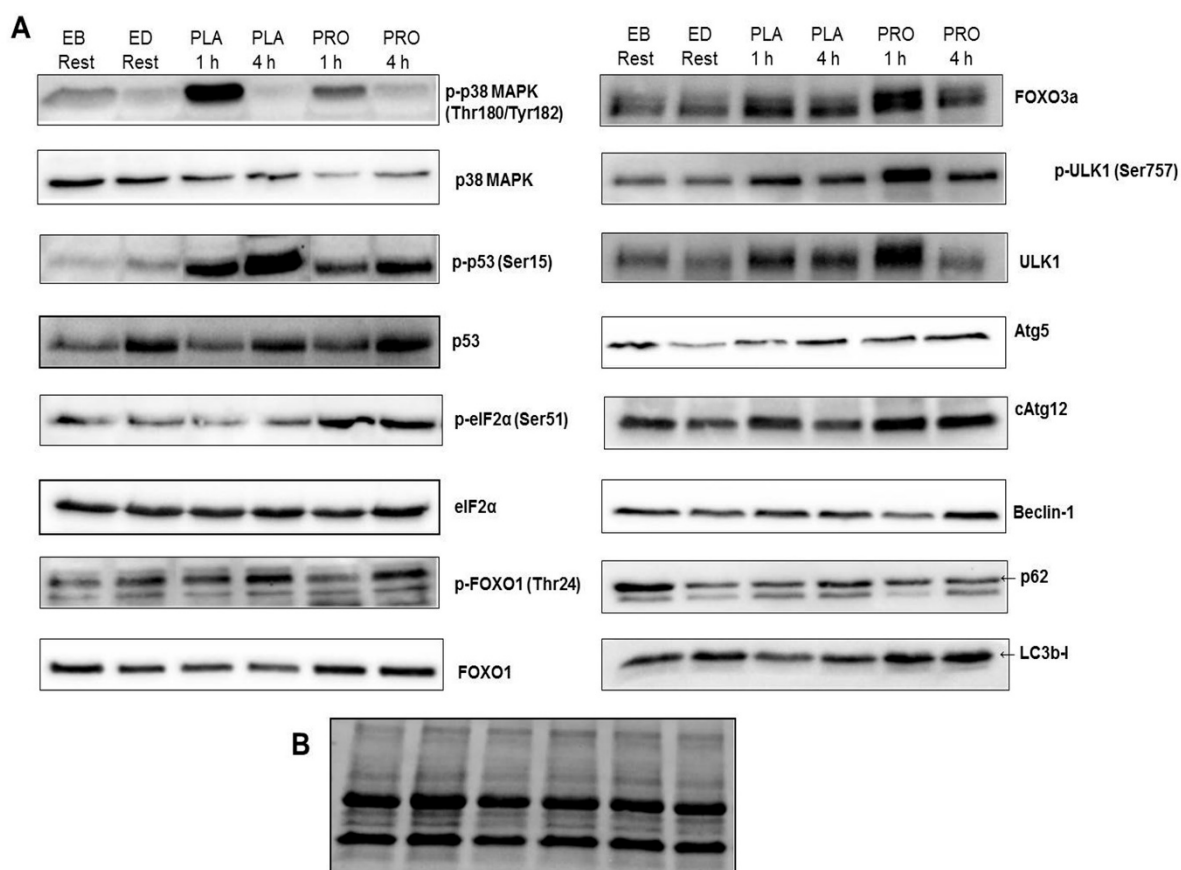
Figure 4.7. Representative blots for all phosphorylated and total proteins (A) and a Stain-Free image of total protein loading (B). Section 3.1 depicts the approximate molecular weight range for the Stain-Free image.

4.5 Discussion

The first novel finding of this study was that both short-term ED and REX commenced after ED in a fasted state and with no nutrient provision during recovery, failed to increase the transcript abundance, phosphorylation state and total protein content of several select molecular markers of autophagy or their upstream regulators. In contrast, Atg5 protein levels decreased following short-term ED, whereas consuming protein after REX increased its conjugated form cAtg12 to levels similar in EB, as well as increasing the total protein abundance of FOXO1 and decreasing p38 MAPK. Moreover, there were no changes in p53^{Ser15} phosphorylation or the gene expression of *SIRT1* and its deacetylase targets *FOXO1* and *PGC-1α* despite large increases in *EGR-1* mRNA abundance post-exercise. This study is

the first to characterise post-absorptive intramuscular autophagy-regulatory responses following an acute bout of REX undertaken in ED in humans.

Short-term energy restriction attenuates markers of autophagy

The constitutive turnover of long-lived proteins, polyubiquitinated aggregates and intracellular organelles by autophagy is critical for preserving the integrity of skeletal muscle mass (Masiero et al. 2009). Considering REX modulates MPB (Phillips et al. 1997) and short-term ED (<21 days) can exacerbate MPB responses (Carbone et al. 2014; Carbone et al. 2013), the expression of autophagy-regulatory genes and proteins following a single bout of REX undertaken in ED were investigated. There were observations of reduced total protein levels of Atg5 and a trend for attenuated Beclin-1, both effectors of autophagosome biogenesis (Yang & Klionsky 2010), following 5 days ED. However, the gene expression, phosphorylation state and total protein levels of several other molecular markers implicated in autophagy were largely unaffected by ED. The original hypothesis was that ED would increase these targets as a means to partition the requisite energy substrate for the maintenance of intracellular homeostasis. Although similar levels of ED (~500-1000 kcal·day^{-1}) in animals (Wohlgemuth et al. 2010) and humans (Wohlgemuth et al. 2011) has previously been shown to increase the expression of Atgs, the duration of energy restriction in these studies were substantially longer (~6 months) than the present investigation. Furthermore, there is evidence to demonstrate that whole-body proteolysis and net protein turnover are attenuated following similar, prolonged restrictions of energy availability (Campbell et al. 2009; Stein et al. 1991), which could suggest a 'threshold' duration of ED is required before autophagy creates a new steady-state protein balance.

5-10 days of ED has been previously reported to reduce post-absorptive rates of MPS (Pasiakos et al. 2010; Areta et al. 2014). Notably, this attenuated response was of a similar magnitude to the ED-induced decline in Atg abundance in the present study and may be a consequence of reduced demands for intracellular amino acids otherwise partitioned by autophagy to maintain MPS. Indeed, autophagy-deficient cells have lower rates of protein synthesis arising from limited amino acid availability (Onodera & Ohsumi 2005). Moreover, $eIF2\alpha^{Ser51}$ phosphorylation, indicative of general protein translational repression (Harding et al. 2000) and activation of an autophagic transcriptional program under certain stresses (including nutrient deprivation) (3), was unchanged following ED. The present findings could suggest that autophagy has a minimal and/or negligible contribution to the acute proteolytic response(s) to short-term periods of moderate energy restriction; rather, the UPS is the predominant system under these circumstances. In this regard, a similar (10 day) ED intervention in healthy adults resulting in a ~60% increase in MPB was accompanied by elevated caspase-3 protein expression (Carbone et al. 2014), while 21 days of ED increased muscle-specific ubiquitin ligase gene expression (Carbone et al. 2013). Another consideration is that autophagy, specifically *de novo* autophagosome biosynthesis, like MPS, is an energy-consuming process (Plomp et al. 1987; Schellens et al. 1988) and thus may have been restrained as a result of reprioritising ATP for alternate cellular processes. Whether ED-induced changes in MPS regulate, or are directly regulated by autophagy, is an area for future investigation.

REX-induced increases in markers of autophagy is augmented by protein ingestion

Compared to the dynamic changes reported for the UPS (Yang et al. 2006; Mascher et al. 2008), there is a paucity of information addressing how REX modulates autophagy and the time course of this response. Two studies have shown decreases in LC3b-II, but not LC3b-I

following REX in young and old individuals (Fry et al. 2013) and REX with protein and carbohydrate co-ingestion (Glynn et al. 2010). Such changes could reflect a reduced autophagic flux after REX as lipidation of cytosolic LC3b-I to LC3b-II results in the latter localising to the inner and outer faces of the autophagosome (Tanida et al. 2005). Moreover, as subjects in these studies were in EB, a more substantial energetic perturbation elicited by exercise-diet interactions may have been required to alter the LC3b-II/LC3b-I ratio. In partial support of this notion, there was a reduction in LC3b-I isoform abundance following ED and REX with placebo ingestion. However, this change occurred without concomitant differences in p62, an ubiquitin-binding protein that bridges substrates to autophagosomes and undergoes degradation itself after interacting with LC3b-II (Pankiv et al. 2007). Therefore, although these results do not preclude enhanced formation of autophagosomes with REX in ED, future work (e.g., immunofluorescence and/or sub-fractionation experiments pinpointing Atg subcellular localisation) *in vivo* human skeletal muscle is required to confirm such regulation. However, a notable finding was the observed increase in the mRNA of the angiogenic marker *VEGF* above both resting conditions with PLA, occurring alongside the changes in LC3b-I. Given that VEGF-mediated angiogenesis is hypothesised to couple oxygen and nutrient delivery to support contractile activity (Arany et al. 2008), and that angiogenic adaptations to exercise is attenuated in mice with a genetic disruption of autophagy (Lira et al. 2013), these results could imply a coordinated autophagic and angiogenic response to mitigate the metabolic demands and subsequent adaptation to REX in ED.

Consuming protein-containing meals during ED can reduce UPS-mediated proteolysis (Carbone et al. 2013), thereby potentially contributing to the 'sparing' of muscle mass loss during conditions of negative EB. Insulin has been reported to exert an inhibitory effect toward intramuscular autophagy in mice (Naito et al. 2013), while in other cell types amino acid availability restricts autophagic flux (Nicklin et al. 2009). Considering the previously

reported insulinotrophic effects of whey (Areta et al. 2014) combined with the aforementioned potential for protein availability to repress the UPS (Carbone et al. 2013), it seemed reasonable to hypothesise that protein ingestion would similarly restrict the exercise-induced increase in autophagy markers in ED. In contrast, REX with PRO partially restored the ED-induced decline in Atg5 by increasing cAtg12 protein abundance above PLA. Similarly, FOXO1, a transcription-independent (Zhao et al. 2010) and dependent (Milan et al. 2015) regulator of autophagy also increased with PRO post-exercise compared to ED and PLA. It is not entirely apparent how PRO mediated increases in the autophagy signalling proteins as these differences were independent of changes in mRNA transcripts of genes involved in autophagy (including corresponding *Beclin-1*, *Atg12*, *LC3b* and *FOXO1*) and the phosphorylation state of FOXO3a, a transcriptional regulator of *Atgs* such as *LC3b* (Mammucari et al. 2007). Nevertheless, Desgeorges and colleagues (2014) previously reported an increase Atg protein content following a starvation stimulus in myotubes that prevailed in the absence of an increase in gene expression, leading the authors to hypothesise that these proteins were translated from a pre-existing pool of mRNA as an abrupt response to the stresses imposed. Considering post-exercise protein ingestion modulates markers of the UPS (Borgenvik et al. 2012; Areta et al. 2013), it is also possible that PRO repressed the activities of skeletal muscle-enriched ubiquitin ligases (e.g., neural precursor cell expressed, developmentally downregulated 4, NEDD4) that tag Atgs for degradation (Kuang et al. 2013). Such a paradigm represents a largely unexplored and intriguing area of future research investigating the effects of energy fluctuations and protein transcriptional and/or translational control.

PRO also increased post-exercise ULK1^{Ser757} phosphorylation, an mTORC1 substrate that inhibits autophagy induction by disrupting AMPK accessibility to other ULK1 amino acid residues (Kim et al. 2011). The observed increase in ULK1 phosphorylation may signify

a temporal restriction of autophagy until a later post-exercise period, as no differences in AMPK phosphorylation between trials post-exercise was reported previously (Areta et al. 2014). Nonetheless, the similar responses in MPS (Areta et al. 2014) and Atg protein abundance with PRO post-exercise suggests a restorative effect toward autophagy regulatory processes when exogenous protein is available in situations of ED. Furthermore, these changes occurred alongside reductions in total p38 MAPK content, which has been shown to facilitate elevated oxidative stress to autophagy-mediated muscle atrophy (McClung et al. 2010). Thus, protein ingestion following REX in ED may stimulate autophagy to ultimately promote a positive net protein balance. While previous findings demonstrate that exogenous rather than intramuscular amino acid availability in EB is a stronger determinant of MPS (Bohe et al. 2003), the present work highlights that autophagy may contribute to the greater total protein turnover and muscle remodelling processes associated with protein ingestion following REX in ED.

REX enhances EGR-1, but not SIRT1 expression in ED

There were large increases in mRNA transcripts encoding for the transcription factor *EGR-1* following REX independent of protein availability. EGR-1 transcriptionally upregulates *SIRT1* in skeletal muscle cells following mechanical stretch (Pardo et al. 2011), and increases in *EGR-1* and *SIRT1* mRNA have been reported following high-intensity endurance exercise (Edgett et al. 2013). Although SIRT1 regulates autophagy by direct deacetylation of various Atgs (Lee et al. 2008), such findings suggest that *EGR-1* gene regulation following exercise is influenced predominantly by contractile/mechanical overload rather than energy status. Although changes in the total protein content (Little et al. 2010b), nuclear activity (Gurd et al. 2011) and/or total intramuscular activity of SIRT1 (Gurd et al. 2010) should not be dismissed, these results demonstrate that *SIRT1* gene expression, along with the expression of

its substrates *PGC-1α*, *FOXO1* and the activity (phosphorylation) of its additional target p53, is unaffected in human skeletal muscle by short-term ED and REX undertaken in ED; these effects seemingly contrast the signalling events following REX performed in the face of low endogenous glycogen availability (Camera et al. 2015).

Autophagy is emerging as a critical mediator of skeletal muscle remodelling and adaptation responses to exercise. This study reports the novel finding that protein levels of Atg5 are attenuated following short-term ED. However, Atg5 activity, as evidenced by cAtg12 abundance, can in part be 'rescued', commensurate with changes in several other autophagy-regulatory proteins, by REX and protein ingestion. Whether this autophagic response is a cause or consequence of the short-term ED-induced attenuation of MPS requires further investigation. Since dysregulation of autophagy has deleterious consequences for the function, quality and mass of skeletal muscle (Masiero et al. 2009), future studies determining how autophagy is regulated during chronic resistance training with high-protein availability and the ultimate impact on turnover-remodelling of muscle proteins will provide valuable mechanistic information for strategies aimed at promoting and maintaining skeletal muscle mass.

Preface to Chapter Five

The results from Chapter Four demonstrate that energy restriction for 5 days attenuated markers of autophagy that could be replenished by REX and protein ingestion, thereby mirroring, in part, the effects of these interventions on MPS responses (Areta et al. 2014). Excess energy availability culminating from alcohol intoxication during recovery from strenuous exercise has been reported to similarly disrupt the maximal amplitude of MPS (Parr et al. 2014). The observation that high-alcohol exposure decreases MPS, but has otherwise negligible effects on proteasome-mediated proteolysis implicate the engagement of alternate degradative pathways induced by alcohol that also represses protein synthesis (Vary et al. 2008; Parr et al. 2014). Indeed, alcohol may stimulate autophagy to facilitate removal of damaged muscle proteins that leads to tissue atrophy (Thapaliya et al. 2014). No studies to date have examined whether post-exercise alcohol intoxication upregulates autophagy-related signalling as a compensatory response to the toxic products of its metabolism and the implications for exercise remodelling in human skeletal muscle.

Chapter Five

5 *Study 2*: Protein Co-ingestion with Alcohol Following Strenuous Exercise Attenuates Alcohol-Induced Intramyocellular Apoptosis and Inhibition of Autophagy

Adapted from:

5.1 Abstract

Alcohol ingestion decreases post-exercise rates of muscle protein synthesis, but the mechanism(s) (e.g., increased protein breakdown) underlying this observation are unknown. Autophagy is an intracellular "recycling" system required for homeostatic substrate and organelle turnover; its dysregulation may provoke apoptosis and lead to muscle atrophy. The acute effects of alcohol ingestion on autophagic cell signalling responses to a bout of concurrent (combined resistance- and endurance-based) exercise were investigated. In a randomised cross-over design, 8 physically active males completed three experimental trials of concurrent exercise with either post-exercise ingestion of alcohol and carbohydrate (12 ± 2 standard drinks; ALC-CHO), energy-matched alcohol and protein (ALC-PRO), or protein (PRO) only. Muscle biopsies were taken at rest and 2 and 8 h post-exercise. Select autophagy-related gene (Atg) proteins decreased compared to rest with ALC-CHO ($P<0.05$), but not ALC-PRO. There were parallel increases ($P<0.05$) in p62 and PINK1, commensurate with a reduction in BNIP3 content, indicating a diminished capacity for mitochondria-specific autophagy (mitophagy) when alcohol and carbohydrate were co-ingested. DNA fragmentation increased in both alcohol conditions ($P<0.05$); however, nuclear AIF accumulation preceded this apoptotic response with ALC-CHO only ($P<0.05$). In contrast, increases in the nuclear content of p53, TFEB and PGC-1α in ALC-PRO were accompanied by markers of mitochondrial biogenesis at the transcriptional (*Tfam*, *SCO2*, *NRF-1*) and translational (COXIV, ATPAF1, VDAC1) level ($P<0.05$). Alcohol ingestion following exercise triggers apoptosis, whereas the anabolic properties of protein co-ingestion may stimulate mitochondrial biogenesis to protect cellular homeostasis.

5.2 Introduction

Exercise sensitises skeletal muscle to exogenous nutrient availability, such that substrate availability interacts with cellular processes regulating protein turnover responses that ultimately form the basis for exercise adaptation (Hawley et al. 2011). While the carbohydrate and protein requirements to promote adaptation to divergent exercise stimuli (i.e., endurance- and resistance-based) are well-characterised (Burke et al. 2011; Phillips & Van Loon 2011), far less is known regarding the impact of alcohol ingestion on exercise response-adaptation processes. Given the widely reported alcoholic drinking practices of professional and recreational athletes (Burke & Read 1988), and the capacity for "binge" alcohol consumption to impair recovery-performance and muscle protein synthesis (MPS) rates following strenuous exercise (Parr et al. 2014; Barnes et al. 2010), elucidating the mechanisms that underpin these detrimental effects of exercised skeletal muscle exposed to alcohol is warranted.

Previous studies in rodents report that large quantities of alcohol dysregulate the stimulation of MPS by growth factors and amino acids, largely due to attenuated signal transduction of the mammalian target of rapamycin (mTOR) complex 1 pathway (Kumar et al. 2002; Lang et al. 2003), a principal modulator of cellular growth. Similarly, alcohol was found to reduce the maximal MPS response to high-force mechanical overload in rodents (Steiner & Lang 2014, 2015a). Recently, alcohol ingested following a single bout of strenuous exercise was shown to attenuate the maximal exercise- and nutrition-induced stimulation of the myofibrillar (contractile) fraction of protein synthesis, despite exogenous protein availability (Parr et al. 2014). While the capacity for alcohol to impair MPS is recognised, its impact upon intramuscular protein degradative pathways is less clear. Acute alcohol exposure had no effect on proteasome-dependent proteolysis in rodents (Vary et al. 2008) and there is no synergistic effect of alcohol toward the transcription of key proteasome-

related ubiquitin ligases following exercise (Parr et al. 2014). Whether autophagy, the constitutive turnover of cellular components such as long-lived organelles (e.g., mitochondria) that protects homeostasis and is required for skeletal muscle integrity (Jokl & Blanco 2016), promotes skeletal muscle catabolism in response to alcohol ingested following an acute exercise bout, is unknown.

Alcohol metabolism produces highly-toxic intermediates that may accelerate cellular metabolism and exacerbate tissue breakdown responses to vigorous contractile activity (Lieber 2005). Furthermore, autophagy is sensitive to the cellular energetic balance and "recycles" nutrient substrate and organelles to maintain cellular viability (Singh & Cuervo 2011). Thus, a post-exercise autophagic response to alcohol may represent a compensatory removal of harmful protein aggregates that can trigger cell death processes (apoptosis) (Adachi & Ishii 2002). Accordingly, the primary aim of this study was to investigate the activation of autophagic cell signalling responses to binge alcohol consumption during recovery from a single bout of combined resistance and endurance ("concurrent") exercise. Due to the anabolic properties of amino acids and their capacity to inhibit autophagic flux (Nicklin et al. 2009), it was hypothesised that the largest autophagic response would occur when alcohol and carbohydrate were co-ingested compared to protein following exercise. A secondary hypothesis was that alcohol would elicit an increased mitophagic (mitochondria-selective autophagy) cell signalling response, since the catabolism of alcohol metabolites predominates in the mitochondria and is a source of reactive oxygen species (ROS) production, which may stimulate mitophagy and provoke muscle protein breakdown responses (Vainshtein et al. 2015a; Qiao et al. 2015).

5.3 Materials and Methods

Subjects

Eight young, healthy, physically active male subjects [age 21.4 ± 4.8 yr, body mass (BM) 79.3 ± 11.9 kg, were recruited from a previous study (Parr et al. 2014). Full subject characteristics, preliminary testing procedures ($\dot{V}O_{2peak}$ and maximal muscular strength), diet and exercise control, and complete details of the experimental design and experimental trials have been reported (Parr et al. 2014). Due to the legal drinking age in Australia, no minors (<18 yr old) were involved in the study. Subjects were advised of any possible risks associated with the study prior to providing written informed consent. The study was approved by the Human Research Ethics Committee of RMIT University (43/11) and was carried out in accordance with the standards set by the latest revision of the *Declaration of Helsinki*.

Experimental Design

The study employed a randomised, counter-balanced crossover design in which each subject completed, on three separate occasions, trials consisting of a bout of concurrent exercise (described subsequently) with either post-exercise ingestion of alcohol and carbohydrate (ALC-CHO), alcohol and protein (ALC-PRO) or protein only (PRO), in which carbohydrate and protein beverages were consumed twice; before and after the alcohol drinking protocol. Each experimental trial was separated by a two week recovery period, during which time subjects resumed their habitual pattern of physical activity.

Experimental Trials

Subjects reported to the laboratory at ~0700 h on the morning of an experimental trial following a 10 h overnight fast and a resting muscle biopsy was taken from the *vastus lateralis* under local anaesthesia (1% lidocaine) using a 5 mm Bergstrom needle modified for suction. Subjects then commenced the concurrent exercise bout which consisted of heavy resistance exercise (8×5 knee extension repetitions at 80% of 1 repetition maximum), followed by 30 min of cycling at ~70% of $\dot{V}O_{2peak}$, and then a high-intensity (10×30 s, 110% PPO) interval cycling bout. Immediately post-exercise and following 4 h of recovery, subjects ingested 500 mL of a carbohydrate (CHO: 25 g maltodextrin) or an isoenergetic protein (PRO: 25 g whey) beverage. Consistent with recommendations for CHO feeding and glycogen resynthesis following training (Burke et al. 2011), an additional CHO-based meal (1.5 g·kg^{-1} BM) was provided immediately after the first post-exercise (2 h) muscle biopsy with a final muscle biopsy obtained 8 h post-exercise. Muscle biopsies were taken from separate incision sites, cleared of visible adipose and/or connective tissue, snap-frozen in liquid nitrogen and stored at -80 °C for subsequent analysis. Subjects commenced drinking alcohol 1 h post-exercise and drinks were consumed in 6 equal volumes of 1 part vodka (~60 mL) to 4 parts orange juice (~240 mL, 1.8 g CHO·kg^{-1} BM) across a 3 h period (12 ± 2 standard drinks consumed), in which alcoholic beverages were consumed within 5 min every 30 min. For PRO trials, subjects still consumed orange juice with a matched volume of water (Figure 5.1).
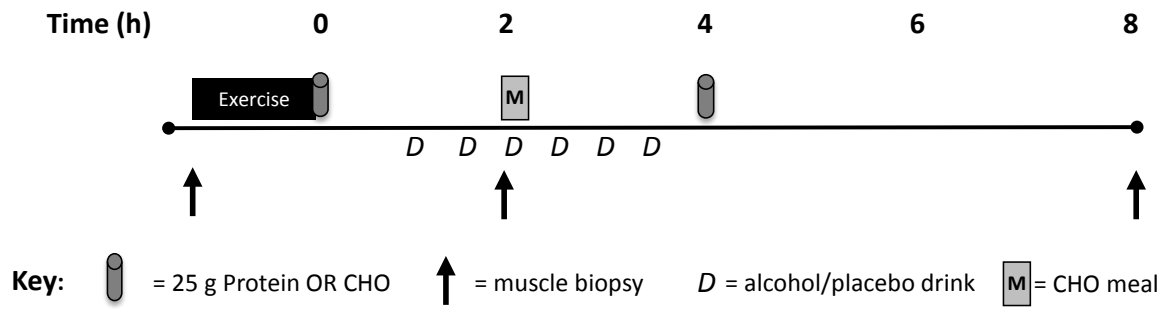
Figure 5.1. Schematic overview of experimental trials comprising Study 2

Analytical Procedures

Skeletal Muscle Sample Preparation

For generation of whole muscle lysates, ~40 mg of skeletal muscle was homogenised 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 16,000 g for 30 min at 4 °C and the supernatant was collected for Western blot analysis. Nuclear and cytoplasmic extracts were prepared using an NE-PER fractionation kit according to the manufacturer's instructions (Thermo Scientific, Rockford, IL). In brief, ~30 mg of skeletal muscle was homogenised on ice, by hand, using a glass Dounce homogeniser in CER-I (cytoplasmic extraction reagent-I) buffer supplemented with protease and phosphatase inhibitors. Homogenised samples were vortexed and incubated on ice for 10 min, after which the CER-II buffer was added. CER-II-containing samples were vortexed and subsequently spun at 16,000 g for 5 min at 4 °C with the supernatants containing cytoplasmic proteins removed and the remaining nuclei-containing pellet resuspended in nuclear extraction reagent buffer (supplemented with inhibitors). Nuclear lysates were subsequently incubated on ice

and vortexed for 15 s every 10 min for a total of 40 min, with a final 10 min centrifugation (16,000 g at 4 ℃) for collection of nuclear supernatants.

Western Blotting

After determination of protein concentration using a BCA protein assay (Pierce, Rockford, USA), all lysates (50 μg and 10 μg of protein for whole muscle and fractionated lysates, respectively) were resuspended in Laemmli sample buffer and loaded into 4–20% Mini-PROTEAN TGX Stain-Free™ Gels (Bio-Rad, California, USA). Following electrophoresis, gels were activated according to the manufacturer's instructions (Chemidoc, Bio-Rad, Gladesville, Australia) and transferred to polyvinylidine fluoride (PVDF) membranes as described in Study 1. After transfer, a Stain-Free image of the PVDF membranes for total protein normalisation was obtained before membranes were rinsed briefly in distilled water and blocked for 1 h with 5% non-fat milk, washed 3 times (5 min each wash) with 10 mM Tris·HCl, 100 mM NaCl and 0.02% Tween 20 (TBST) and incubated with a primary antibody diluted in TBST (1:1,000) overnight at 4 °C on a shaker. Membranes were incubated for 1 h the next day with a secondary antibody diluted in TBST (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 time points for each subject were run singularly on the same gel. Antibodies against phospho-ULK1 Ser[317] and Ser[757] (no. 12753 and no. 6888, respectively), Atg4b (no. 5299), Atg5 (no. 2630), cAtg12 (no. 4180), Beclin-1 (no. 3738), LC3b (no. 2775), p62 (no. 5114), NEDD4 (no. 2740), PINK1 (no. 6946), Parkin (no. 2132), BNIP3 (no. 44060), AIF (no. 5318), PARP1 (no. 9542), caspase-3 (no. 9662), TFEB (no. 4240), phospho-AMPK[Thr172] (no. 2531), AMPKα (no. 2532), phospho-p53[Ser15] (no.

9284), p53 (no. 2527), COXIV (no. 4850), Mitofusin-2 (no. 11925), H2B (no. 8135) and GAPDH (no. 2118) were purchased from Cell Signalling Technology (Danvers, MA). Antibodies directed against PGC-1α (ab54481), ATPAF1 (ab101518), VDAC1 (ab14734) and an additional LC3b antibody (ab48394) were purchased from Abcam (Melbourne, Australia). For all proteins, volume density of each target band was normalised to the total protein loaded into each lane (Figure 5.2A) using Stain-Free™ technology (Bio-Rad, California, USA) (Gurtler et al. 2013). Purity of the cytoplasmic and nuclear fractions was determined by immunoblotting for the glycolytic enzyme GAPDH and the nuclear histone 2B, respectively (Figure 5.2B). Due to limited tissue size, analysis of fractionated samples was restricted for some time points: For the majority of proteins measured, ALC-CHO 2 h ($n = 7$), ALC-PRO 2 h ($n = 6$) and 8 h ($n = 7$), and PRO 2 h ($n = 6$) and 8 h ($n = 7$) time points were restricted. There was $n = 5$ for some time points of Parkin (ALC-PRO 2 h, PRO 8 h), PARP1 (ALC-PRO 2 h, PRO 2 h), phospho-AMPK^{Thr172} and ATPAF1 (both PRO 8 h). Due to difficulties in quantification (non-specific binding), cytoplasmic p53 analysis was also based on $n = 5$.

A

Nuclear

Cytoplasmic

Whole muscle

B

	N	C	N	C	
15 kDa					H2B
37 kDa					GAPDH
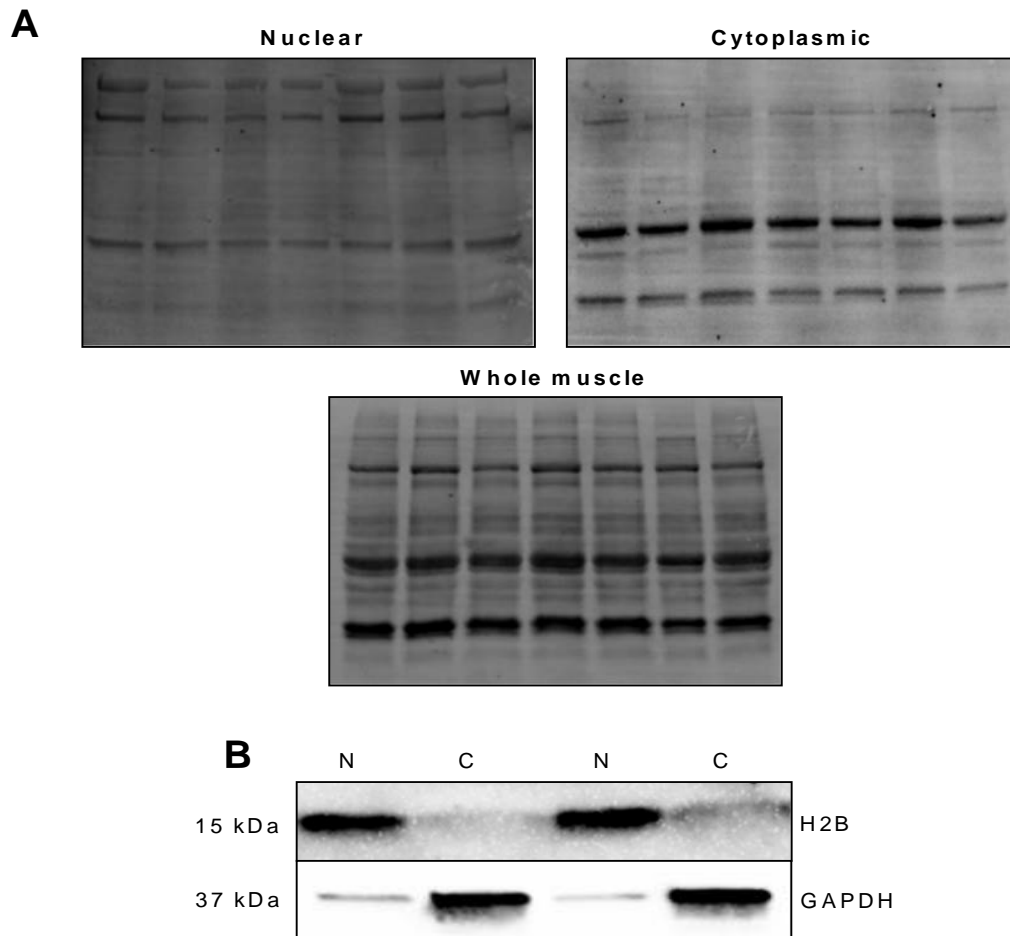
Figure 5.2. Representative Stain-Free images used for total protein normalisation (A). Section 3.1 depicts the approximate molecular weight range on these images. Purity of nuclear (N) and cytoplasmic (C) fractions were determined by immunoblotting for histone 2B (H2B) and GAPDH, respectively (B)

RNA Extraction, Quantification, Reverse Transcription and Real-Time PCR

Skeletal muscle tissue RNA extraction was performed using a TRIzol-based kit according to the manufacturer's protocol (Invitrogen, Melbourne, Australia, Cat. No. 12183–018A). Briefly, ~20 mg of frozen skeletal muscle tissue was homogenised in TRIzol with chloroform added to form an aqueous RNA phase. This RNA phase was then eluted through a spin cartridge with extracted RNA quantified using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Scoresby, Australia) according to the manufacturer's protocol. Reverse

transcription and real-time Polymerase Chain Reaction (RT-PCR) was performed as described in Study 1. Quantification of mRNA in duplicate was performed using a CFX96 Touch™ Real-Time PCR Detection System (Bio Rad, California, USA). TaqMan-FAM-labelled primer/probes for *Atg12* (Hs01047860), *BECN1* (Hs00186838), *LC3b* (Hs00797944), *SQSTM1/p62* (Hs01061917), *Atg4b* (Hs00367088), *BNIP3* (Hs00969291), *SCO2* (Hs04187025), *SESN-2* (Hs00230241), *PUMA* (Hs00248075), *Bax* (Hs00180269), *PGC-1α* (Hs01016719), *Tfam* (Hs00273372) and *NRF-1* (Hs01031046) were used. *GAPDH* (Hs99999905) has been validated as an exercise housekeeping gene (Jemiolo & Trappe 2004) and was used to normalise threshold cycle (CT) values. *GAPDH* values were stably expressed between conditions (data not shown). The relative amount of mRNA was calculated using the relative quantification (ΔΔCT) method (Livak & Schmittgen 2001).

Cell Death ELISA

Detection of DNA damage and cell death (apoptosis) was performed using a cell death detection ELISA (Roche Diagnostics, Mannheim, Germany). The cell death ELISA quantitatively determines apoptotic DNA fragmentation by measuring the cytoplasmic histone-associated mono- and oligonucleosomes. Briefly, even concentrations (120 µg) of nuclei-free cytoplasmic lysates were loaded as an antigen source to an anti-histone monoclonal antibody fixated to the walls of microplate modules. Lysates were loaded prior to the addition of an anti-DNA secondary antibody conjugated to peroxidase. The amount of peroxidase retained in the immunocomplex was determined photometrically by incubating with 2,2′-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) as a substrate on a plate shaker (300 rpm) for ~15 min at room temperature (20 ºC). Absorbance was measured at a wavelength of 405 nm using a SpectraMax Paradigm plate reader (Molecular Devices,

Sunnyvale, CA, USA). Samples were measured in duplicate on the same plate and absorbance values were normalised to μg of protein loaded in the assay per sample. Due to limited cytoplasmic lysate availability sample analysis was restricted for ALC-CHO 2 h (n = 7), ALC-PRO 2 h (n = 6), ALC-PRO 8 h (n = 7), PRO 2 h (n = 5) and PRO 8 h (n = 6).

GSH/GSSG Ratio

Detection of oxidative stress was performed using a GSH/GSSG Ratio Detection Assay Kit (Abcam, Melbourne, Australia). Cytoplasmic lysates were first deproteinised by adding trichloroacetic acid (TCA) to the samples in a 1:10 dilution. Samples were then incubated on ice (15 min) and briefly centrifuged (5 min, 12,000 g at 4 ℃) with the supernatant removed and neutralised of excess TCA using a neutralising solution (Abcam, Melbourne, Australia). The assay was performed using standards for reduced (GSH) and oxidised (GSSG) glutathione. Samples (n = 4) were incubated for ~45 min at room temperature (20 ℃) in a Thiol Green Stock solution diluted in assay buffer (GAM) and a 25X GSSG Probe diluted in GAM solution for the detection of reduced and oxidised glutathione, respectively. Fluorescence was measured at an excitation/emission wavelength of 490/520 nm using a SpectraMax Paradigm plate reader (Molecular Devices, Sunnyvale, CA, USA). Samples were measured in duplicate on the same plate and fluorescence values were normalised to μg of protein loaded in the assay per sample.

Statistical Analysis

Data were analysed using two-way repeated measures analysis of variance (ANOVA) with Student-Newman-Keuls post-hoc analysis (time × treatment) performed when an overall

statistically significant difference in group means of a particular comparison was found (SigmaPlot for Windows; Version 12.5). Significance was set to $P<0.05$ and all data are presented as mean ± standard deviation (SD). When tests for normality and/or equal variance failed, data were log-transformed and statistical inferences were made based on these data.

5.4 Results

Autophagy Regulatory Proteins

Autophagy involves the formation of vesicles termed autophagosomes that sequester and deliver cellular constituents to lysosomes for degradation in a process regulated by autophagy-related gene (Atg) proteins (Mizushima et al. 2011). AMP-activated protein kinase (AMPK)-targeted phosphorylation of Atg1 or Unc-51-like kinase 1 (ULK1) at Ser^{317} that activates autophagy was unchanged with any exercise-nutrient stimulus (data not shown). In contrast, $ULK1^{Ser757}$ phosphorylation, a site targeted by mTOR that inhibits autophagy induction (Kim et al. 2011), increased (P<0.05) with PRO above rest and each alcohol treatment (data not shown). Whole muscle abundance of Atg4b, Atg5, the conjugated form of Atg5 (cAtg12) and Beclin-1 all decreased compared to rest at 8 h post-exercise in ALC-CHO only (P<0.05; Figure 5.3A-D), while Atg5 and cAtg12 values at 8 h post-exercise were greater in ALC-PRO than ALC-CHO (P<0.05). All Atg proteins except Beclin-1 were higher than ALC-CHO in PRO at 8 h post-exercise (P<0.05). An indirect marker of autophagosome formation is an increase in the lipidated membrane-bound form of light chain 3b (LC3b-II) and a decrease in p62 (Tanida & Waguri 2010). There were no differences in the non-lipidated LC3b-I isoform with any exercise-nutrient condition (Figure 5.3E), although LC3b-II decreased below rest at 2 h (P<0.05) and remained attenuated by 8 h post-exercise in ALC-CHO (P=0.052; Figure 5.3F). LC3b-II similarly decreased below rest at 8 h with PRO

(P<0.05). p62 increased above rest at 8 h post-exercise in ALC-CHO (P<0.01; Figure 5.3G), while similar increases also occurred between 2-8 h of exercise recovery in ALC-PRO (P<0.05). These findings altogether suggest that alcohol co-ingested with carbohydrate, but not protein, inhibited autophagy. To gain insight into whether this inhibition culminated from elevated Atg degradation by the proteasome, measurements were obtained for the ubiquitin ligase neural precursor cell expressed developmentally downregulated 4 (NEDD4), which has been shown to target Beclin-1 for proteasomal degradation (Platta et al. 2012). However, NEDD4 declined below rest at 8 h post-exercise with ALC-CHO only (P<0.05; Figure 5.3H), suggesting that the alcohol-induced reduction in Atgs was probably not attributable to their proteasomal breakdown.
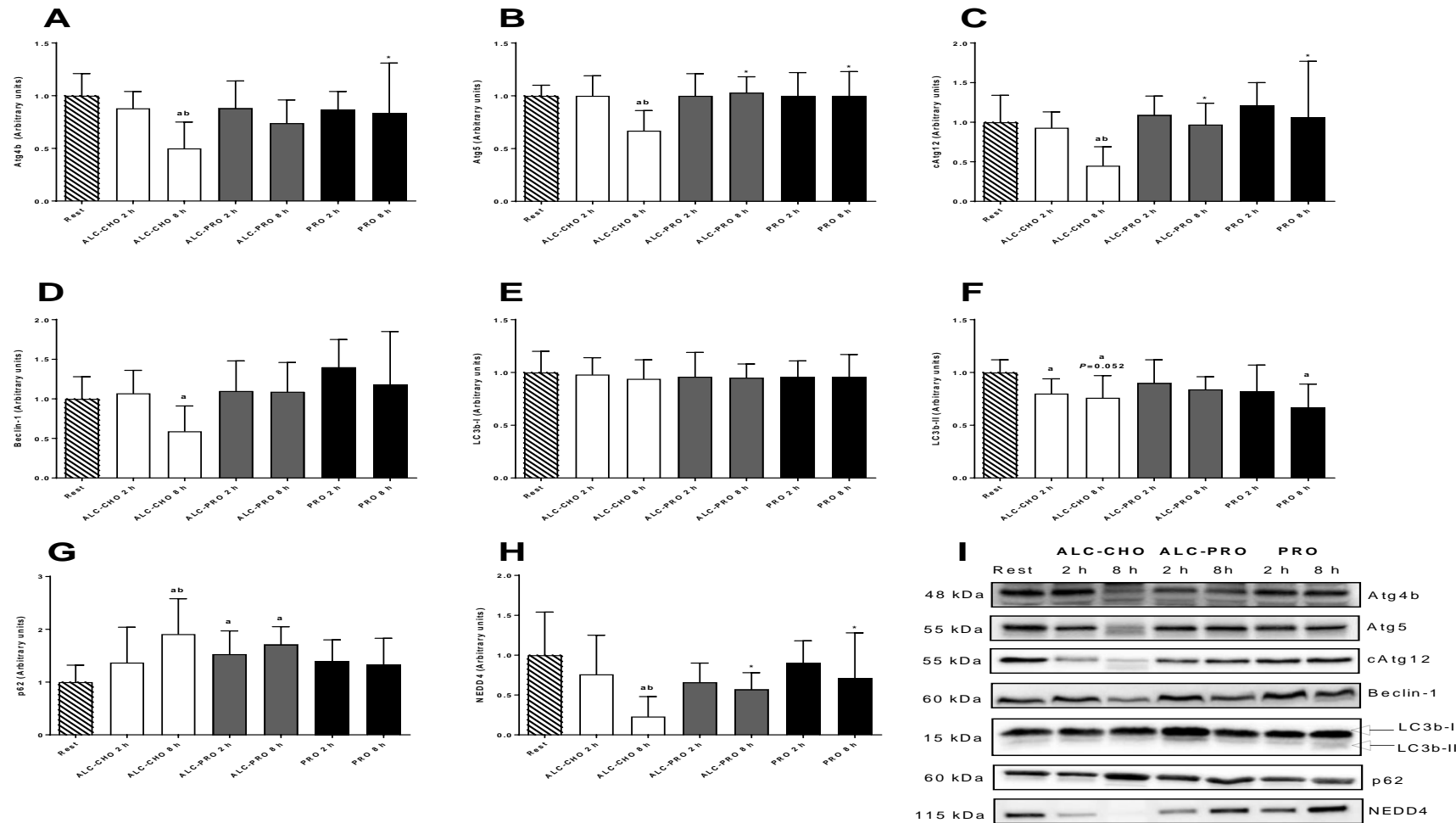
Figure 5.3. Whole muscle abundance of autophagy regulatory proteins Atg4b (A), Atg5 (B), cAtg12 (C), Beclin-1 (D), LC3b-I (E), LC3b-II (F), p62 (G), and the ubiquitin ligase NEDD4 (n = 7 for ALC-CHO time points and ALC-PRO 2 h only; H)

Figure 5.3. Continued

Analysed at rest and following a single bout of concurrent exercise with ingestion of either alcohol and carbohydrate (ALC-CHO), alcohol and protein (ALC-PRO), or protein only (PRO). I, representative images for all proteins. Data were analysed using a 2-way ANOVA with repeated measures and Student-Newman-Keuls post-hoc analysis. Values are presented (mean ± SD) as a fold change relative to resting values. Significantly different ($P<0.05$) vs. (a) rest, (b) ALC-CHO 2 h, and (*) 8 h between treatments.

Autophagy Regulatory Genes

There were no changes in the mRNA transcripts of *Atg12*, *BECN1*, *LC3b* and *SQSTM1/p62*. However, *Atg4b* increased (P<0.05) above rest at 8 h following exercise in both alcohol treatments (data not shown).

Mitochondria-Specific Autophagy (Mitophagy)

PTEN-induced putative protein kinase-1 (PINK1) and Parkin regulate canonical mitophagy. There were large increases in PINK1 above rest for ALC-CHO and PRO at 8 h post-exercise (P<0.01; Figure 5.4A). However, cytoplasmic Parkin only increased at 8 h with PRO above ALC-CHO (P<0.05; Figure 5.4B). Another mitophagy-specific protein, Bcl-2/adenovirus E1B 19 kDa-interacting protein-3 (BNIP3), decreased below rest at 8 h post-exercise in ALC-CHO (P<0.05; Figure 5.4C). BNIP3 was higher than both ALC-CHO and PRO treatments with ALC-PRO at 8 h post-exercise, an effect paralleled by similar temporal increases in its gene expression (P<0.05; Figure 5.4D). Given the previously mentioned reduction in "general" autophagy, these findings suggest that mitophagy was also inhibited by alcohol/carbohydrate co-ingestion post-exercise.

Figure 5.4. Mitophagy-related proteins PINK1 (A), Parkin (B), BNIP3 (*n* = 7 for ALC-CHO 8 h and ALC-PRO 2 h only; C), and *BNIP3* gene expression (*n* = 7; D)

Analysed at rest and following a single bout of concurrent exercise with ingestion of either alcohol and carbohydrate (ALC-CHO), alcohol and protein (ALC-PRO), or protein only (PRO). E, representative images for all proteins. Data were analysed using a 2-way ANOVA with repeated measures and Student-Newman-Keuls post-hoc analysis. Values are presented (mean ± SD) as a fold change relative to resting values. Significantly different (P<0.05) vs. (a) rest, (b) ALC-CHO 2 h, (f) PRO 2 h, and (*) 8 h between treatments.

103

DNA Fragmentation and Apoptotic Signalling

To determine whether attenuated autophagic/mitophagic responses to ALC-CHO reflected activation of apoptotic (cell death) events, measurements of fragmented DNA in cytoplasmic lysates, as a marker of cellular apoptosis, were performed. DNA fragmentation increased significantly by ~185% above rest at 8 h post-exercise with ALC-CHO only ($P<0.05$; Figure 5.5A). Detection of DNA fragmentation at 8 h in both alcohol treatments was greater than PRO ($P<0.05$). Apoptosis can be executed by caspase-dependent and -independent pathways, the latter involving poly (ADP-ribose) polymerase 1 (PARP1) triggering the mitochondria-to-nuclear translocation of apoptosis inducing factor (AIF) (Yu et al. 2002). Nuclear abundance of AIF doubled above rest during early (2 h) exercise recovery in ALC-CHO ($P<0.05$; Figure 5.5B), but declined sharply thereafter by 8 h ($P<0.01$). Within the cytoplasm, AIF levels with ALC-PRO and PRO were greater than ALC-CHO at 8 h post-exercise ($P<0.05$; Figure 5.5C). Full-length (~116 kDa) nuclear PARP1 decreased below resting levels by 8 h in ALC-CHO only ($P<0.05$; Figure 5.5D). As a result, PARP1 was greater than ALC-CHO with ALC-PRO and PRO at this 8 h time point ($P<0.05$). Immunoreactive ~89 kDa bands of PARP1, reduced proteolytic targets of caspase-3, were undetectable in nuclear lysates. Furthermore, we could not detect bands for the cleaved, active (~17 kDa) caspase-3 in whole muscle homogenates. Notably, pro-caspase-3 levels were greater than ALC-CHO in response to ALC-PRO and PRO at 8 h post-exercise ($P<0.05$; Figure 5.5E). These findings suggest that alcohol-induced DNA fragmentation was elicited by caspase-independent pathways.
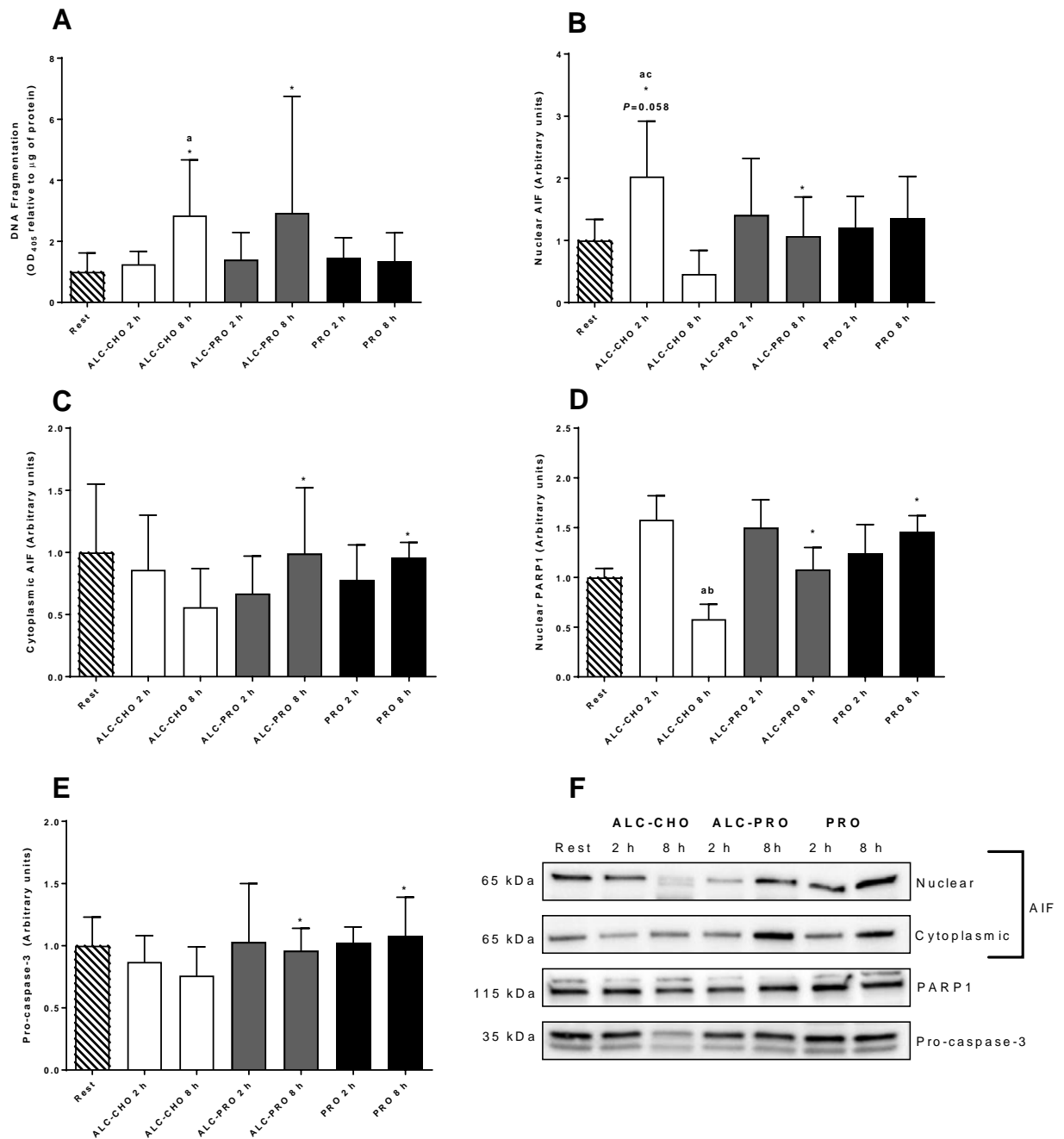
Figure 5.5. Apoptotic DNA fragmentation (A), nuclear (B) and cytoplasmic AIF (C), nuclear PARP1 (D), and whole muscle pro-caspase-3 ($n = 7$ for ALC-CHO time points and ALC-PRO 2 h only; E)

Analysed at rest and following a single bout of concurrent exercise with ingestion of either alcohol and carbohydrate (ALC-CHO), alcohol and protein (ALC-PRO), or protein only (PRO). F, representative images for all proteins. Data were analysed using a 2-way ANOVA with repeated measures and Student-Newman-Keuls post-hoc analysis. Values are presented (mean ± SD) as a fold change relative to resting values. Significantly different (P<0.05) vs. (a) rest, (b, c) ALC-CHO 2 and 8 h, and (*) 8 h between treatments.

105

Nuclear and Cytoplasmic TFEB, PGC-1α, AMPK and p53

The apoptogenic effects of alcohol led us to investigate changes in the nuclear and cytoplasmic levels of several proteins highly-sensitive to changes in cellular energy availability. Nuclear and cytoplasmic levels of transcription factor EB (TFEB), a transcriptional regulator of autophagy, decreased below rest in ALC-CHO at 8 h post-exercise ($P<0.05$; Figure 5.6A, B). Nuclear TFEB at 8 h in ALC-PRO was greater than the ALC-CHO ($P<0.001$) and PRO ($P<0.01$) conditions. Following 2 h of exercise recovery in both alcohol treatments, nuclear PPARγ-coactivator-1α (PGC-1α) doubled above rest ($P<0.05$; Figure 5.6C). However, its nuclear abundance returned to basal levels by 8 h for ALC-CHO only ($P<0.01$). Cytoplasmic PGC-1α in ALC-PRO increased above rest and ALC-CHO at 8 h ($P<0.05$; Figure 5.6D). While there were no differences in nuclear AMPKα at any time point (data not shown), cytoplasmic phospho-AMPKα^{Thr172} increased above rest at 8 h in ALC-CHO only ($P<0.05$; Figure 5.6E). The apoptogenic p53 increased in the nucleus above rest in ALC-PRO and PRO at 8 h post-exercise ($P<0.05$; Figure 5.6F). Within cytoplasmic fractions, p53 with each alcohol treatment at 8 h increased above rest and PRO ($P<0.05$; Figure 5.6G), of which the largest effect occurred for ALC-CHO ($P<0.01$). In addition, whole muscle p53^{Ser15} phosphorylation was highest in ALC-CHO after exercise, increasing above rest at 8 h ($P<0.001$) and above ALC-PRO and PRO treatments ($P<0.05$; Figure 5.6H).
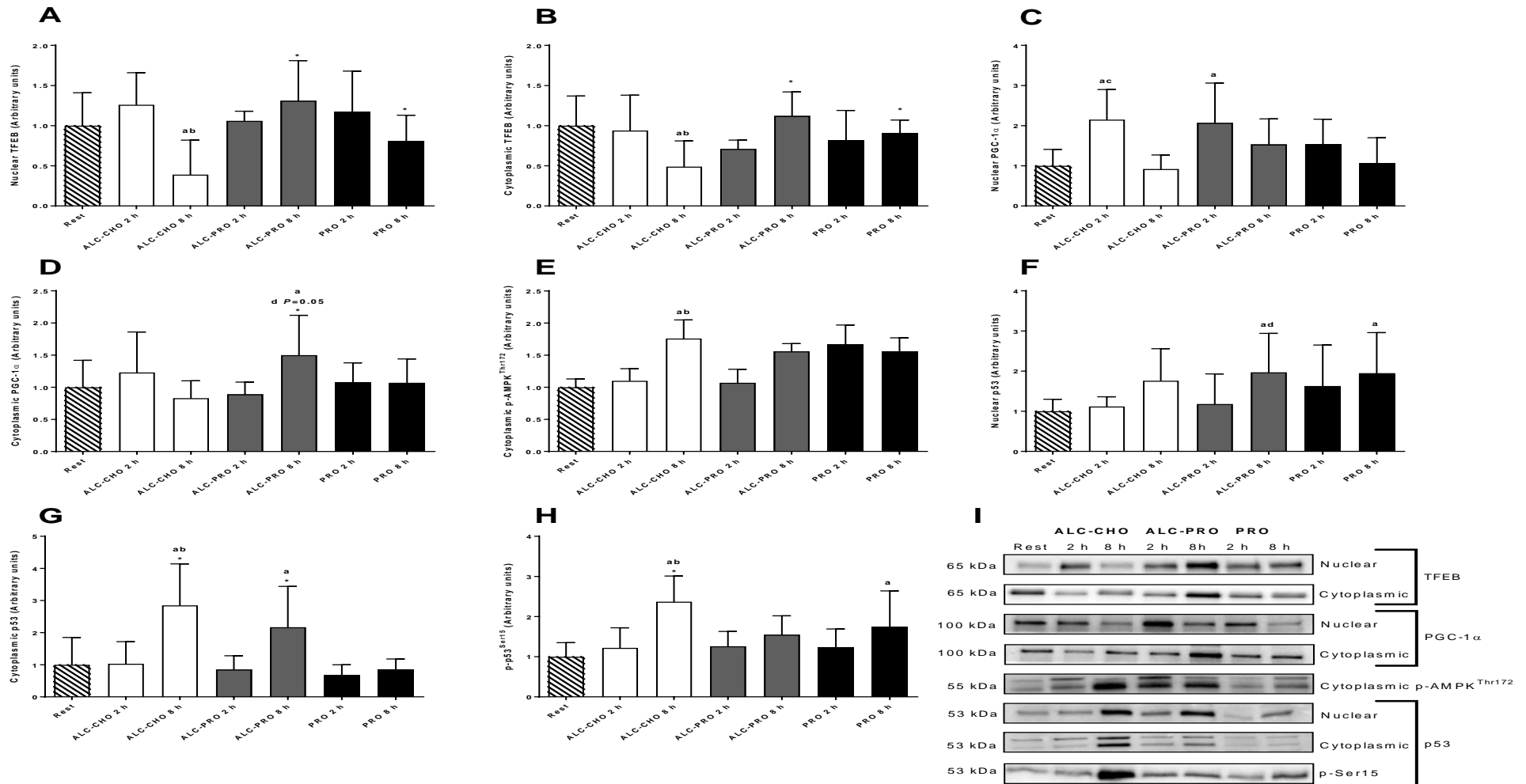
Figure 5.6. Nuclear (A) and cytoplasmic TFEB (B), nuclear (C) and cytoplasmic PGC-1α (D), cytoplasmic phospho-AMPKα^{Thr172} (E), nuclear (F) and cytoplasmic p53 (G), and whole muscle phospho-p53^{Ser15} (H)
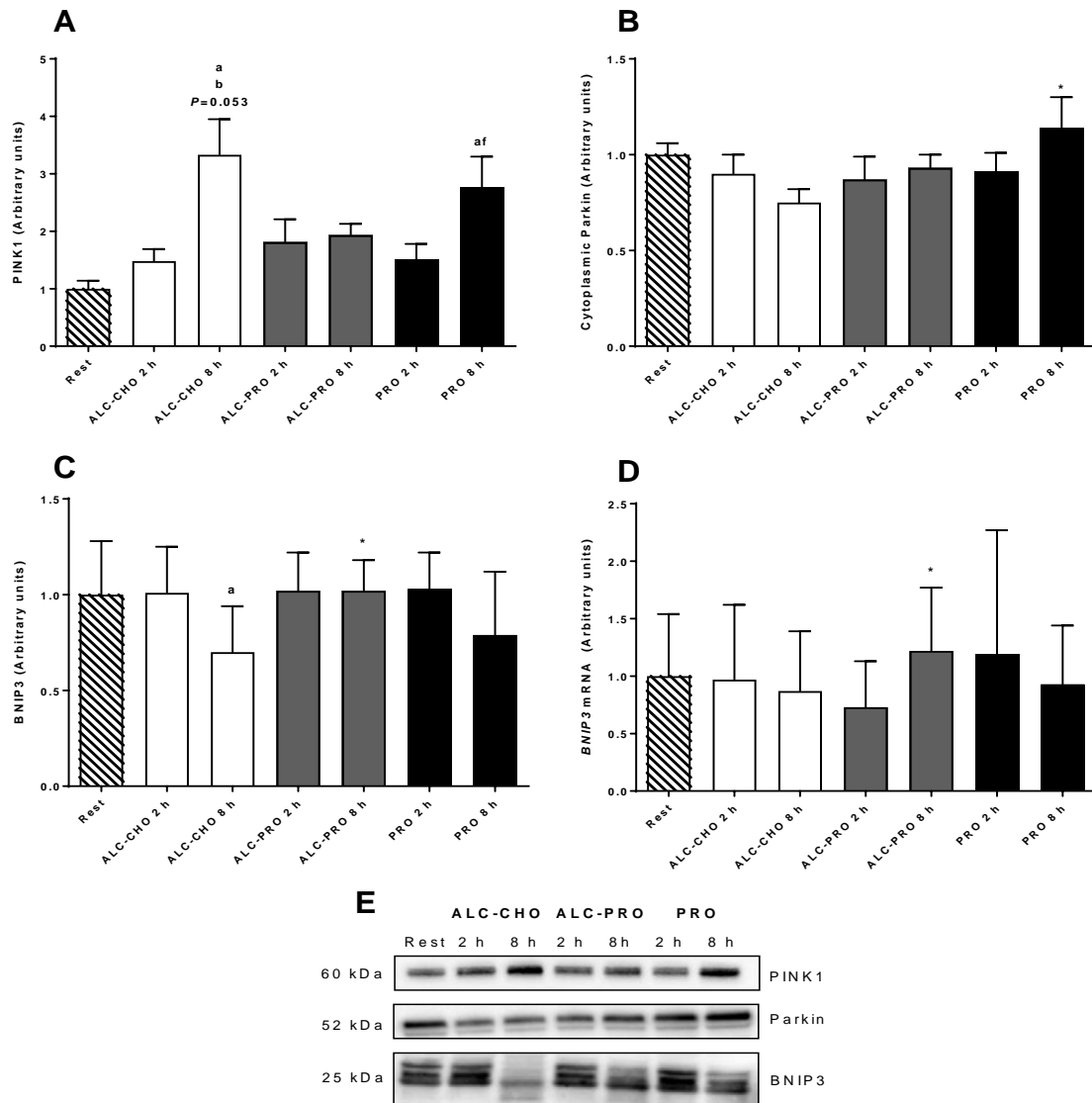
107

Figure 5.6. Continued

Analysed at rest and following a single bout of concurrent exercise with ingestion of either alcohol and carbohydrate (ALC-CHO), alcohol and protein (ALC-PRO), or protein only (PRO). I, representative images for all proteins. Data were analysed using a 2-way ANOVA with repeated measures and Student-Newman-Keuls post-hoc analysis. Values are presented (mean ± SD) as a fold change relative to resting values. Significantly different (P<0.05) vs. (a) rest, (b, c) ALC-CHO 2 h and 8 h, (d) ALC-PRO 2 h, and (*) 8 h between treatments.

p53- and PGC-1α-Target Genes and Oxidative Stress

Subsequent analyses focussed on select p53/PGC-1α transcriptional targets that are involved in cell-fate (i.e., survival or death) responses to changes in metabolic homeostasis (Sen et al. 2011). There were differential responses of p53-target genes to post-exercise alcohol ingestion. *SCO2* (synthesis of cytochrome *c* oxidase 2) expressing a protein regulating assembly of the respiratory chain cytochrome *c* oxidase (COX) complex, increased above rest with ALC-PRO at 8 h post-exercise (P<0.05; Figure 5.7A). *SESN-2* is an endogenous antioxidant (Budanov et al. 2004) and was also elevated at this time point with ALC-CHO compared to 2 h (P<0.05; Figure 5.7B). *PUMA* is a pro-apoptotic gene involved in mitochondrial ROS generation (Chipuk et al. 2005; Liu et al. 2005) and increased above rest in all treatments (P<0.05; Figure 5.7C), whereby the largest effect occurred for ALC-CHO (P<0.001). In contrast, there were no changes for the pro-apoptotic *Bax* following any exercise-nutrient stimulus (data not shown). These changes in *SESN-2* and *PUMA* suggested alcohol may have induced an increase in ROS production. Therefore, measurements were performed in cytoplasmic lysates of the ratio of reduced to oxidised glutathione (GSH/GSSG), a major endogenous antioxidant. However, there were no significant changes in the GSH/GSSG ratio with any exercise-nutrient stimulus (Figure 5.7D). PGC-1α can affect its own transcription (Jager et al. 2007) and there were large increases in *PGC-1α* gene expression during exercise recovery in all treatments with the largest effect prevailing 2 h post-exercise (P<0.001; Figure 5.7E). Mitochondrial biogenesis-related PGC-1α-target genes similarly revealed divergent responses to the experimental conditions. *Tfam* (mitochondrial transcription factor A) increased above rest at 8 h post-exercise in both alcohol treatments (P<0.05; Figure 5.7F), whereby ALC-PRO demonstrated the largest effect (P<0.01).

NRF-1 (nuclear respiratory factor-1) increased above rest at 8 h after exercise in response to ALC-PRO alone (P<0.05; Figure 5.7G).
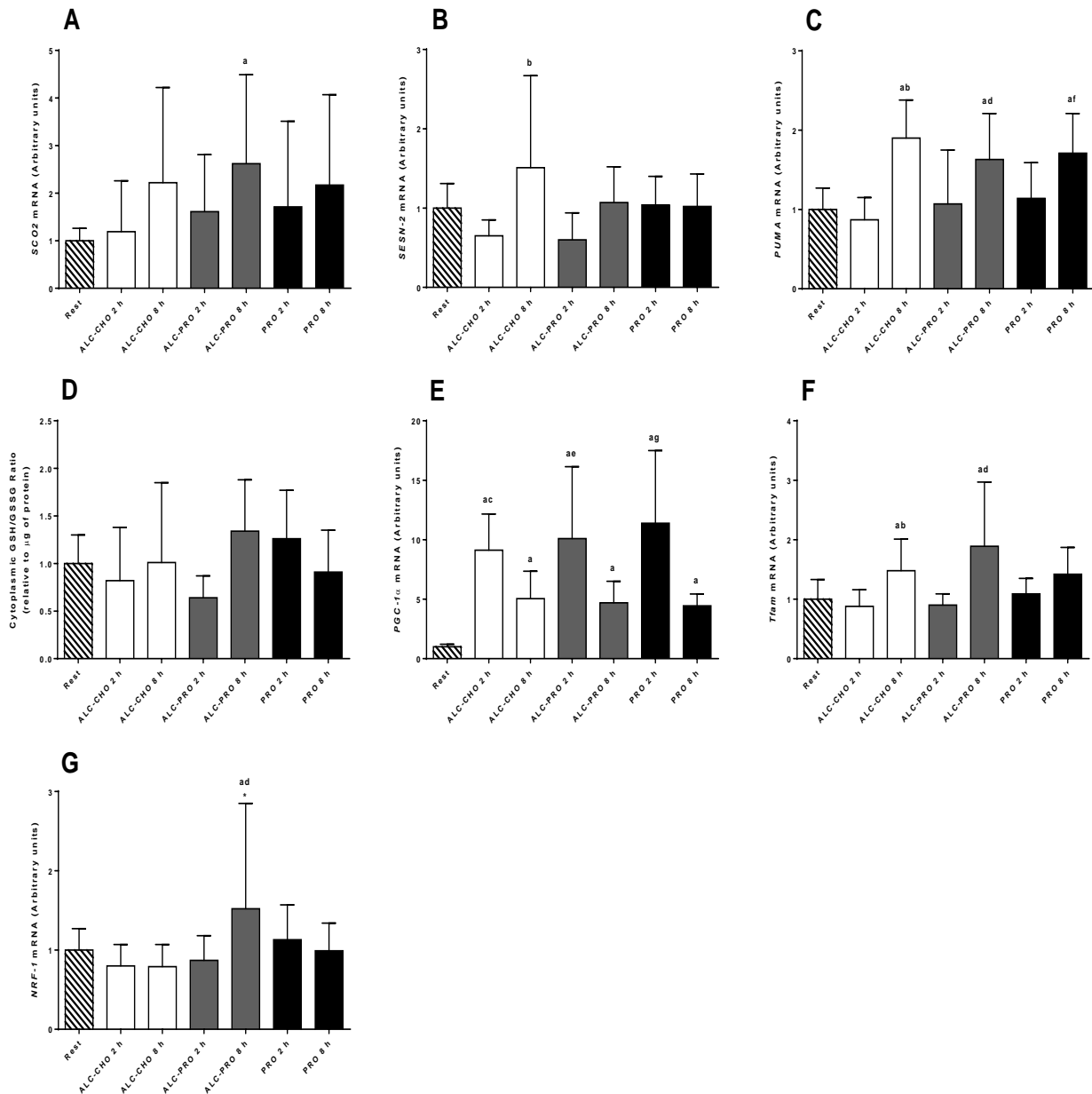
Figure 5.7. mRNA expression of *SCO2* (A), *SESN-2* (B) and *PUMA* (C), the cytoplasmic reduced (GSH) to oxidised (GSSG) glutathione ratio ($n = 4$; D), and *PGC-1α* (E), *Tfam* (F) and *NRF-1* (G) mRNA

Analysed at rest and following a single bout of concurrent exercise with ingestion of either alcohol and carbohydrate (ALC-CHO), alcohol and protein (ALC-PRO), or protein only (PRO). Data were analysed using a 2-way ANOVA with repeated measures and Student-Newman-Keuls post-hoc analysis. Values are expressed relative to GAPDH and presented (mean ± SD; mRNA $n = 7$) as a fold change relative to resting values. Significantly different ($P<0.05$) vs. (a) rest, (b, d, f) 2 h within treatments, (c, e, g) 8 h within treatments, and (*) 8 h between treatments.

Mitochondrial Proteins

Suspecting that the transcriptional induction of SCO2, Tfam and NRF-1 reflected a mitochondrial biogenesis response to post-exercise alcohol/protein ingestion, measurements were conducted for the abundance of several key proteins regulating mitochondrial function. At 8 h post-exercise with ALC-PRO, there were increases above rest in the cytoplasmic content of COX subunit IV; these changes were greater than ALC-CHO and PRO ($P<0.01$; Figure 5.8A). Similarly, the respiratory chain F_1-ATP synthase complex assembly factor 1 (ATPAF1) increased above rest at 8 h post-exercise in ALC-PRO only ($P<0.05$; Figure 5.8B) and this change was greater than ALC-CHO ($P<0.001$). ATPAF1 was also elevated in PRO above ALC-CHO between 2-8 h of exercise recovery and above ALC-PRO at 2 h only ($P<0.05$). Voltage-dependent anion channel-1 (VDAC1), an abundant mitochondrial regulator of substrate trafficking, was higher than ALC-CHO and PRO treatments at 8 h post-exercise in response to ALC-PRO ($P<0.01$; Figure 5.8C). Whole muscle levels of the mitochondrial membrane fusion protein Mitofusin-2 decreased below rest at 8 h in ALC-CHO ($P<0.05$; Figure 5.8D). Mitofusin-2 levels in ALC-PRO and PRO were greater than ALC-CHO at 8 h post-exercise ($P<0.05$). Altogether these data suggest that high-protein availability with alcohol stimulates mitochondrial biogenesis.
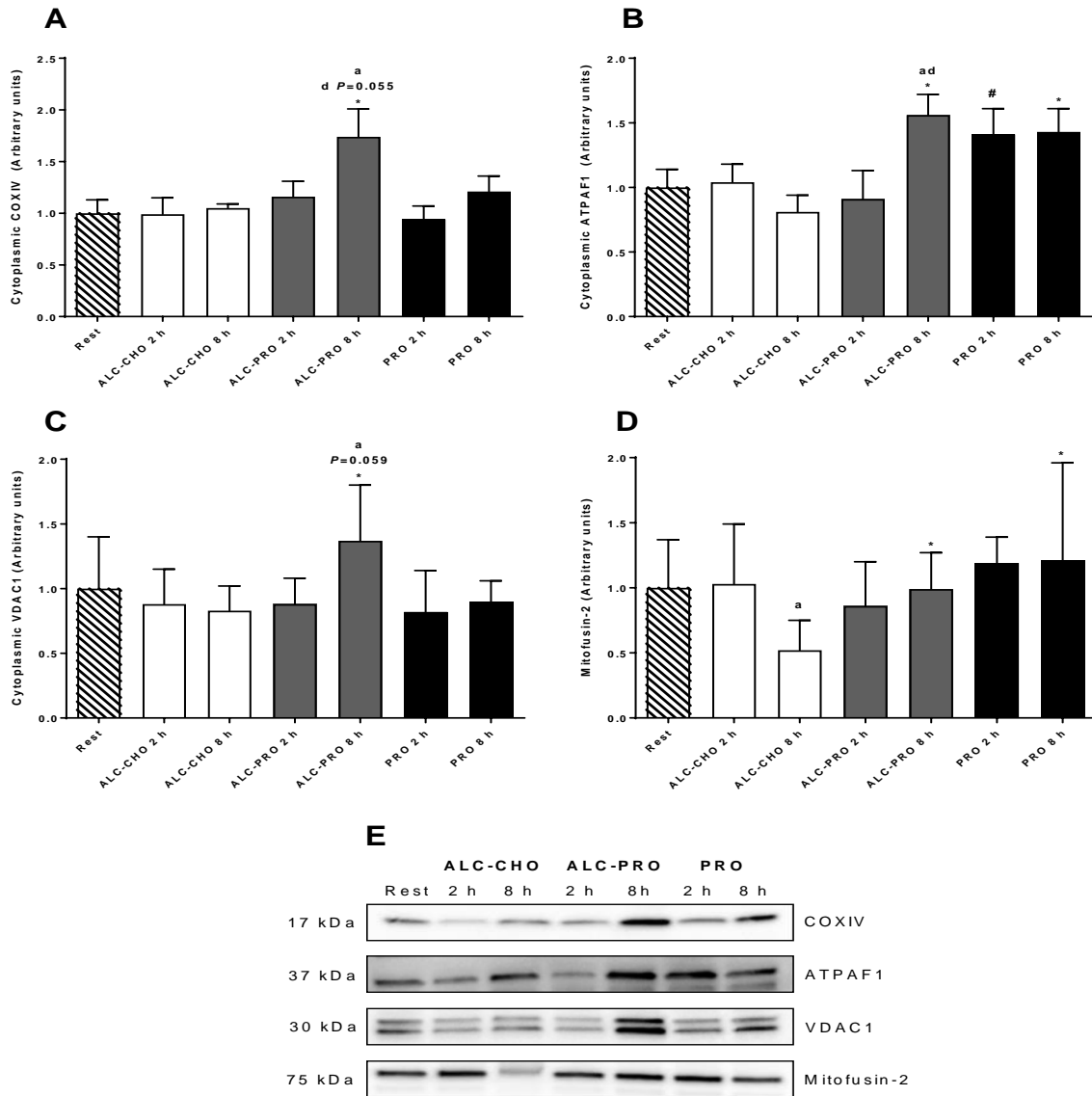
Figure 5.8. Cytoplasmic levels of mitochondrial proteins COXIV (A), ATPAF1 (B), VDAC1 (C), and whole muscle Mitofusin-2 (*n* = 7 for ALC-CHO time points and ALC-PRO 2 h only; D)

Analysed at rest and following a single bout of concurrent exercise with ingestion of either alcohol and carbohydrate (ALC-CHO), alcohol and protein (ALC-PRO), or protein only (PRO). E, representative images for all proteins. Data were analysed using a 2-way ANOVA with repeated measures and Student-Newman-Keuls post-hoc analysis. Values are presented (mean ± SD) as a fold change relative to resting values. Significantly different (P<0.05) vs. (a) rest, (d) ALC-PRO 2 h, and (#) 2 h and (*) 8 h between treatments.

5.5 Discussion

This is the first study to characterise the pro-apoptotic effects of acute binge alcohol consumption in human skeletal muscle following exercise. The main finding was that alcohol co-ingested with carbohydrate (i.e., before and after the alcohol drinking protocol) following a single bout of strenuous concurrent exercise represses autophagy and triggers intramyocellular apoptosis as indicated by DNA fragmentation. In contrast, energy-matched protein consumption attenuated alcohol-induced apoptotic responses following exercise and was accompanied by increases in markers of mitochondrial biogenesis. This apoptotic response may have been the result of alcohol imposing an additional metabolic stress to a cellular environment already disrupted by prior exercise. However, the anabolic properties of protein that increase mitochondrial protein synthesis may lower the magnitude of apoptotic events when co-ingested with alcohol.

Post-exercise ingestion of alcohol and carbohydrate dysregulates autophagy

It was previously demonstrated that alcohol consumption following a strenuous bout of exercise repressed maximal rates of myofibrillar protein synthesis in human skeletal muscle compared to consuming protein-only beverages during recovery (Parr et al. 2014). The observation that acute alcohol exposure failed to augment proteasome-dependent protein breakdown (Vary et al. 2008) led us to investigate whether aberrant activation of autophagy, an alternate lysosome-dependent degradative pathway sensitive to cellular bioenergetics (Singh & Cuervo 2011), may play a role in the post-exercise alcohol-induced repression of myofibrillar protein synthesis by degrading intracellular substrate and inducing a net negative protein balance. In contrast to one of the original hypotheses, by 8 h following exercise (4 h after ingesting the final alcoholic beverage),

114

several Atgs implicated in the biogenesis of autophagic vesicles (autophagosomes) along with the nuclear and cytoplasmic content of TFEB, a transcriptional regulator of autophagy (Settembre et al. 2011), were consistently attenuated (below resting values) with alcohol and carbohydrate ingestion, but not when alcohol was consumed with protein. Corresponding gene expression (except *Atg4b*) was largely unaffected at any time point, while NEDD4, an ubiquitin ligase that can "tag" Atgs such as Beclin-1 for proteasomal degradation (Platta et al. 2012), followed the same alcohol/carbohydrate-only pattern of post-exercise decline. These findings were surprising as substantial increases in autophagy alongside a reduction in proteasomal activity have previously been observed in skeletal muscle of alcoholic cirrhosis patients (Thapaliya et al. 2014), suggesting that chronic alcohol exposure ultimately elevates skeletal muscle autophagy for constitutive removal of damaged cellular compartments.

Alcohol metabolism is energetically expensive and produces highly-toxic intermediates such as acetaldehyde and acetate (Lieber 2005), the latter of which has been shown to be taken up by skeletal muscle following its release from the splanchnic region in response to an acute ethanol infusion (Jorfeldt & Juhlin-Dannfelt 1978). Alcohol metabolism provokes mitochondrial production of ROS that can signal for the mitophagic removal of these organelles (Bailey et al. 1999). Canonical mitophagy involves PINK1 accumulating on the surface of damaged/depolarised mitochondria where it recruits and activates the ubiquitin ligase Parkin (Kane et al. 2014; Vives-Bauza et al. 2010). Ubiquitinated mitochondrial proteins are subsequently delivered to autophagosomes by the bridging protein p62, which in turn undergoes degradation itself (Geisler et al. 2010). Alternatively, BNIP3 is a hypoxia-sensitive mitophagy receptor containing an LC3-interacting motif that facilitates direct autophagosomal engulfment of mitochondria (Hanna et al. 2012). Co-ingestion of alcohol and carbohydrate after exercise

elicited a dysregulated mitophagic response as evidenced by parallel increases in PINK1 and p62 and a reduction in BNIP3. Given that alcohol led to the consistent reduction of Atgs, the molecular "machinery" required for autophagic digestion of mitochondria, these data suggest that post-exercise alcohol intoxication prevented the disposal of potentially damaged mitochondria. Notably, when the intensity of a given stressor (i.e., alcohol) overwhelms the protective capabilities of mitophagy, intrinsic cell death (apoptotic) pathways can be activated (Marino et al. 2014). Findings of alcohol ingestion disrupting autophagy along with an apparent, alcohol-induced downregulation of proteasome-dependent proteolysis, led us to investigate whether induction of apoptotic processes were responsible for this degradation of signalling proteins.

Post-exercise ingestion of alcohol triggers intramyocellular apoptosis

Alcohol with carbohydrate and also alcohol with protein co-ingestion triggered apoptotic DNA fragmentation, as revealed by the detection of post-exercise mono- and oligonucleosomes in cytoplasmic (nuclei-free) lysates. However, the largest apoptotic response prevailed for alcohol/carbohydrate and was preceded by AIF nuclear accumulation. AIF normally resides in the mitochondrial intermembrane space (Susin et al. 1999), but upon mitochondrial outer membrane permeabilisation (MOMP), AIF rapidly redistributes to the nucleus where it has been shown to elicit large-scale DNA fragmentation (Daugas et al. 2000a). Hyperactivity of PARP1, a DNA repair enzyme, signals to AIF to facilitate its nuclear import as an alternative to the canonical cytochrome *c*/caspase-dependent pathway of mitochondrial apoptosis (Kolthur-Seetharam et al. 2006; Yu et al. 2002). Whether PARP1 was responsible for initially signalling to AIF is unclear, as it followed a similar pattern to AIF, decreasing sharply during late (8 h)

116

exercise recovery with alcohol/carbohydrate. In addition, DNA fragmentation was not detected until 8 h post-exercise and was paralleled by increases in the cytoplasmic abundance of p53, a well-characterised effector of apoptosis. p53 promotes apoptosis by transcription-dependent and –independent mechanisms, the latter involving MOMP, cytochrome *c* release and caspase activation (Chipuk et al. 2004; Dumont et al. 2003; Mihara et al. 2003). Despite increased cytoplasmic p53, precursor caspase-3 levels were lowest after exercise in alcohol/carbohydrate and its cleaved, active fragment was undetectable and there was no evidence for nuclear cleavage of PARP1, a major caspase-3 substrate (Lazebnik et al. 1994); these effects seemingly contrast denervation-induced apoptotic signalling in rodent skeletal muscle (Siu & Alway 2005, 2006). Thus, these findings suggest that AIF nuclear import is the initial, caspase-independent driver of post-exercise alcohol-induced apoptosis. Degradation of nuclear PARP1 would also limit DNA repair capacity and its decay may have perpetuated the p53-independent apoptotic response. Another consideration is that the observed apoptotic response could also affect the myonuclei of myogenic cells (i.e., satellite cells) that are activated in response to vigorous exercise, thereby impairing regenerative recovery, particularly from the mechanical demands imposed by resistance exercise contraction (Petrella et al. 2008).

Aberrant mitophagy signalling observed in the alcohol/carbohydrate condition raises the possibility that the metabolic burden of alcohol added to an intracellular environment already disrupted by contractile stress (i.e., substrate depletion, altered redox state, Ca^{2+} fluctuations etc.) overwhelmed mitochondrial metabolism. In this regard, the potential ATP depletion and ROS generated as a result of mitochondrial alcohol metabolism may have triggered MOMP, release of AIF and initiation of apoptosis, which are well-characterised features of alcohol-induced liver injury (Adachi & Ishii 2002). In support of this hypothesis, phosphorylation of cytoplasmic

AMPKα^{Thr172} and downstream (whole muscle) p53^{Ser15} that reflects a response to diminished energy availability (Jones et al. 2005) peaked with alcohol/carbohydrate ingestion. This increased p53 activity could account for the largest alcohol/carbohydrate-induced mRNA expression of *PUMA* and *SESN-2*, p53-inducible genes involved in mitochondrial ROS generation (Chipuk et al. 2005; Liu et al. 2005) and scavenging (Budanov et al. 2004), respectively. As a DNA damage-sensitive antioxidant, *SESN-2* may have been upregulated to quench excessive ROS and combat further tissue damage. However, there were no differences in the ratio of reduced (GSH) to oxidised (GSSG) glutathione, decreases of which reflect oxidative stress (Powers et al. 2010). Direct mitochondrial measurements of ROS such as hydrogen peroxide may be a more accurate reflection of oxidative stress in human skeletal muscle (Sahlin et al. 2010). Nevertheless, changes in redox state arising from alcohol metabolism could have posttranslationally modified (i.e., oxidatively degraded) intracellular signalling proteins, including precursor caspase-3, which prevents its apoptotic activity (Zeigler et al. 2003). Indeed, limited ATP availability facilitates AIF-driven apoptosis (Daugas et al. 2000b; Nicotera et al. 2000). Future time course studies are required to ascertain whether ATP depletion and/or mitochondrial ROS differentially activate apoptotic signal transduction following exercise and alcoholic intoxication.

Alcohol and protein co-ingestion may stimulate mitochondrial biogenesis

The capacity for an increase in exogenous protein availability to prevent cellular damage (e.g., preservation of Atgs) and attenuate the magnitude of alcohol-induced apoptotic DNA fragmentation was associated with an apparent activation of mitochondrial biogenesis. PGC-1α is the transcriptional "master regulator" of mitochondrial anabolism and increased rapidly in the

nucleus following exercise when alcohol was consumed, deteriorating thereafter when carbohydrate was co-ingested. Alcohol co-ingested with protein otherwise facilitated nuclear retention of PGC-1α and promoted its cytoplasmic accumulation along with raising nuclear levels of TFEB, which in addition to regulating autophagosome and lysosome abundance, is a coactivator of PGC-1α and governs mitochondrial turnover (i.e., undulations of synthesis and breakdown) (Scott et al. 2014). Hence, because autophagy (including mitophagy) was neither up- nor downregulated with alcohol and protein co-ingestion, the TFEB response presumably favoured mitochondrial biogenesis. Indeed, PGC-1α-inducible genes (*NRF-1*, *Tfam*) and the p53 transcriptional target *SCO2* that promote mitochondrial anabolism (Hood 2001), increased to the greatest extent when alcohol and protein were co-ingested. Although large *PGC-1α* mRNA responses to exercise followed for all treatments, its preferential accumulation at the protein level with alcohol/protein and resultant transcriptional response (e.g., *NRF-1* upregulation) suggests that this effect was compensatory to counter the effects of alcohol exposure, since measureable increases in PGC-1α protein and rates of mitochondrial protein synthesis typically occur 18-24 h after acute exercise (Baar et al. 2002; Perry et al. 2010; Burd et al. 2012). In support of this postulate, mitochondrial proteins required for ATP production (COXIV, ATPAF1) and ATP trafficking (VDAC1) all increased following exercise to the greatest extent with alcohol and protein co-ingestion, whereas Mitofusin-2 levels, an outer mitochondrial membrane fusion protein, were unchanged, suggesting that these nascent mitochondrial proteins may have been import-incorporated into existing organelles.

Alcohol-induced ROS production is particularly damaging to mitochondrial DNA (mtDNA) due to its close proximity to the respiratory chain, and alcohol exposure has been shown to prevent mtDNA-encoded translation of proteins encoding for subunits of respiratory complexes

(Cunningham et al. 1990). Thus, it is tempting to speculate that alcohol and protein-induced mitochondrial biogenesis represented a homeostatic matching of ATP synthesis with ROS formation to relieve mtDNA and cellular damage, thereby lowering the overall apoptotic response (i.e., reducing oxidative damage to cellular proteins etc.). The reason(s) for exogenous protein and not carbohydrate eliciting this protective response against alcohol despite matched energy content is unknown, but could be related to branched-chain amino acids (BCAAs), of which whey protein is enriched, harbouring an intrinsic capacity to induce skeletal muscle mitochondrial biogenesis when mitochondrial function is impaired (D'Antona et al. 2010). The previous finding of attenuated myofibrillar protein synthesis with alcohol/protein co-ingestion may have culminated from a concomitant stimulation of mitochondrial protein synthesis, in turn restricting available amino acid substrate for maximal stimulation of the myofibrillar fraction (Parr et al. 2014). Of note is that in a parallel study, lower blood alcohol levels from 6-8 h of exercise recovery with alcohol/protein versus alcohol/carbohydrate were reported (Parr et al. 2014). Although the mechanism(s) of this protein-induced reduction in blood alcohol is unknown, these findings suggest that protein availability reduced skeletal muscle uptake of alcohol and its metabolites (e.g., acetate) thus, in part, relieving mitochondria from the burden of alcohol catabolism and inevitable ROS formation. It is also unclear whether protein availability before or after alcohol ingestion, or the combined effects of consuming two beverages (50 g total protein), is the catalyst for attenuating alcohol-inflicted cellular damage. Nonetheless, timing and distribution of exogenous protein used in the current study otherwise aligns with optimal anabolic feeding strategies that elevate plasma aminoacidemia to levels likely necessary to counter post-exercise alcohol-induced intramuscular toxicity (Areta et al. 2013; Parr et al. 2014). Future studies investigating rates of mitochondrial protein synthesis as well as mitochondrial

respiration following combined exercise- and alcohol-induced intracellular stress are required to confirm this thesis.
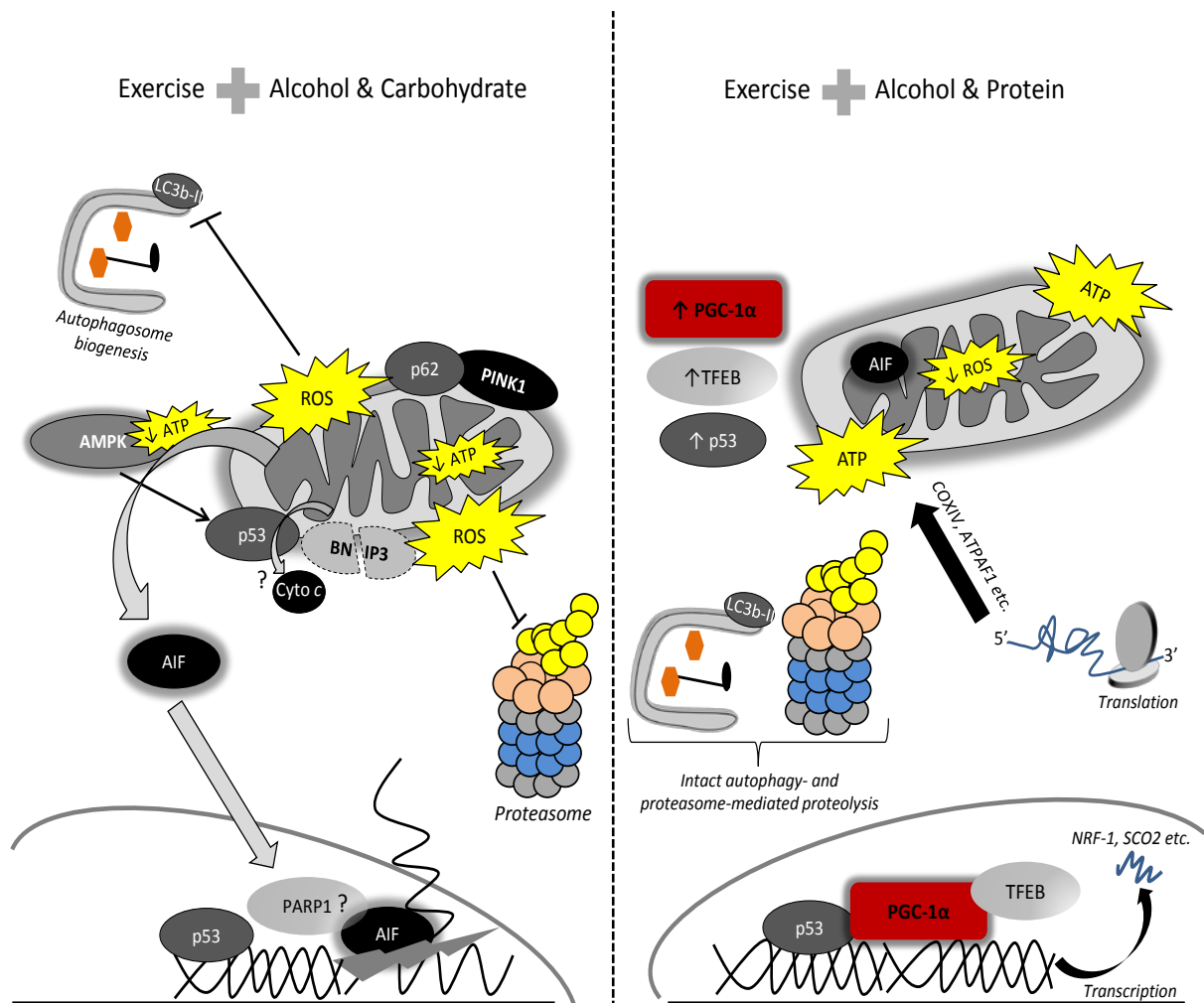
Figure 5.9. Hypothetical model of the effects of alcohol co-ingested with carbohydrate versus protein following exercise training

Following strenuous exercise, binge alcohol consumption co-ingested with carbohydrate overwhelms mitochondrial respiration, leading to a reduction in cellular ATP levels and overproduction of reactive oxygen species (ROS) that causes oxidation of proteins implicated in cellular turnover (i.e., autophagy- and proteasome-mediated proteolysis). Consequently, failed clearance of damaged mitochondria, as indicated by a reduction in BNIP3 and increase in PINK1 and p62, and persistent ROS emissions could augment mitochondrial membrane permeability and trigger release of apoptogenic factors; notably, AIF (which may be recruited to the nucleus by PARP1) and cytochrome *c*, yet the latter requires confirmation. AIF nuclear translocation triggers apoptotic DNA fragmentation. In contrast, protein availability, potentially owing to its enrichment of branched-chain amino acids, stimulates an abrupt, mitochondrial anabolic response regulated by PGC-1α, p53 and TFEB. Ultimately, the transcription of mRNA and translation of proteins integral to mitochondrial biogenesis is a homeostatic countermeasure (i.e., by increasing cellular ATP availability, attenuating ROS formation and preserving constitutive degradative pathways) to the metabolic burden imposed by alcohol introduced to an already disrupted, by prior exercise, cellular environment.

In conclusion, this study has provided novel information showing that alcohol ingestion instigates cellular apoptosis following strenuous exercise. Alcohol with exogenous protein availability appeared to engender skeletal muscle with an abrupt, mitochondrial anabolic response that may have combated the stress imposed by alcohol metabolism (Figure 5.9), an effect likely mediated by the intrinsic anabolic properties of BCAAs enriched in the whey protein beverages. While the effects of consuming alcohol alone after exercise are currently unknown, the post-exercise feeding patterns of carbohydrate and protein employed in the present investigation are consistent with recommendations for optimal nutrition following exercise training (Burke et al. 2011; Phillips & Van Loon 2011). As such, these data reveal several potential mechanisms unravelling how binge drinking practices could compromise sports- and exercise training recovery-adaptation (Barnes et al. 2010).

Preface to Chapter Six

Similar to the deleterious effects of alcohol intoxication on skeletal muscle protein turnover, previous evidence *in vivo* has demonstrated that excess lipid availability attenuated the maximal growth potential of skeletal muscle in response to mechanical overload (Sitnick et al. 2009). In humans, Stephens and colleagues (2015) recently reported that 7 h of an intralipid infusion diminished the muscle protein synthetic response to amino acid feeding through downregulation of mTORC1 signalling. However, little is known regarding the impact of high-fat availability on skeletal muscle autophagy, which is surprising given the intimate relationship at the lysosome between protein synthetic and degradative processes. Nonetheless, a previous study demonstrated that chronic lipid overload had negligible effects toward various markers of skeletal muscle autophagy (Turpin et al. 2009). In contrast, in non-skeletal muscle cells, persistent lipid exposure alters membrane composition of autophagic vesicles, consequently diminishing rates of fusion and perturbing autophagic flux (Koga et al. 2010). To date, no study has analysed cell signalling responses regulating protein turnover responses to an acute lipid overload in humans and whether exercise can rescue any potential dysregulations imposed upon the cellular protein turnover machinery.

Chapter Six

6 *Study 3*: Exercise Sensitises Skeletal Muscle to the Intramyocellular Anabolic Effects of Protein Ingestion Following an Intralipid Infusion

6.1 Abstract

High-systemic fat availability attenuates protein feeding-induced intramuscular anabolic mTORC1 signalling. Whether the combined effects of exercise and protein ingestion can 'rescue' this inhibition is unknown. In a parallel-groups design, middle-aged sedentary males (n = 28) matched for fat-free mass and body mass index received either a 5 h saline infusion (n = 9), an intravenous 20% lipid infusion (n = 9), or a lipid infusion with concomitant exercise (n = 10). Muscle biopsies were taken immediately after the infusions, at which time a protein beverage was ingested. Further biopsies were taken 2 and 4 h after protein ingestion. Lipid infusions increased plasma free fatty acid concentrations from ~0.4 to ~2 mmol·L^{-1} and were elevated above control for the duration of the infusions. The greatest intramuscular mTORC1 signalling response was observed in the exercise and lipid trial (P<0.05). Phosphorylation of eIF2α at Ser^{51} was substantially elevated in response to lipid emulsions independent of exercise (P<0.05). Lipid alone triggered an elevated autophagic response with an increase in the LC3b-II/I ratio, a marker of autophagosome biosynthesis, and a reduction in the autophagosomal substrate-bridging protein p62 (P<0.05). Exercise did, however, promote a reduction in established markers of mitochondrial content including various proteins comprising complexes of the respiratory chain (P<0.05) and citrate synthase activity. There was also a concomitant increase (P<0.05) in several proteins implicated in mitochondrial autophagy (mitophagy), suggesting that the combined stresses of lipid availability and strenuous exercise was accounted for by abrupt disposal of mitochondria in an autophagosome-independent manner. Exercise retains the sensitivity of skeletal muscle to the anabolic properties of amino acids and promotes rapid mitochondrial turnover (mitophagy) in the face of high-fat availability.

6.2 Introduction

Atrophy of skeletal muscle is an inevitable consequence of aging. However, both the onset and/or severity of this process (termed sarcopenia) are exacerbated by inactivity and fat-rich diets. This situation results in an unfavourable accumulation of fat mass relative to muscle mass, termed 'sarcopenic obesity' (Parr et al. 2013). From a biological perspective, sarcopenic obesity may be associated with intrinsic defects in the intracellular systems regulating skeletal muscle protein turnover. For example, incomplete synthesis of nascent muscle proteins in response to a meal, or diminished constitutive rates of degradation of long-lived cellular proteins. To investigate this paradigm *in vivo*, Stephens and colleagues (2015) used an intravenous lipid emulsion to elevate systemic free fatty acids (FFAs) and found that, compared to a saline control, the muscle protein synthetic response to administered amino acids was blunted in young healthy males (Stephens et al. 2015). The findings from that study demonstrate that even in pre-sarcopenic individuals, high-FFA availability can cause physiological resistance to anabolic stimuli. Mechanistically, such a lipid-induced repression of muscle protein synthesis has been associated with an inability to maximally activate protein translational signalling through the mammalian (also termed mechanistic) target of rapamycin complex 1 (mTORC1) pathway (Stephens et al. 2015; Sitnick et al. 2009).

Activation of mTORC1 by amino acids and contractile stimuli take places primarily at the surface of the lysosome (Jacobs et al. 2014). The lysosome is also the terminal point of autophagy, a degradative system that catabolises cellular products (e.g., protein macromolecules) that are subsequently utilised as reduced precursor amino acids for protein synthesis (Mizushima & Klionsky 2007; Yu & Long 2015). Hence, proper synergy between these opposing, yet interrelated systems of protein synthesis and breakdown is required to preserve both the size and

quality of skeletal muscle throughout the lifespan (Masiero et al. 2009; Bujak et al. 2015). Autophagy is responsible for constitutive turnover of damaged/superfluous mitochondria (mitophagy) (Wang & Klionsky 2011). Previous models of elevating systemic FFAs have generated conflicting findings: high-FFA availability has been shown to both increase (Hancock et al. 2008; Li et al. 2016) and decrease (Sparks et al. 2005; Richardson et al. 2005) markers of skeletal muscle mitochondrial mass/function, suggesting that lipid-availability may modulate mitophagy-mediated mitochondrial turnover. Therefore, the aim of this study was to investigate the separate and combined effects of an intralipid infusion-induced increase in circulating FFAs and exercise on intramuscular protein turnover and responses to protein feeding in a cohort of subjects susceptible to developing sarcopenic obesity. It was hypothesised that compared to a saline control condition, the lipid infusion would diminish the anabolic cell signalling response to protein feeding, whereas exercise would 'rescue' this impairment. It was also hypothesised that, due to the capacity for FFAs to cause oxidative stress [e.g., mitochondrial reactive oxygen species (ROS) production] (Lambertucci et al. 2008), intralipid would upregulate a compensatory autophagic response that would also be attenuated by exercise.

6.3 Materials and Methods

Subjects

Twenty-nine sedentary, overweight, but otherwise apparently healthy male subjects volunteered to participate in this study. Subjects were excluded if they 1) regularly (>3 times per week) engaged in strenuous physical activity, 2) were smokers, or 3) taking medication(s) at the time of the study that contravened any of the outcome measurements. Subjects were advised of any possible risks associated with the study prior to providing written informed consent. The study was approved by the Human Research Ethics Committee of ACU (2016-53H) and was undertaken in accordance with the standards set by the latest revision of the *Declaration of Helsinki*. One subject was excluded from analysis due to fasting blood glucose values on the morning of an experimental trial exceeding 6.0 mmol·L^{-1}. Therefore, twenty-eight subjects [age 41 ± 1 yr; body mass (BM) 95.0 ± 2.4 kg; body mass index (BMI); 29.5 ± 0.6 kg·m^{-2}] were included in the final analyses.

Experimental Design

The study employed a parallel-groups design in which subjects were allocated to a single experimental intervention where they received either a saline control infusion (control; $n = 9$), an intravenous lipid infusion (lipid; $n = 9$), or the same lipid infusion with the concomitant performance of exercise (exercise/lipid; $n = 10$). Group stratification was by fat-free mass (FFM), BMI and age, and the physiological parameters of participants in each group are provided in Table 6.1.

Table 6.1. Participant characteristics

	Saline ($n = 9$)	Lipid ($n = 9$)	Exercise/Lipid ($n = 10$)
Age (y)	39.2 ± 2.5	41.1 ± 2.4	40.9 ± 2.2
Body Mass (kg)	91.9 ± 4.0	94.3 ± 5.7	99.0 ± 2.9
BMI (kg·m^{-2})	29.4 ± 0.8	29.2 ± 1.3	30.0 ± 1.0
Fat Mass (kg)	27.5 ± 2.2	26.9 ± 3.4	31.5 ± 1.9
Fat Free Mass (kg)	61.3 ± 2.6	64.4 ± 2.8	63.8 ± 1.5
$\dot{V}O2_{peak}$ (ml·kg^{-1}·min^{-1})	36.67 ± 2.70	38.15 ± 2.91	32.52 ± 1.72
$\dot{V}O2_{peak}$ (L·min^{-1})	3.37 ± 0.28	3.51 ± 0.22	3.19 ± 0.15
PPO (W)	245 ± 20	251 ± 13	227 ± 12
Leg Extension 1-RM (kg)	125 ± 8	120 ± 9	130 ± 7

All values represent mean $\pm$ standard error of the mean (SEM). Data were analysed using a one-factor ANOVA. No significant differences were found for any variable between groups ($P > 0.05$).

Physiological Testing and Diet and Exercise Control

At least one week prior to an experimental trial, subjects undertook assessments of body composition, peak oxygen uptake ($\dot{V}O_{2peak}$) and peak power output (PPO) and maximum strength of the quadriceps. Body composition was determined with whole-body dual-energy X-ray absorptiometry (DXA) analysis (GE Lunar iDXA Pro, enCORE software Version 16), while $\dot{V}O_{2peak}$, PPO and maximum strength of the quadriceps were determined as described in Section 3.3. For the 48 h prior to an experimental trial, subjects were instructed to refrain from participating in any strenuous physical activity and to abstain from consuming any caffeine or alcohol. Subjects were provided with pre-packaged food and drinks to be consumed as the final, high-carbohydrate meal (3 g carbohydrate·kg^{-1} BM, 0.3 g fat·kg^{-1} BM, 0.5 g protein·kg^{-1} BM) the night before an experimental trial.

Experimental Trials

Subjects reported to the laboratory at ~0700 h on the morning of an experimental trial following a ~10-12 h overnight fast. After resting in the supine position for ~15 min, a Teflon catheter was inserted into the antecubital vein of each forearm and a baseline blood sample (~4 mL) was obtained. A 20% intravenous lipid (Baxter, NSW, Australia) infusion (intralipid; 20% soybean oil, 1.2% egg yolk phospholipids, 2.25% glycerin and water for infusion) was subsequently administered at a rate of 1.5 mL·min^{-1} for 5 h, or a saline (0.9%) control infusion at the identical rate for the same duration with blood samples taken every hour throughout the infusions. Heparin (Pfizer, NSW, Australia), an anticoagulant, was infused in combination with intralipid to catalyse the release of FFAs from the emulsion into the circulation. Following an initial bolus injection (200 U), heparin was infused at a continuous rate (600 U·h^{-1}) alongside intralipid for the 5 h period. Immediately after the commencement of intralipid, subjects in the exercise group undertook a concurrent exercise bout comprised of 30 min of cycling at ~60% of $\dot{V}O_{2peak}$ followed by resistance exercise (7 × 5 knee extension repetitions, 80% of 1 RM, with an additional 8th set at the same lifting intensity performed to volitional fatigue). Subjects in the non-exercising groups rested. Concurrent exercise was selected due to the large intramuscular signalling and protein turnover responses to divergent exercise stimuli that occur in the untrained state (Wilkinson et al. 2008). Upon completion of the 5 h lipid or saline infusion, an initial muscle biopsy was obtained from the *vastus lateralis* under local anaesthesia (1% lignocaine) using a 5 mm Bergstrom needle modified for suction. Immediately following the first muscle biopsy, subjects in each group ingested 500 mL of a whey protein beverage (30 g, WPI; Swisse, Victoria, Australia) and rested throughout a 4 h period, during which time blood samples were taken every hour. Additional muscle biopsies were obtained 2 and 4 h after protein ingestion

(Figure 6.1). Biopsies were taken from separate incision sites (~2-3 cm apart), cleared of any visible adipose and/or connective tissue, snap-frozen in liquid nitrogen and stored at −80 ℃ for subsequent analysis. Blood samples were centrifuged at 4 ℃ (1,500 g, 10 min) and aliquots of plasma were stored at −80 ℃.

Figure 6.1. Schematic overview of experimental trials comprising Study 3

Analytical Procedures

Blood Analyses

Plasma FFA concentration was analysed using a nonesterified fatty acid (NEFA) kit (Wako Pure Chemical Industries, Osaka, Japan). Blood glucose concentrations were determined using a YSI (model 2900) Biochemistry Analyser (Xylem, QLD, Australia). Plasma insulin, tumour necrosis factor α (TNF-α) and interleukin-6 (IL-6) were measured utilising commercially available and

customised Milliplex human magnetic bead panels (Millipore, Massachusetts, USA) according to the manufacturer's instructions. Analytes were quantified in duplicate using the Magpix system using xPONENT 4.2 software. Two quality controls of designated ranges were run with each assay to ensure validity of the data generated.

Western Blotting

Approximately 30 mg of skeletal muscle was homogenised to generate whole muscle lysates 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 16,000 g for 30 min at 4 °C and the supernatant was subsequently collected. After determination of protein concentration using a BCA protein assay (Pierce, Rockford, USA), all lysates were resuspended in Laemmli sample buffer and loaded for electrophoresis into Stain-Free™ Gels (Bio-Rad, California, USA) or, for the optimal detection of each protein detected using the OXPHOS antibody cocktail (described subsequently), lysates were loaded into 12% polyacrylamide gels. Protein transfer to polyvinylidine fluoride (PVDF) membranes was performed as described in Study 1. Following transfer, a Stain-Free image of the PVDF membranes for total protein normalization was obtained before membranes were blocked for ~1 h with 5% non-fat milk and washed with 10 mM Tris·HCl, 100 mM NaCl and 0.02% Tween 20 (TBST). Membranes were then incubated overnight at 4 °C with a primary antibody diluted in TBST (1:1,000). Membranes were incubated for 1 h the next day with a secondary antibody diluted in TBST (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 time points for each subject were run singularly on the same gel. Antibodies against phosphorylated (p)-4E-BP1$^{Thr37/46}$ (no. 2855), 4E-BP1 (no. 9644), p-ACC^{Ser79} (no. 11818), ACC (no. 3662), p-Akt at Thr^{308} and Ser^{473} (no. 9275 and 9271, respectively), Akt (pan) (no. 4691), p-AMPK^{Thr172} (no. 2531), AMPKα (no. 2532), cAtg12 (no. 4180), p-DRP1^{Ser616} (no. 3455), DRP1 (no. 8570), p-eEF2^{Thr56} (no. 2331), eEF2 (no. 2332), p-eIF2α^{Ser51} (no. 9721), eIF2α (no. 9722), GAPDH (no. 2118), LC3b (no. 2775), Mitofusin-2 (no. 11925), p-mTOR^{Ser2448} (no. 2971), mTOR (no. 2972), p62 (no. 5114), p-p70S6K^{Thr389} (no. 9205), p70S6K (no. 9202), Parkin (no. 2132), PINK1 (no. 6946), p-PRAS40^{Thr246} (no. 2640), PRAS40 (no. 2610), p-Raptor^{Ser792} (no. 2083), Raptor (no. 2280), p-rpS6$^{Ser235/236}$ (no. 2211), rpS6 (no. 2217), p-TSC2^{Thr1462} (no. 3611), TSC2 (no. 3612), p-ULK1 at Ser^{317} and Ser^{757} (no. 12753 and no. 6888, respectively), and ULK1 (no. 8054) were purchased from Cell Signalling Technology (Danvers, MA). Antibodies directed against citrate synthase (ab96600), VDAC1 (ab14734), an additional LC3b antibody (ab48394) and a human mitochondrial OXPHOS antibody cocktail (ab110411) were purchased from Abcam (Melbourne, Australia). With the exception of the OXPHOS cocktail that was normalised to GAPDH, volume density of each target band for all proteins (n = 8-10 per time point) was normalised to the total protein loaded into each lane (Figure 6.2) using Stain-Free™ technology (Bio-Rad, California, USA) (Gurtler et al. 2013).
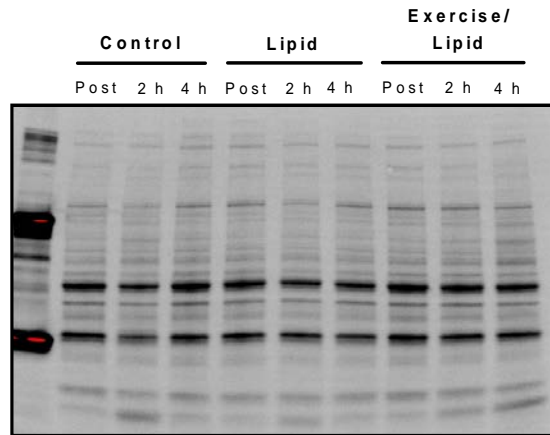
Figure 6.2. Representative Stain-Free image used for protein normalization (refer to Section 3.1 for the molecular weight ladder)

Citrate Synthase Activity

Citrate synthase activity was determined as previously described (Granata et al. 2016). In brief, samples were analysed in duplicate by loading 5 µL of whole skeletal muscle lysate (prepared at a concentration of 2 mg·mL^{-1}) into each well of a 96-well microtiter plate containing a reagent cocktail of 25 µL of 1 mM DTNB [5,5′-Dithiobis(2-nitrobenzoic acid)], 40 µL of 3 mM Acetyl CoA and 165 µL of 100 mM Tris buffer (pH 8.3). Following the addition of 15 µL of Oxaloacetic acid (10 mM) to each well, absorbance was measured at 30 s intervals for 5 min at a wavelength of 412 nm using a SpectraMax Paradigm plate reader (Molecular Devices, Sunnyvale, CA). Citrate Synthase activity is expressed as µmol per minute, per gram of muscle protein.

Statistical Analysis

Data were analysed using two-way analysis of variance (ANOVA) with Student-Newman-Keuls post-hoc analysis (time × treatment) performed when an overall statistically significant difference in group means of a particular comparison was found (SigmaPlot for Windows; Version 12.5). Subject characteristics and areas under the curve (AUC) for blood metabolites were analysed using a one-factor ANOVA with the same post-hoc analysis. Significance was set to P<0.05 and all data are presented as mean ± SEM. When tests for normality and/or equal variance failed for Western blot densitometry values, data were log-transformed where appropriate and statistical inferences were made based on these data.

6.4 Results

Plasma FFA, Glucose and Insulin Concentrations

Lipid infusions increased plasma FFA levels above baseline with no differences between exercise and non-exercise groups, reaching a peak concentration of ~2.0 mmol·L^{-1} after 4 h (P<0.001; Figure 6.3A). As a result, there was no difference for the AUC for FFAs between the two lipid conditions. As intended, FFA levels were significantly higher (P<0.001) than the control condition for the duration of the intralipid infusions. These values returned to baseline and were comparable to control 2 h after cessation of lipid infusions. Exercise/lipid increased plasma insulin levels above lipid and control groups 3 h into the infusions (P<0.05; Figure 6.3B), which also lead to an augmented insulin response to protein ingestion (P<0.05). These differences produced a greater insulin AUC compared to control for exercise/lipid (P<0.05). Although no differences between all treatments were found in the plasma glucose AUC (Figure

6.3C), plasma glucose was elevated ($P<0.05$) above lipid and control conditions 3 h into the infusion in exercise/lipid. Plasma glucose was also lower 4 h into the control infusion compared with exercise/lipid alone ($P<0.05$). Notably, the increase in plasma insulin 60 min after ingestion of protein in all experimental conditions was independent of changes in glucose concentration.
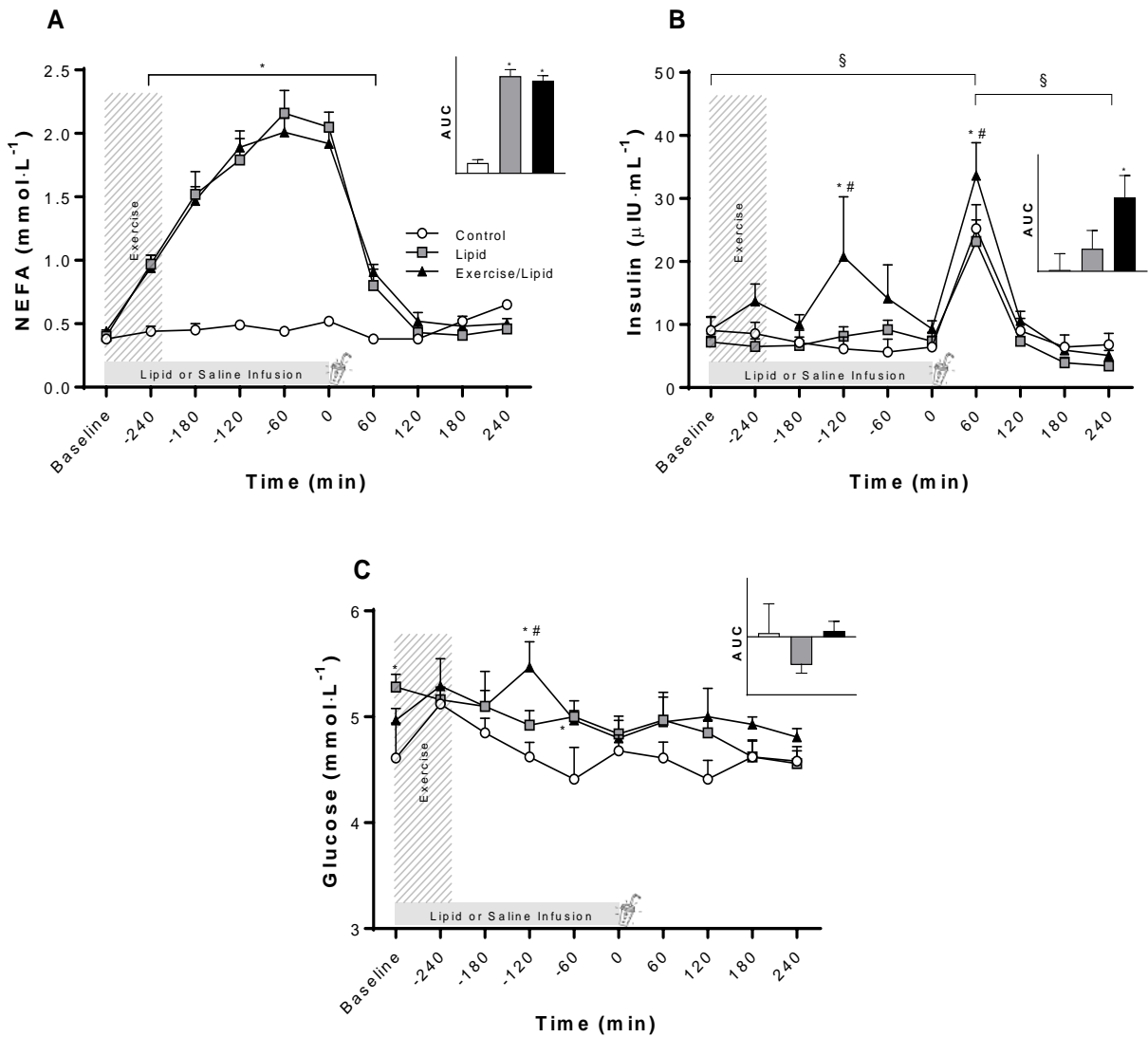
Figure 6.3. Plasma non-esterified free fatty acids (NEFA; A), insulin (B) and glucose (C) analysed throughout a 5 h saline control infusion or an intralipid emulsion with or without the addition of exercise. All groups consumed 30 g of whey protein immediately after the infusion

Data were analysed using a 2-way ANOVA with repeated measures and Student-Newman-Keuls post-hoc analysis (1-way ANOVA was used to analyse AUCs). Data are presented (mean ± SEM). Significant effect of time compared with all other time points (§), or differences between treatments: (*) saline or (#) lipid (P<0.05).

Plasma Cytokines

Plasma IL-6 concentration was increased in the saline infusion compared to intralipid treatment after 4 h ($P<0.05$). There were no other significant differences in circulating IL-6 or TNF-α at any time (Figure 6.4A, B).
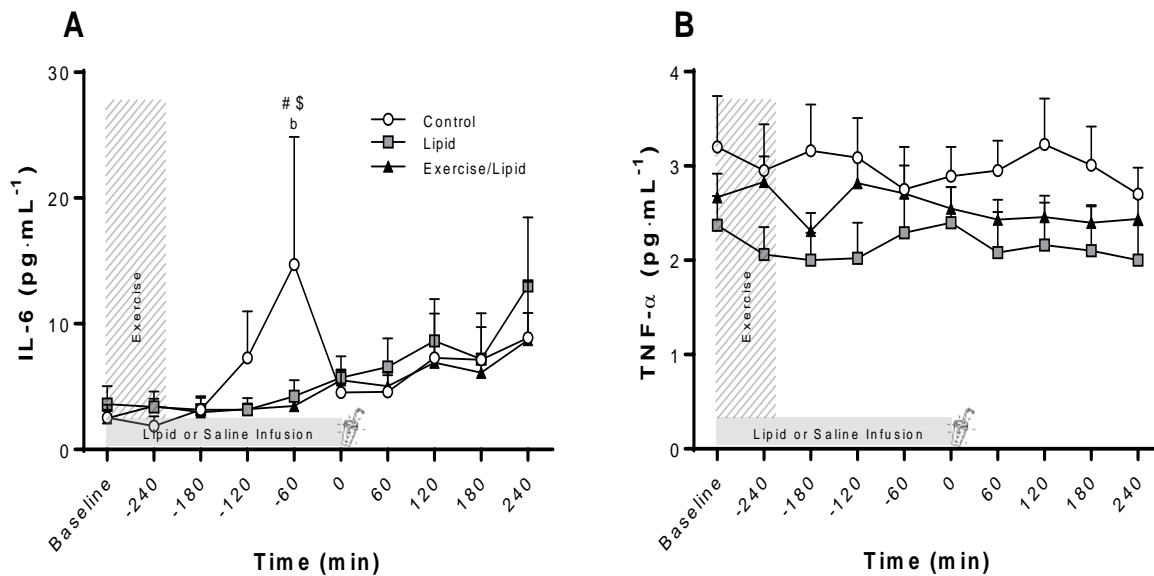
Figure 6.4. Plasma interleukin-6 (IL-6; A) and tumour necrosis factor-α (TNF-α; B) analysed throughout a 5 h saline control infusion or an intralipid emulsion with or without the addition of exercise. All groups consumed 30 g of whey protein immediately after the infusion

Data were analysed using a 2-way ANOVA with repeated measures and Student-Newman-Keuls post-hoc analysis. Data are presented (mean ± SEM). Significant effect of time compared with (b) -240 min, or differences between treatments: (#) lipid or ($) exercise/lipid (P<0.05).

Akt/mTORC1 Signalling

Akt phosphorylation at Thr^{308} and Ser^{473} residues, indicative of complete activation of the protein kinase (Alessi et al. 1996), was increased above control after the lipid infusion (P<0.05) and elevated further when exercise was performed (P<0.01; Figure 6.5A, B). This Akt response declined incrementally from 2 h after protein ingestion. In contrast, Akt^{Ser473} phosphorylation was elevated 2 h after protein ingestion in control (P<0.01) and had returned to post-infusion levels by 4 h. Akt substrate of 160 kDa $(AS160)^{Thr642}$ phosphorylation followed essentially a similar pattern to Akt, increasing to the largest extent in exercise/lipid after the infusion and declining thereafter (P<0.05; Figure 6.5C). Similarly, phosphorylation by Akt of negative regulators of mTORC1 (Vander Haar et al. 2007; Inoki et al. 2002), tuberin (or $TSC2^{Thr1462}$) and proline-rich Akt substrate of 40 kDa $(PRAS40^{Thr246})$ was augmented by exercise/lipid and declined after protein ingestion (P<0.05; Figure 6.5D, E). Unlike the reduction in Akt signalling 2 h after protein ingestion, $mTOR^{Ser2448}$ phosphorylation was amplified at this time in exercise/intralipid, as well as in non-exercise groups, yet its phosphorylation persisted for an additional 2 h in exercise/lipid only (P<0.05; Figure 6.5F).
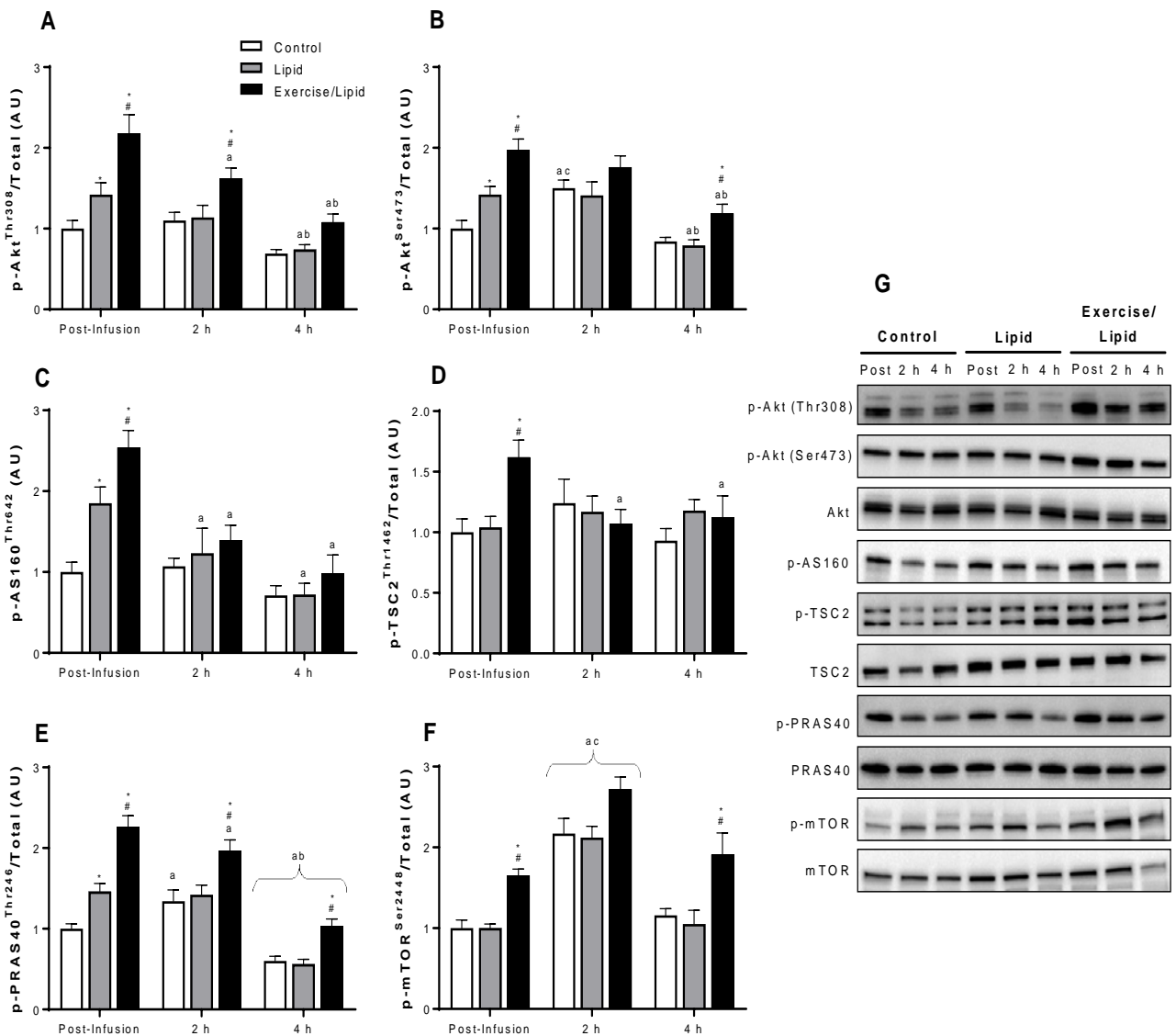
Figure 6.5. Phospho-Akt at Thr^{308} (A), Ser^{473} (B), and phosphorylation of its downstream effectors $AS160^{Thr642}$ (C), $TSC2^{Thr1462}$ (D), $PRAS40^{Thr246}$ (E) and $mTOR^{Ser2448}$ (F) relative to the total abundance of their corresponding protein analysed in response to a 5 h saline control infusion or an intralipid emulsion with or without the addition of exercise. All groups consumed 30 g of whey protein immediately after the post-infusion biopsy. G, representative images for all proteins

Data were analysed using a 2-way ANOVA with repeated measures and Student-Newman-Keuls post-hoc analysis. Data are presented (mean $\pm$ SEM) as a fold change relative to post-infusion values in control. Significant effect of time compared with (a) post-infusion, (b) 2 h, (c) 4 h, or differences between treatments: (*) saline or (#) lipid ($P<0.05$).

141

Following protein ingestion, phosphorylation of the mTORC1 substrate eukaryotic initiation factor (eIF) 4E-binding protein-1 (4E-BP1) at $Thr^{37/46}$ was attenuated in lipid compared to control (2 h) and exercise/intralipid (4 h) (P<0.05; Figure 6.6A). Protein ingestion promoted phospho-activation of additional mTORC1 effectors, the ribosomal protein S6 (rpS6) p70 kinase (p70S6K) (Thr^{389}, a direct substrate of mTORC1) and rpS6 ($Ser^{235/236}$), whereby the largest effects (P<0.05; Figure 6.6B, C) prevailed for exercise/intralipid. p70S6K phosphorylation post-lipid infusion revealed a strong tendency (P=0.068) to decrease compared to exercise/lipid. Eukaryotic elongation factor 2 (eEF2) phosphorylation at Thr^{56} that causes attenuated translation elongation was lower at 2 h in exercise/intralipid compared to non-exercise conditions (P<0.01; Figure 6.6D).
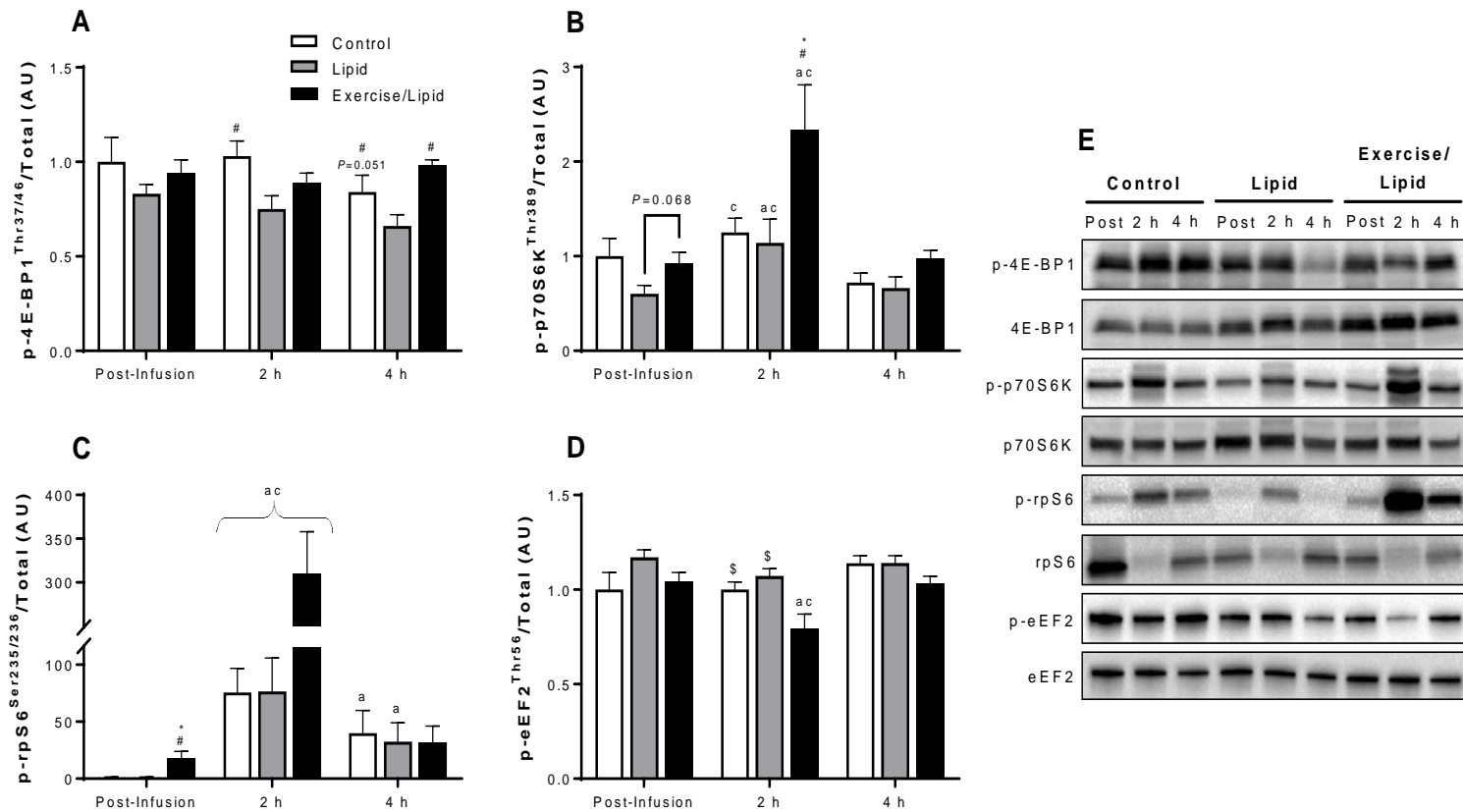
Figure 6.6. Phosphorylation of mTORC1 effectors 4E-BP1[Thr37/46] (A), p70S6K[Thr389] (B), rpS6[Ser235/236] (C) and eEF2[Thr56] (D) relative to the total abundance of their corresponding protein analysed in response to a 5 h saline control infusion or an intralipid emulsion with or without the addition of exercise. All groups consumed 30 g of whey protein immediately after the post-infusion biopsy. E, representative images for all proteins

Data were analysed using a 2-way ANOVA with repeated measures and Student-Newman-Keuls post-hoc analysis. Data are presented (mean ± SEM) as a fold change relative to post-infusion values in control. Significant effect of time compared with (a) post-infusion, (c) 4 h, or differences between treatments: (*) saline, (#) lipid or ($) exercise/lipid ($P<0.05$).

143

AMPK Signalling

There were no differences in the phosphorylation state of the cellular energy sensor AMP-activated protein kinase (AMPK) at Thr^{172}, or of its substrates regulating oxidative metabolism (acetyl-CoA carboxylase; ACC^{Ser79}) and protein synthesis (regulatory associated protein of mTOR; $Raptor^{Ser792}$) were found when normalised to their corresponding total protein (data not shown). In contrast, when expressed relative to total cellular protein (using Stain-Free normalisation), phosphorylation of ACC and Raptor, but not AMPK, were attenuated after protein ingestion in lipid alone and in both lipid groups, respectively ($P<0.05$; Figure 6.7A-C).
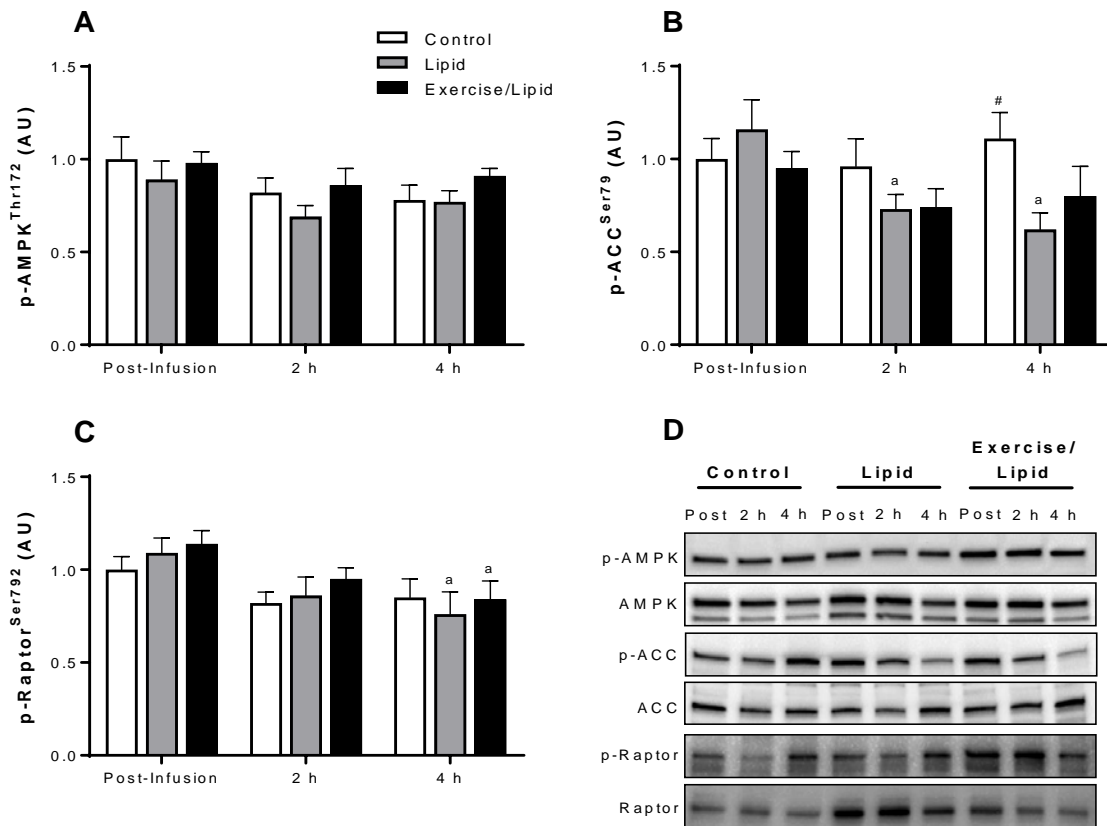
Figure 6.7. Phospho-AMPK at Thr[172] (A) and phosphorylation of its substrates ACC[Ser79] (B) and Raptor[Ser792] (C) relative to total cellular protein analysed in response to a 5 h saline control infusion or an intralipid emulsion with or without the addition of exercise. All groups consumed 30 g of whey protein immediately after the post-infusion biopsy. D, representative images for all proteins

Data were analysed using a 2-way ANOVA with repeated measures and Student-Newman-Keuls post-hoc analysis. Data are presented (mean ± SEM) as a fold change relative to post-infusion values in control. Significantly different ($P < 0.05$) vs. (a) post-infusion or between treatments: (#) lipid ($P < 0.05$).

Autophagy

The autophagy-related gene (Atg) protein Unc-51-like kinase 1 (ULK1) regulates proximal steps of autophagy induction (Kim et al. 2011). ULK1 inhibitory (mTORC1-mediated) phosphorylation at Ser^{757} was greater in control and exercise/lipid than lipid alone ($P<0.01$) 2 h after protein ingestion (Figure 6.8A). AMPK-catalysed Ser^{317} (pro-autophagy) phosphorylation of ULK1 also increased ($P<0.05$) above post-infusion levels in control at 2 h ($P<0.05$; Figure 6.8B), which was partly attributable to a corresponding reduction in its total protein content ($P<0.05$; data not shown). Phosphorylation of eIF2α at Ser^{51} leads to autophagy activation (B'Chir et al. 2013; Kouroku et al. 2007). Phospho-eIF2α at Ser^{51} was significantly elevated above control ($>100\%$, $P<0.001$), independent of exercise, in response to lipid emulsions (Figure 6.8C). When normalised to its total protein, increases in phospho-eIF2α was ~40% greater than control ($P<0.05$; Figure 6.8D).
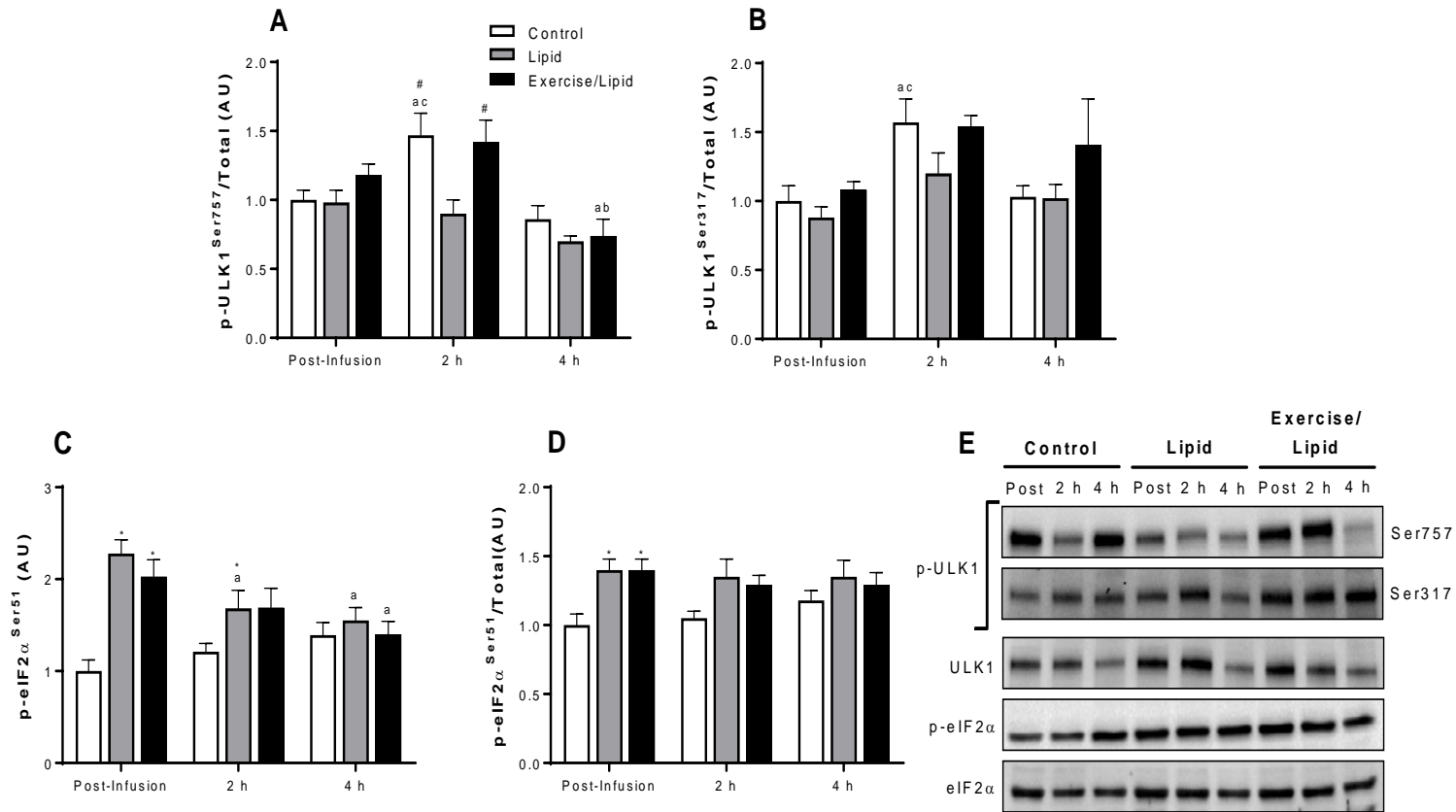
Figure 6.8. Phospho-ULK at Ser^{Ser757} (A), Ser^{Ser317} (B) and eIF2α^{Ser51} normalised to total cellular protein (C) and the abundance of its corresponding total protein (D) analysed in response to a 5 h saline control infusion or an intralipid emulsion with or without the addition of exercise. All groups consumed 30 g of whey protein immediately after the post-infusion biopsy. E, representative images for all proteins

Data were analysed using a 2-way ANOVA with repeated measures and Student-Newman-Keuls post-hoc analysis. Data are presented (mean ± SEM) as a fold change relative to post-infusion values in control. Significant effect of time compared with (a) post-infusion, (b) 2 h, (c) 4 h, or differences between treatments: (*) saline or (#) lipid ($P<0.05$).

An indirect surrogate of autophagy is the ratio of lipidated microtubule-associated protein 1 light chain 3b (LC3b-II) to non-lipidated LC3b-I. This ratio was elevated in lipid (compared to exercise/lipid) immediately post-infusion and was commensurate with an attenuation of p62 ($P<0.05$; Figure 6.9A-D). Despite a fall in the LC3b-II/I ratio and concomitant rise in p62 within each lipid group following protein ingestion, the ratio was the most pronounced (i.e., elevated above the exercise condition) in lipid alone across the 2-4 h biopsy sampling period ($P<0.05$; Figure 6.9C). The Atg5-conjugated form of Atg12 (cAtg12) was increased above post-infusion values from 2-4 h in lipid and at 4 h in exercise/lipid, resulting in a greater response compared with control ($P<0.05$; Figure 6.9E).
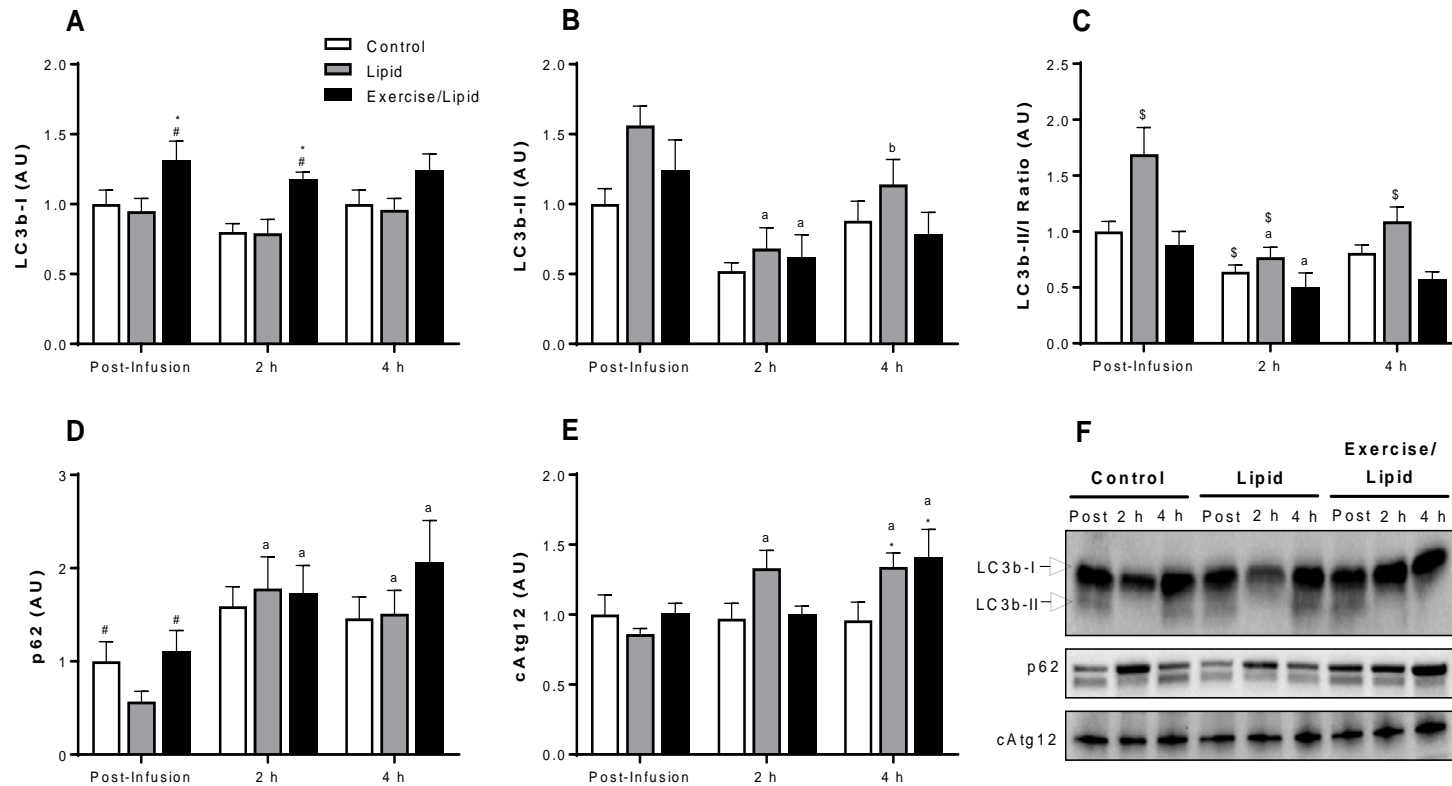
Figure 6.9. Markers of increased autophagic flux: LC3b-I (A), LC3b-II (B), the LC3b-II/I ratio (C), p62 (D) and conjugated Atg12 (cAtg12; E) analysed in response to a 5 h saline control infusion or an intralipid emulsion with or without the addition of exercise. All groups consumed 30 g of whey protein immediately after the post-infusion biopsy. F, representative images for all proteins

Data were analysed using a 2-way ANOVA with repeated measures and Student-Newman-Keuls post-hoc analysis. Data are presented (mean ± SEM) as a fold change relative to post-infusion values in control. Significant effect of time compared with (a) post-infusion, (b) 4 h, or differences between treatments: (*) saline, (#) lipid or ($) exercise/lipid (P<0.05).

149

Mitochondrial Content

An antibody directed against five individual subunits of each respiratory chain complex was utilised as an indirect marker of mitochondrial content in human skeletal muscle (Larsen et al. 2012). Relative to the control group, exercise/lipid led to a consistent reduction in four out of the five respiratory proteins measured (only complex II remained unaffected). The largest effect ($P<0.05$) occurred immediately post-infusion (Figure 6.10A-E). Complex V (ATP5A) was attenuated by lipid post-infusion, in addition to the protein abundance of citrate synthase for each lipid group ($P<0.05$; Figure 6.10G). Compared to control, there was a trend for citrate synthase activity ($P=0.068$) to decline in exercise/lipid, but not lipid alone ($P=0.135$), 4 h after protein ingestion (Figure 6.10H).
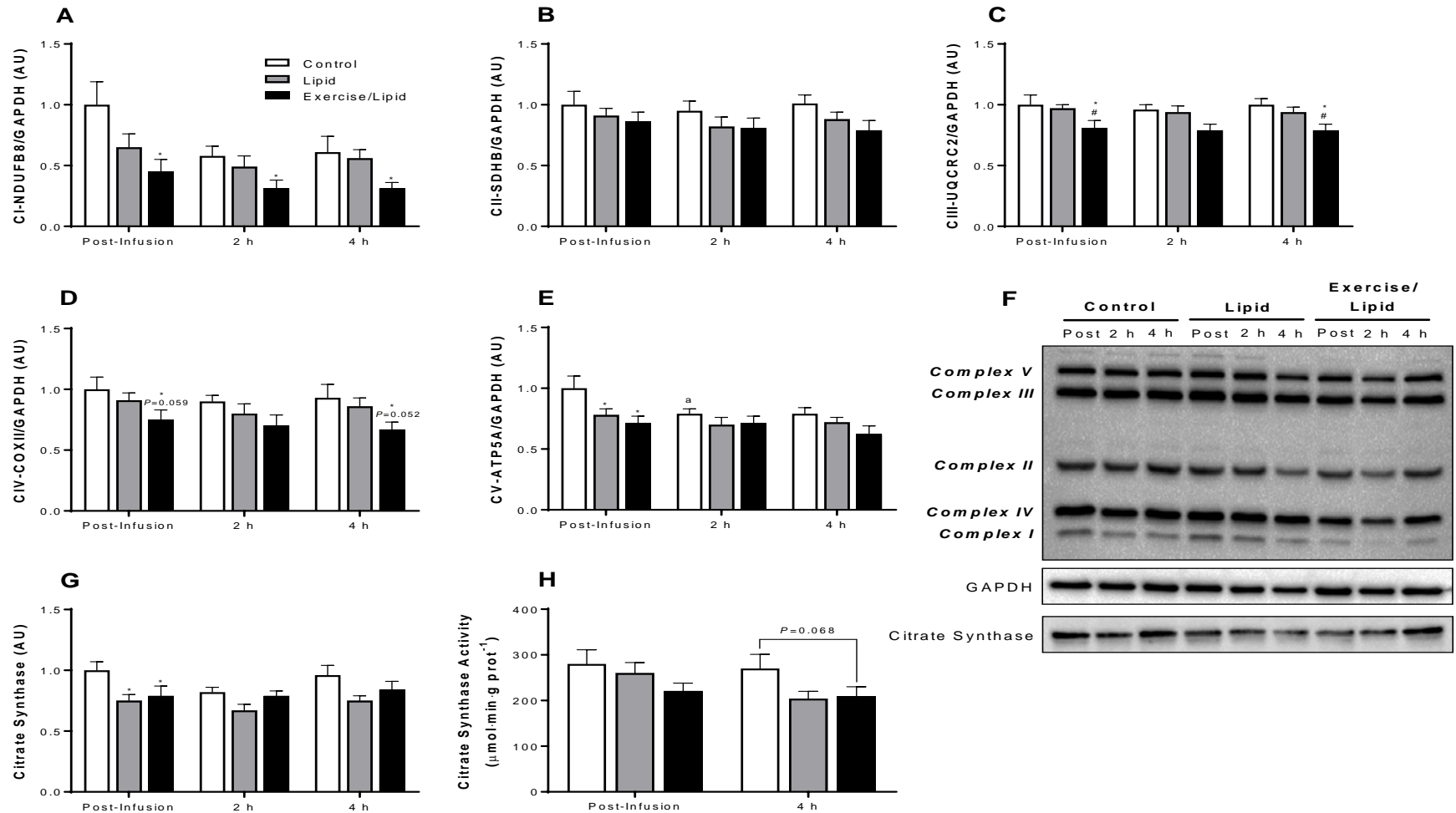
Figure 6.10. Markers of mitochondrial content: protein abundance of subunits comprising complexes I-V of the respiratory chain (A-E), the matrix enzyme citrate synthase (G) and citrate synthase activity (n = 7-10 per time point; H) analysed in response to a 5 h saline control infusion or an intralipid emulsion with or without the addition of exercise. All groups consumed 30 g of whey protein immediately after the post-infusion biopsy. F, representative images for all proteins

Figure 6.10. Continued

Data were analysed using a 2-way ANOVA with repeated measures and Student-Newman-Keuls post-hoc analysis. Data are presented (mean ± SEM) as a fold change relative to post-infusion values in control. Significant effect of time compared with (a) post-infusion, or differences between treatments: (*) saline or (#) lipid (P<0.05).

Mitochondrial Autophagy, Fusion and Fission

Mitochondria-specific autophagy (mitophagy) is governed by PINK1 (PTEN-induced putative kinase 1) and Parkin. PINK1 levels were increased following protein ingestion in all experimental groups (P<0.01; Figure 6.11A). There was a tendency (P=0.057) for PINK1 abundance to increase further above lipid alone at 4 h with exercise/lipid. Parkin was increased at all time points (P<0.05) in exercise/lipid above other experimental conditions (Figure 6.11B). The abundant outer mitochondrial membrane protein voltage-dependent anion channel 1 (VDAC1) increased (P=0.017; Figure 6.11C) in exercise/lipid above the other experimental conditions (4 h). Levels of the mitochondrial fusion protein Mitofusin-2 remained unchanged in exercise/lipid, but was attenuated (P<0.05) 4 h after the infusion in lipid alone (Figure 6.11D). Finally, post-infusion levels of the pro-mitochondrial fission phosphorylation of dynamin-related protein (DRP1) at Ser^{616} showed a tendency (P=0.055) to increase above post-infusion levels at 2 h in lipid only (Figure 6.11E). DRP1 total protein abundance was attenuated below control in each lipid group after the infusions, and declined further with protein ingestion in lipid alone (P<0.05; Figure 6.11F).
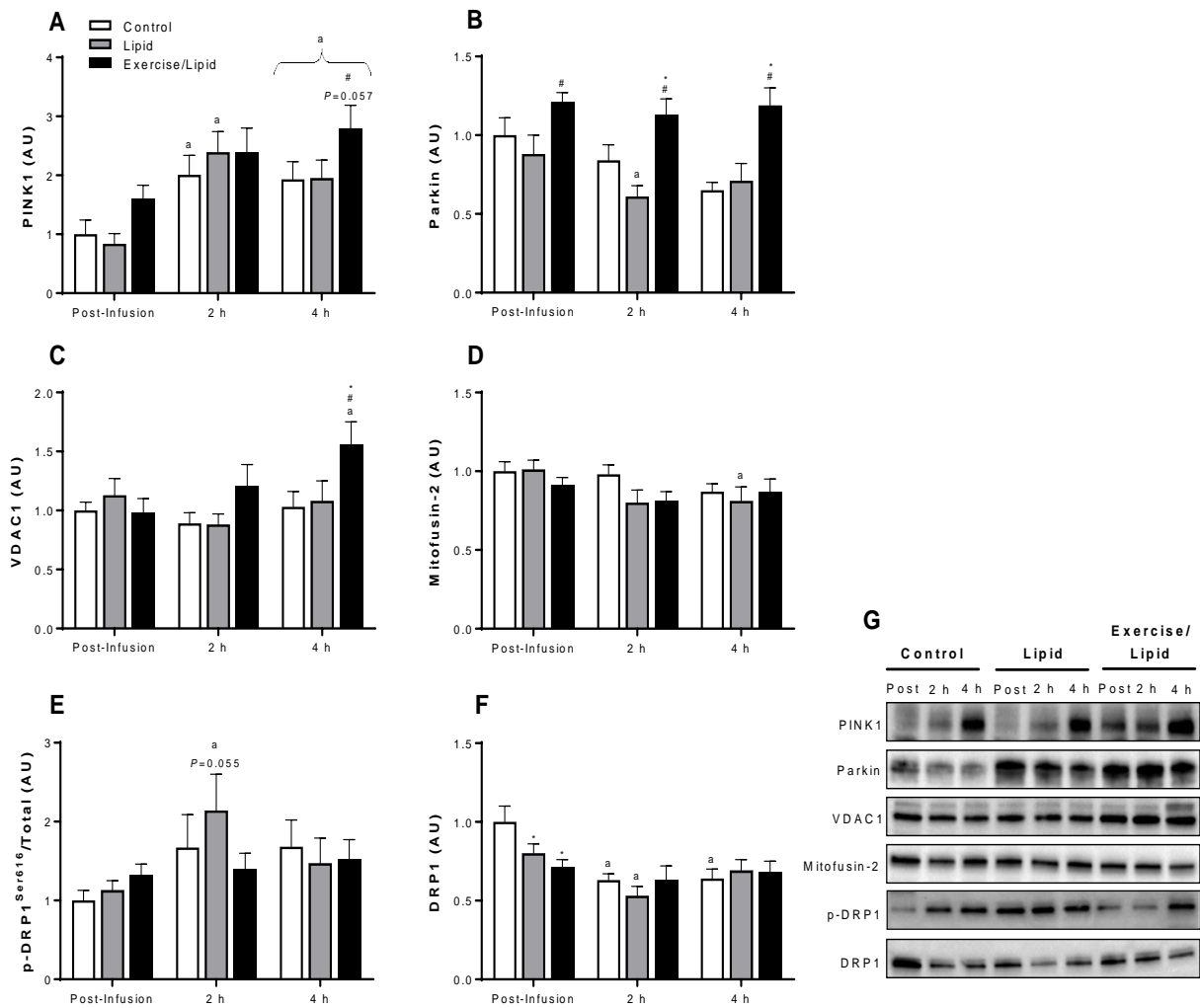
Figure 6.11. Markers of mitochondrial turnover/dynamics: PINK1 (A), Parkin (B), VDAC1 (C), Mitofusin-2 (D) and phospho- (E) and total-DRP1 (F) analysed in response to a 5 h saline control infusion or an intralipid emulsion with or without the addition of exercise. All groups consumed 30 g of whey protein immediately after the post-infusion biopsy. G, representative images for all proteins

Data were analysed using a 2-way ANOVA with repeated measures and Student-Newman-Keuls post-hoc analysis. Data are presented (mean ± SEM) as a fold change relative to post-infusion values in control. Significant effect of time compared with (a) post-infusion, or differences between treatments: (*) saline or (#) lipid ($P<0.05$).

6.5 Discussion

This is the first study to characterise the effects of high-systemic fat availability combined with exercise on muscle protein turnover responses in humans susceptible to developing sarcopenic obesity. The first finding was that exercise undertaken at the onset of an intralipid infusion sensitised skeletal muscle to the anabolic properties of amino acid ingestion. In addition, exercise attenuated the lipid-induced induction of cellular markers of autophagy, which facilitated a potentiated translational signalling response to protein feeding. Exercise also promoted concomitant disposal of mitochondrial respiratory chain and matrix proteins in a manner involving activation of PINK1/Parkin signalling, but distinct from canonical, autophagosome-dependent mitochondrial autophagy (mitophagy). Hence, any potential lipid- and/or contractile-induced damage to mitochondria can be compensated for in an energy-efficient manner to prioritise energy availability for alternate cellular processes such as protein translation.

Exercise sensitises skeletal muscle to amino acids independent of lipid-induced Akt signalling

Recently, Stephens and colleagues (2015) reported that a 10% lipid emulsion infused for 7 h diminished the muscle protein synthetic response to a single (21 g) feeding of amino acids that in part, was attributable to a defect in mTORC1-directed phosphorylation of 4E-BP1, but independent of upstream Akt activity. That investigation employed a hyperinsulinemic-euglycemic clamp in which elevated FFA availability triggered insulin resistance and impaired glucose disposal (Stephens et al. 2015). The results of Stephens et al. (2015) suggest that acute, lipid-induced anabolic resistance to feeding is distinct from defects in proximal insulin/Akt signalling. In the present study, insulin and glucose were 'unclamped,' but infusion of intralipid elevated Akt signalling, an effect that was even greater after exercise. Peak Akt substrate

phosphorylation (and thus inhibition) of negative regulators (TSC2, PRAS40) of mTORC1 was observed immediately after the lipid infusion and decayed following ingestion of 30 g of whey protein. FFAs can stimulate insulin release (Itoh et al. 2003), which probably explains the elevated Akt response to the lipid emulsions. However, the mechanism by which exercise augmented insulin/Akt signalling is unknown, but may, in part, relate to a transient increase in blood glucose that occurred during the lipid emulsion and resultant phosphorylation of the Akt target AS160, which is implicated in glucose uptake (Kramer et al. 2006).

Consistent with these data (Stephens et al. 2015), phosphorylation of 4E-BP1 in response to protein ingestion was reduced by increased lipid availability, indicating that amino acid-induced 4E-BP1 phosphorylation in skeletal muscle is highly sensitive to elevations in circulating FFAs. In that regard, it is possible that ingestion of greater amounts of protein (>30 g) is necessary to overcome the repressive effects of increased lipid availability toward 4E-BP1 phosphorylation, an event that leads to translation of mRNAs encoding translation factors and ribosomal proteins (Thoreen et al. 2012). p70S6K regulates both protein translation initiation and capacity (which involves augmenting cellular ribosomal mass) (Jastrzebski et al. 2007) and its phosphorylation on Thr[389] is an accepted readout of mTORC1 signalling (Goodman et al. 2011). Although there was a non-significant (P=0.068) reduction in p70S6K phosphorylation after the lipid infusion, similar high-fat feeding-induced reductions (P=0.08) in its activity have been reported in humans following endurance-based exercise (Hammond et al. 2016). However, in that investigation there was no manipulation of exogenous protein availability. Conversely, in the present study, the addition of aerobic- and resistance-based exercise to the lipid infusion augmented protein feeding-induced stimulation of mTORC1 signalling (e.g., p70S6K and eEF2 phosphorylation) above the saline control infusion. Thus, even in the face of elevated plasma FFAs, exercise

sensitises the intramuscular translational machinery to the anabolic properties of ingested amino acids.

Lipid availability stimulates autophagosome biogenesis independent of AMPK signalling

Canonical autophagy involves endogenous biosynthesis of autophagosomes, double-membrane-bound vesicles that indiscriminately engulf cellular material, or segregate specific subcellular compartments (e.g., mitochondria) for lysosomal degradation (Mizushima & Klionsky 2007). Using immunoblotting, this process can be indirectly estimated by an increase in the autophagosomal membrane-bound LC3b-II (relative to cytosolic LC3b-I) and a reduction in p62 (Tanida & Waguri 2010). Indeed, these changes in LC3b and p62 were evident following the lipid infusion and were abrogated by prior exercise. Moreover, the autophagic response was independent of AMPK-regulated activation of ULK1, a proximal effector of autophagy (Kim et al. 2011). Exposing divergent cell types to individual fatty acids (e.g., palmitate) has been shown to both activate (Hebbachi & Saggerson 2012) and inhibit (Wu et al. 2007) AMPK signalling. Thus, because AMPK-mediated activation of autophagy reflects an obligatory homeostatic response to conditions of diminished nutrient availability, the elevated autophagic flux probably culminated from an alternate upstream cue(s). Notably, upregulation of autophagy coincided with elevated phosphorylation of eIF2α at Ser[51], a classical response to endoplasmic reticulum (ER) stress that attenuates protein translation and can be provoked by high-lipid availability (Harding et al. 1999; Deldicque et al. 2011). However, it is worth noting that the increase in phospho-eIF2α manifested after both lipid infusions. This increase in phosphorylation status (~40%) is mechanistically relevant, as only a small elevation in eIF2α phosphorylation in the order of ~20-30% is necessary to attenuate protein translation (Holcik & Sonenberg 2005). ER

stress-induced eIF2α phosphorylation has been shown to enhance LC3b-II accumulation and autophagosome biosynthesis (Chu et al. 2015; Kouroku et al. 2007), while separate work in skeletal muscle cells revealed that ER stress also attenuates amino acid-induced mTORC1 activation (Deldicque et al. 2011). Therefore, the elevated autophagic response to lipid alone could indicate a downstream attempt to ameliorate ER stress, but whether this is to the detriment of protein translation is unknown and needs to verified experimentally (e.g., by determining muscle protein synthesis). Nonetheless, exercise appears to circumvent any potential lipid-induced diminution of protein translation by sensitizing mTORC1 signalling to the ingested amino acids.

Exercise/lipid promotes mitophagy independent of autophagosome biogenesis

Mitochondrial respiration supplies up to 90% of cellular energy with consequential leakage of electrons forming reactive oxygen species (ROS), the production of which is exacerbated by the addition of lipid metabolites to mitochondria (Garcia-Ruiz et al. 1997). ROS are potent cues for mitochondrial autophagy (mitophagy) (Qiao et al. 2015). Although no direct measures of oxidative stress were obtained in the present investigation, several markers of mitochondrial content and quality control were analysed in whole tissue homogenates as surrogates for mitochondrial turnover. Unexpectedly, exercise/lipid caused the largest reduction in mitochondrial content (Larsen et al. 2012), commensurate with the induction of mitophagy as evidenced by the accumulation of PINK1 and Parkin. In response to stress (e.g., oxidative), PINK1 becomes stabilised on the outer membrane of damaged mitochondria where it recruits Parkin (Kim et al. 2008; Vives-Bauza et al. 2010), an E3 ubiquitin ligase which ubiquitinates a host of mitochondrial substrates including, but not limited to, VDAC1 and in particular,

Mitofusin-2, whose proteasomal degradation is postulated to precede mitophagic removal of the entire organelle (Chan et al. 2011; Geisler et al. 2010; Gegg et al. 2010). This pathway eventually recruits autophagosomes to consume the sequestered organelles, as proteolysis of Mitofusin-2 disables the capacity for mitochondria to undergo fusion and escape degradation. Given that exercise/lipid suppressed the induction of autophagosome biogenesis and had little bearing on the abundance of Mitofusin-2, the present results suggest the engagement of an alternate form of PINK1/Parkin-dependent mitochondrial degradation. Support for this notion is the observation that VDAC1 accumulated alongside PINK1 and Parkin with exercise/lipid. Recent evidence highlights a role for VDAC1 in aiding PINK1 for the recruitment of cytosolic Parkin to the surface of damaged mitochondria (Sun et al. 2012).

As so-called 'master regulators' of mitochondrial quality control, PINK1/Parkin have been shown to coordinate novel forms of autophagosome-independent mitochondrial degradation, whereby single-membrane mitochondrial-derived vesicles bud off mitochondria carrying a small cargo of respiratory chain and matrix proteins (McLelland et al. 2014), or mitochondria are captured within early endosomes that eventually fuse with lysosomes (Hammerling et al. 2017). The latter pathways operate independently of DRP1 activity (that remained unchanged in exercise/lipid), an instigator of mitochondrial fission that constricts and severs mitochondria producing two daughter organelles with uneven membrane potential, with the defective unit digested by mitophagy (Twig et al. 2008). As previously discussed, because mitochondria supply the bulk of cellular energy, the removal of mitochondrial proteins potentially damaged by the combination of exercise- and lipid-induced oxidative stress may have transpired via the most energy-efficient means. This hypothesis is based on the fact that elevations in autophagic flux,

arising from the energy cost of manufacturing autophagosomes and degradation of cellular constituents (Plomp et al. 1987), could restrict the maximal anabolic potential of skeletal muscle.
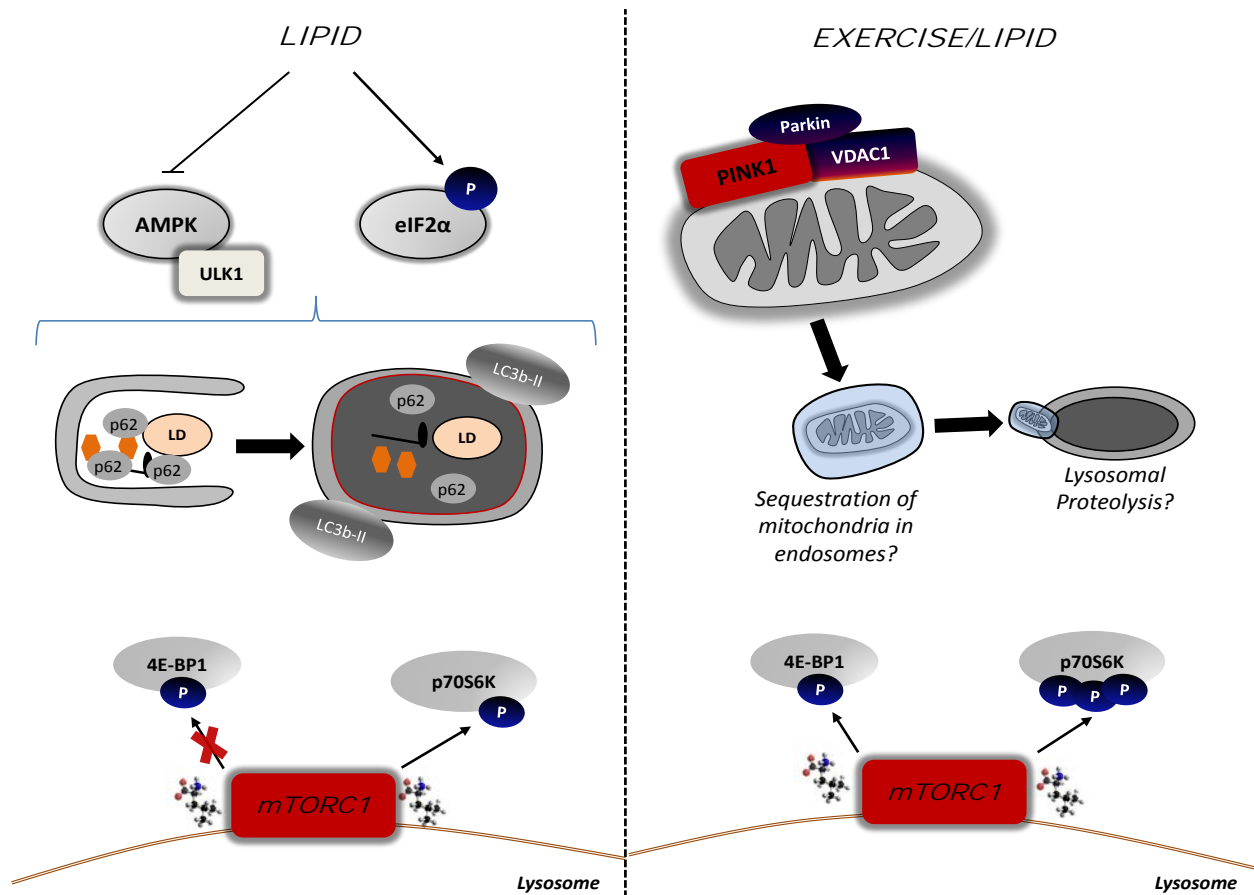
Figure 6.12. Hypothetical model of intralipid (left) and intralipid with the addition of exercise on protein turnover responses to protein ingestion in skeletal muscle

Lipid availability provokes an elevated, presumably compensatory autophagic flux, downstream of ER (eIF2α-mediated), but not energy stress (AMPK), to dispose of damaged cellular aggregates and lipid droplets (LD). However, this response is to the detriment of mTORC1-mediated anabolic responses to amino acid availability, impinging in particular on the phosphorylation of 4E-BP1. In contrast, exercise performed during the lipid emulsion results in the PINK1/Parkin (and possibly VDAC1)-mediated turnover of mitochondrial organelles affected by the combined intracellular stresses of lipid infiltration and exercise-induced bioenergetics. Such a route of mitochondrial turnover is energetically efficient, bypassing the energy demands of autophagosome biogenesis and therefore prioritizing energy utilization for the protein translational machinery (i.e., amino acid-dependent mTORC1 signalling).

In conclusion, results from this study provide novel data to demonstrate that the diminished anabolic signalling response to protein ingestion in the face of high-circulating FFAs can be 'rescued' when exercise is undertaken at the onset of an intralipid infusion. Moreover, independent of evidence for autophagosome biosynthesis, exercise promoted a reduction in mitochondrial content in a manner potentially regulated by classical surveyors of mitochondrial quality control, PINK1 and Parkin. These findings raise the possibility that mitochondrial organelles damaged as a result of lipid-induced oxidative stress, are disposed of by energy-efficient means to sustain the energy-consuming protein translational capacity of skeletal muscle cells subjected to strenuous exercise and protein ingestion in the face of elevated circulating FFAs (Figure 6.12). While high-FFA availability independent of exercise upregulated eIF2α Ser[51] phosphorylation, indicative of a diminished protein translational response to cellular 'stresses' (e.g., lipid-induced), exercise stimulated a compensatory and augmented mTORC1 signalling response that coincided with restraining the induction of autophagy, thereby prolonging the anabolic potential of the cell following protein ingestion. Taken collectively, our results reinforce the potency of combining strenuous exercise with high-quality protein availability to combat the deleterious effects of excess lipids toward skeletal muscle turnover-remodelling.

Chapter Seven

7   Summary and Future Directions

The primary aim of this thesis was to investigate autophagy and protein turnover responses to divergent forms of exercise and nutritional stimuli in human skeletal muscle. The principal findings demonstrate that in the face of diminished energy availability, or alternatively, excess sources of energy (alcohol, fat) that have otherwise 'toxic' effects toward cellular function, molecular remodelling responses of skeletal muscle tissue to vigorous exercise stimuli is optimised (or remains intact) with sufficient availability of high-quality protein. The studies directly arising from this thesis have been investigated on a heterogeneous population; specifically, healthy, young, male and female participants with divergent levels of physical fitness, as well as middle-aged males categorised as susceptible to developing sarcopenic obesity. Accordingly, the results have wider implications for a larger number of the general population within contrasting environments, such as 1) athletes and non-athletes deliberately restricting dietary energy availability in an attempt to alter body composition, 2) recreational and professional athletes who 'binge' drink alcohol following team sports, or 3) pre-sarcopenic individuals consuming a high-fat diet and maintaining a sedentary lifestyle.

Restricting energy availability combined with resistance-based exercise was initially selected as an appropriate model to activate autophagy signalling (Chapter 4), whereas the potentially detrimental availability of nutrients in the form of alcohol and fat were utilised to disrupt intramuscular metabolism (Chapters 5 and 6, respectively) and pinpoint how autophagy reacts to such homeostatic disturbances. The first study (Chapter 4) determined changes in autophagic cell signalling following a 5 day period of dietary energy deficit (ED) and resistance exercise (REX) performed in ED with and without protein ingestion. The results revealed that

proteins implicated in the biogenesis of autophagic vesicles were attenuated following ED, but replenished when protein was ingested during recovery from a single bout of REX. These changes were consistent with the previously reported modulation of myofibrillar protein synthesis under the same experimental conditions (Areta et al. 2014), suggesting that autophagy is intimately involved in the regulation of protein synthesis, especially in the context of changes in cellular energy availability and contraction-induced bioenergetics.

A limitation of this study was the narrow window of biopsy sampling (only up until 4 h post-exercise). Hence, future investigations incorporating longer analyses for at least 24 h [the duration of protein synthetic responses following REX and protein ingestion (Burd et al. 2011)] will help to uncover the contribution from autophagy to acute adaptive remodelling responses to the aforementioned exercise and nutritional stimuli. Because protein turnover is controlled by the cycling of regulatory proteins on and off the lysosome, establishing their abundance and/or interaction partners in response to exercise/nutritional stimuli would be a significant step toward gaining a more comprehensive mechanistic understanding of autophagy in human skeletal muscle. Future work would therefore benefit from novel forms of biochemical analyses such as lysosomal enrichment protocols combined with immunofluorescence analyses. It would also be worth ascertaining whether a larger degree of energy restriction (i.e., $>\sim1000$ kcal·day^{-1}) upregulates autophagy and induces a net negative protein balance.

In contrast to the effects of energy restriction on autophagy, high-alcohol availability has been shown to augment autophagy in a manner largely independent of the proteasome (Thapaliya et al. 2014; Vary et al. 2008). Therefore, under the experimental conditions of strenuous 'concurrent' (combined resistance- and endurance-based) exercise and post-exercise alcohol intoxication with and without protein co-ingestion (Parr et al. 2014), Study 2 analysed

the regulation of autophagy and resultant implications for cellular homeostasis following these stimuli. Autophagy was found to be attenuated by alcohol and carbohydrate co-ingestion, leading to cell death processes that were ameliorated, in part, by alcohol and protein co-ingestion. The capacity for protein availability to protect cellular homeostasis in the face of alcohol toxicity was attributable to an abrupt, yet compensatory, mitochondrial anabolic response, suggesting the anabolic properties of amino acids enriched in the whey protein beverages were harnessed for rapid synthesis of mitochondrial proteins to combat alcohol-provoked mitochondrial toxicity.

The results of this study have ramifications for a large proportion of the population, both athletes and non-athletes alike, who often consume large amounts of alcohol following exercise training and/or team sport activities. While it is advisable that any individual participating in vigorous physical activity should refrain from alcohol intoxication following exercise bouts and/or sports performance, the anabolic effects of protein ingestion may dampen the deleterious effects of alcohol metabolism. Thus, in the face of high-alcohol availability, protein is imperative for facilitating a "bare-minimum" (i.e., elevated, but incomplete amplification of protein turnover) of recovery-adaptation. Future work is required to ascertain the specific dose of alcohol that elicits cellular damage and apoptotic responses. Moreover, it would also be prudent to determine the abundance of autophagy regulatory and mitochondrial proteins the day after consuming alcohol post-exercise and whether any correlations exist between these markers and indices of physical performance (e.g., muscular strength, aerobic capacity etc.). Additionally, examination of mitochondrial respiration and/or tracer-derived measurements of mitochondrial, in combination with myofibrillar, protein synthesis would be highly valuable for ascertaining how alcohol or its metabolites affects the function and/or synthesis of different subcellular compartments and what role autophagy, in particular mitophagy, has in this response.

Study 3 (Chapter 6) used increased fat availability as the nutritional perturbation to cellular homeostasis with and without the addition of exercise. In this study, an older but otherwise healthy cohort was selected due to their susceptibility to developing sarcopenic obesity that can be exacerbated by fat-rich diets (Parr et al. 2013). It was found that lipid availability induced by an intralipid emulsion diminished the anabolic (mTORC1-dependent) cell signalling response to protein ingestion that was circumvented by exercise undertaken during the emulsion. This observation is in agreement with previous work highlighting the potency of strenuous exercise stimuli in augmenting the anabolic response to amino acids (Burd et al. 2011). When considering the mechanism(s) of lipid-induced anabolic resistance to protein feeding, it is worth noting that both amino acids and contractile overload promote mTORC1 localisation to the lysosome where it is directly activated (Jacobs et al. 2014). mTORC1 can also be 'primed' at these sites to subsequently translocate to other membrane-bound subcellular organelles (Manifava et al. 2016). Therefore, a plausible hypothesis is that elevated fatty acid concentrations alter membrane composition of lysosomes and/or other putative sites of mTORC1 regulation. This perturbation to membrane lipid composition has been shown to affect the function of other membrane-bound cellular compartments, including, but not limited to, the plasma membrane and mitochondria (Cazzola et al. 2004; Cunha et al. 2008; Kahle et al. 2015). Therefore, future work is required to ascertain if similar changes occur to alternate cellular membranous structures that consequently impair protein translational responses to physiological stimuli.

In this study it was also identified that intralipid triggered an upregulated autophagic flux, which was repelled by concomitant exercise performance. However, in contrast to the prediction that this autophagic response may specifically target defective mitochondria with lipid, but not exercise/lipid, the opposite prevailed. Specifically, in the absence of evidence for autophagosome

biogenesis, mitochondrial content was attenuated by exercise/lipid in a manner possibly regulated by classical markers of mitochondrial turnover. Since the entire process of classical mitophagy is energetically costly, necessitating energy availability to manufacture autophagosomes (e.g., *de novo* synthesis of the double-membrane), a hypothesis was pointed to whereby mitochondria potentially damaged by lipid are disposed of through an energy-efficient, autophagosome-independent pathway. Such a route of mitochondrial degradation may permit prioritisation of alternate, energy-costly cellular processes promoted by exercise stimuli, not the least of which include protein translation and proteolysis of myofibrillar proteins that dominate early phases of exercise recovery (Phillips et al. 1997; Yang et al. 2006). Mitochondrial fractionation of skeletal muscle and subsequent proteomic analysis would help to pinpoint novel proteins with a role in disposing of mitochondrial organelles in an autophagosome-dependent and -independent manner.

Indeed, such a form of proteomic analysis, but in combination with other fractionation techniques (i.e., separating myofibrillar from mitochondrial proteins) would shed light on the regulation of protein turnover in human skeletal muscle in the context of all three of the exercise-nutrient interactions investigated in this thesis. A recent phosphoproteomic analysis conducted in rodent skeletal muscle following resistance-like stimuli identified several putative, mechanically sensitive regulators of muscle protein synthesis (Potts et al. 2017), while similar work in humans successfully mapped the endurance exercise phosphoproteome, and in doing so, discovered a novel AMPK substrate implicated in mitochondrial function (Hoffman et al. 2015). The global analytical nature of an "omics" approach is fast emerging as a feasible way of mapping the biological complexity of protein turnover responses to exercise in human skeletal muscle (Hoffman 2017), and could represent an exciting avenue for the future mechanistic validation of

autophagy in response to exercise-nutrient interactions. Beyond measuring changes in the phosphoproteome that are transient in nature, conducting a time course study of analysis (i.e., sequential biopsies at select time points across a 24-48 h period of recovery) would illuminate crosstalk between protein synthetic and degradative pathways and prevailing changes in protein abundance. This approach would be unprecedented for unravelling the complex interplay between protein turnover pathways in human skeletal muscle and would provide direct evidence for the intimate role for autophagy in the regulation of protein synthesis. In addition, deuterium oxide is a contemporary method used to label amino acids *in vivo* and determine day-to-day rates of synthesis and breakdown of individual skeletal muscle proteins (Hesketh et al. 2016). Thus, this method could alternatively be used to measure changes in protein abundance in response to 'real-world' interventions of exercise-nutrient interactions and correlate these changes with the activation (or lack thereof) of autophagy.

In conclusion, when considering all of the variables tested, in each individual study comprising this work, the results can be simplistically summarised as follows:

The optimal exercise-nutrient interaction for promoting turnover-remodelling of skeletal muscle, in humans, is high-protein availability.

Chapter Eight

8 List of References

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Chapter Nine

9   Research Portfolio Appendix

Publications and Statement of Contributions

1. **Smiles WJ**, Hawley JA, Camera DM. Effects of skeletal muscle energy availability on
   protein turnover responses to exercise. **J Exp Biol. 2016 Jan;219(Pt 2):214-25;**

Contribution statement: WJS was primarily responsible for the literature search and summary,
and the first draft of the review. DMC was involved in summarising, drafting and writing the
manuscript. JAH was involved in the initial concept, manuscript preparation and final proofing.

Approximate percentage contributions: W.J. Smiles 60%; J.A. Hawley 10%; D.M. Camera 30%.

I acknowledge that my contribution to the above paper is 60%.

W.J. Smiles: Date: 21/06/2017

As principal supervisor of this paper, I certify that the above contributions are true and correct:

J.A. Hawley: Date: 21/06/2017

2. Camera DM, **Smiles WJ**, Hawley JA. Exercise-induced skeletal muscle signalling pathways and human athletic performance. **Free Radic Biol Med. 2016 Sep;98:131-43;**

Contribution statement: WJS and DMC were primarily responsible for the literature search and summary, and the first draft of the review. WJS and DMC were involved in summarising, drafting and writing the manuscript. WJS, JAH and DMC were all involved in the initial concept, manuscript preparation and final proofing.

Approximate percentage contributions: D.M. Camera 45%; W.J. Smiles 35%; J.A. Hawley 20%.

I acknowledge that my contribution to the above paper is 35%.

W.J. Smiles: Date: 21/06/2017

As principal supervisor of this paper, I certify that the above contributions are true and correct:

J.A. Hawley: Date: 21/06/2017

3. Camera DM and **Smiles WJ**. Autophagy, exercise and lifestyle modification. In: Autophagy and Cardiometabolic Diseases: From Mechanisms to Molecules to Medicine (Invited Review). **Elsevier; Accepted for publication.**

Contribution statement: WJS and DMC were responsible for the literature search and summary, and the first draft of the review. WJS and DMC were involved in summarising, drafting and writing the manuscript. WJS and DMC were involved in the initial concept, manuscript preparation and final proofing.

Approximate percentage contributions: D.M. Camera 55%; W.J. Smiles 45%.

I acknowledge that my contribution to the above book chapter is 45%.

W.J. Smiles: Date: 21/06/2017

As principal supervisor of this paper, I certify that the above contributions are true and correct:

D.M. Camera: Date: 21/06/2017

4. **Smiles WJ**, Areta JL, Coffey VG, Phillips SM, Moore DR, Stellingwerff T, Burke LM, Hawley JA, Camera DM. Modulation of autophagy signalling with resistance exercise and protein ingestion following short-term energy deficit. **Am J Physiol Regul Integr Comp Physiol. 2015 Sep;309(5):R603-12;**

Contribution statement: WJS was involved in study design and completed analysis of tissue samples, data collection and statistical analyses, and manuscript writing. JLA and VGC were involved in study design and primarily responsible for ethical approval and experimental trials. VGC was involved in manuscript writing. DMC assisted with trial completion, data analysis and manuscript preparation. LMB, TS, DRM and SMP were involved in study design and grant submission. JAH was involved in study design, grant submission and manuscript preparation.

Approximate percentage contributions: W.J. Smiles 53%; J.L. Areta 10%; V.G. Coffey 10%; S.M. Phillips 2%; D.R. Moore 2%; T. Stellingwerff 2%; L.M. Burke 1%; J.A. Hawley 5%; D.M. Camera 15%.

I acknowledge that my contribution to the above paper is 53%.

W.J. Smiles: Date: 21/06/2017

As principal supervisor of this paper, I certify that the above contributions are true and correct:

J.A. Hawley: Date: 21/06/2017

5. **Smiles WJ**, Parr EB, Coffey VG, Lacham-Kaplan O, Hawley JA, Camera DM. Protein co-ingestion with alcohol following strenuous exercise attenuates alcohol-induced intramyocellular apoptosis and inhibition of autophagy. **Am J Physiol Endocrinol Metab. 2016 Nov 1;311(5):E836-E849;**

Contribution statement: WJS was involved in study design, completed analysis of tissue samples, data collection and statistical analyses, and manuscript writing. EBP was involved in study design and primarily responsible for ethical approval and experimental trials, as well as manuscript edits. VGC was involved in study design and experimental trials and manuscript editing. OLK was involved in manuscript preparation. JAH was involved in grant submission and manuscript writing. DMC assisted with trial completion, data analysis and manuscript preparation.

Approximate percentage contributions: W.J. Smiles 60%; E.B. Parr 15%; V.G. Coffey 7%; O. Lacham-Kaplan 2%; J.A. Hawley 2%; D.M. Camera 14%.

I acknowledge that my contribution to the above paper is 60%.

W.J. Smiles: Date: 21/06/2017

As principal supervisor of this project, I certify that the above contributions are true and correct:

J.A. Hawley: Date: 21/06/2017

6. **Smiles WJ**, van Loon, LJ, Hawley JA, Camera DM. Exercise sensitises skeletal muscle to the intramyocellular anabolic effects of protein ingestion following an intralipid infusion.

In preparation.

Contribution statement: WJS was involved in the study design, was primarily responsible for receiving ethical approval and experimental trials, as well as analysis of tissue samples, data collection, statistical analysis, and manuscript preparation. LJVL was involved in study design. JAH was involved in grant submission and study design. DMC was involved in the study design, assisted in ethical clearance, experimental trials and analysis of tissue and blood samples, and was responsible alongside JAH for the final edits of the manuscript.

Approximate percentage contributions: W.J. Smiles 73%; L.J. van Loon 2%; J.A. Hawley 5%; D.M. Camera 20%.

I acknowledge that my contribution to the above paper is 73%.

W.J. Smiles: Date: 21/06/2017

As principal supervisor of this project, I certify that the above contributions are true and correct:

J.A. Hawley: Date: 21/06/2017

Additional Publication

7. **Smiles WJ**, Conceição MS, Telles GD, Chacon-Mikahil MP, Cavaglieri CR, Vechin FC, Libardi CA, Hawley JA, Camera DM. Acute effects of low-intensity cycling with blood flow restriction on metabolic signalling in human skeletal muscle. **Eur J Appl Physiol. 2017; 2017 Jan 25;**

Contribution statement: WJS was involved in study design and analysis samples, data collection and statistical analyses, and manuscript writing. MSC was responsible for ethical approval and experimental trials, and assisted in manuscript writing. GD Telles was involved in data collection. MPCM, CRC, FCV and CAL were involved in study design. JAH was involved in grant submission and final edits of the paper. DMC was involved in manuscript preparation.

Approximate percentage contributions: W.J. Smiles 40%; M.S. Conceição 35%; G.D. Telles 5%; M.P. Chacon-Mikahil 2%; C.R. Cavaglieri 2%; F.C. Vechin 2%; C.A. Libardi 2%; J.A. Hawley 5%; D.M. Camera 7%.

I acknowledge that my contribution to the above paper is 40%.

W.J. Smiles: Date: 21/06/2017

As principal supervisor of this project, I certify that the above contributions are true and correct:

J.A. Hawley: Date: 21/06/2017

Conference Presentations

1. **Smiles WJ**, Van Loon LJ, Hawley JA, Camera DM. Exercise sensitises skeletal muscle to the intramyocellular anabolic effects of protein ingestion following an intralipid infusion. **Cell Symposia, Exercise Metabolism** – Gothenburg, 2017 (Poster Presentation).

Contribution statement: This poster presentation was based on the work from Chapter 5. The poster was designed and presented by WJS. JAH and DMC reviewed the poster and provided feedback.

2. **Smiles WJ**, Parr EB, Coffey VG, Lacham-Kaplan O, Hawley JA, Camera DM. Protein co-ingestion with alcohol following strenuous exercise attenuates alcohol-induced intramyocellular apoptosis and inhibition of autophagy. **Exercise and Sport Science Australia, Research to Practice** – Melbourne, April 2016 (Oral Presentation).

Contribution statement: This presentation was based on work from Chapter 4. The presentation was designed and delivered by WJS. JAH, OLK and DMC reviewed the presentation and provided feedback.

3. **Smiles WJ**, Areta JL, Coffey VG, Phillips SM, Moore DR, Stellingwerff T, Burke LM, Hawley JA, Camera DM. Modulation of autophagy signalling with resistance exercise and protein ingestion following short-term energy deficit. **Cell Symposia, Exercise Metabolism** – Amsterdam, 2015 (Poster Presentation).

Contribution statement: This poster presentation was based on the work from Chapter 3. The poster was designed and presented by WJS. JAH and DMC reviewed the poster and provided feedback.

Individual Papers

9.1 Published papers which form the basis of Chapter Two

Camera DM and **Smiles WJ**. Autophagy, exercise and lifestyle modification. In: *Autophagy and Cardiometabolic Diseases: From Mechanisms to Molecules to Medicine* (Invited Review). Elsevier; Accepted for publication.

Camera DM, **Smiles WJ**, Hawley JA. Exercise-induced skeletal muscle signalling pathways and human athletic performance. *Free Radic Biol Med*. 2016 Sep; 98:131-43.

Due to copyright restrictions, the published version of this journal article is not available here. Please view the published version online at:

http://www.sciencedirect.com/science/article/pii/S0891584916000502

Smiles WJ, Hawley JA, Camera DM. Effects of skeletal muscle energy availability on protein turnover responses to exercise. *J Exp Biol*. 2016 Jan; 219 (Pt 2): 214-25.

Due to copyright restrictions, the published version of this journal article is not available here. Please view the published version online at:

http://jeb.biologists.org/cgi/pmidlookup?view=long&pmid=26792333

9.2 Published paper which forms the basis of Chapter Four

Smiles WJ, Areta JL, Coffey VG, Phillips SM, Moore DR, Stellingwerff T, Burke LM, Hawley JA, Camera DM. Modulation of autophagy signalling with resistance exercise and protein ingestion following short-term energy deficit. *Am J Physiol Regul Integr Comp Physiol*. 2015 Sep; 309 (5): R603-12.

Due to copyright restrictions, the published version of this journal article is not available here. Please view the published version online at:

http://ajpregu.physiology.org/cgi/pmidlookup?view=long&pmid=26136534

9.3 Published paper which forms the basis of Chapter Five

Smiles WJ, Parr EB, Coffey VG, Lacham-Kaplan O, Hawley JA, Camera DM. Protein co-ingestion with alcohol following strenuous exercise attenuates alcohol-induced intramyocellular apoptosis and inhibition of autophagy. *Am J Physiol Endocrinol Metab*. 2016 Nov 1; 311 (5): E836-E849.

Due to copyright restrictions, the published version of this journal article is not available here. Please view the published version online at:

http://ajpendo.physiology.org/cgi/pmidlookup?view=long&pmid=27677502

Study 3: Letter to Participants and Consent Forms

ACU Human Ethics Committee Approval Number: 2016-53H

Project Title: Increased Fat Availability and Effects on Muscle Growth Responses after Exercise

Risk Level: Low Risk

Date Approved: 21/04/2016

Ethics Clearance End Date: 1/12/2016

Modification to Protocol:

Date Approved: 30/05/2016

The Chair of the Human Research Ethics Committee has approved the following modification(s):

- Administration of heparin which is to be co-administered with the intralipid.

PARTICIPANT INFORMATION LETTER

PROJECT TITLE: Increased Fat Availability and Effects on Muscle Growth Responses after Exercise

PRINCIPAL INVESTIGATOR: Prof. John Hawley and Dr. Donny Camera

STUDENT RESEARCHER: Mr. William Smiles

STUDENT'S DEGREE: PhD

Dear Participant,

You are invited to participate in a research project conducted by the Australian Catholic University (ACU), Melbourne, titled 'Effects of Fat Availability on Muscle Growth Responses after Exercise', as you are male, aged 35-55 years with a Body Mass Index (BMI) of 27-32 who is non-diabetic.

This Participant Information Sheet/Consent Form tells you about the research project. It explains the tests and research involved. This information will help you decide whether you wish to take part in the research. Please read this document carefully and do not hesitate to ask questions about anything that you do not understand or would like to know more about. Before deciding whether or not to take part, you may want to talk about the research it with a relative, friend or local doctor. Participation in this research is voluntary. Hence, if you don't wish to take part, you don't have to. Furthermore, you will receive the best possible care whether or not you take part. If you decide you want to take part in the research project, **you will be asked to sign the consent section.** By signing it you are informing us that you:

• Understand what you have read

• Consent to take part in the research project

• Consent to the tests and research that are described

• Consent to the use of your personal and health information as described.

You will be given a copy of this Participant Information and Consent Form to keep.

What is the project about?

The ingestion of excess energy, particularly fat, is largely responsible for the current epidemic of lifestyle-related diseases such as obesity, diabetes and sarcopenia (the loss of muscle mass with increasing age). Sarcopenia is worsened by low levels of physical activity and particularly in overweight or obese individuals through an increase in body fat and decrease in muscle mass. However, performing resistance and aerobic exercise in combination with the ingestion of high-quality protein sources (e.g.: steak, chicken, whey protein shake, etc.) has been extensively shown to enhance and/or maintain muscle mass.

This has important health implications for reducing the risk of developing sarcopenia, obesity or other debilitating health conditions such as diabetes.

Accordingly, the aim of this research is to investigate whether high fat levels in the blood decreases the muscle growth response to exercise and consuming protein after exercise.

Who is undertaking the project?

This project is being conducted by Mr. William Smiles and will form the basis for the degree of Doctorate at ACU. Mr. Smiles is supervised by Professor John Hawley and Dr. Donny Camera. Professor Hawley is the Head of this group and directs and supervises all of the applied exercise research. Mr. Smiles and Dr. Camera are responsible for coordinating research trials involved in this project. The project has been approved by the ACU Human Research Ethics Committee and is supported and funded by a Collaborative Research Network (CRN) project grant.

Are there any risks associated with participating in this project?

- Muscle Biopsies

This study involves sampling of skeletal muscle tissue. As a result, you may experience a dull pain for up to 24 hours after the biopsy procedure, but will be able to return to normal activity, including full exercise training after 24 hours. It is common for subjects to experience some mild muscle soreness over the next 48 hours; however, this should not restrict movement. In some rare cases, mild bruising has occurred but these symptoms disappear within a week. Although the possibility of infection, scarring and significant bruising is quite small, if by any chance it does occur, please inform us immediately. Finally, in order to participate in the study, a biopsy screening form must be completed by the participant. **For further information, refer to the biopsy information sheet attached to this letter.**

- Catheter Administration

Catheterisation (for infusions and blood sampling) of participants is slightly discomforting and can lead to the possibility of bruising and infection. The use of sterile, disposable catheters, syringes, swabs, etc. will reduce the possibility of infection. Also, qualified and experienced staff reduces the likelihood of bruising, as this is typically caused by poor vein-puncture techniques. Although the possibility of infection, vein blockage and significant bruising is small, if by chance it does occur, consult your doctor immediately and please inform the researcher(s).

- Intralipid-Heparin Infusion

The intralipid infusion is used to increase fat in the blood. Risks with an intralipid infusion with heparin sodium are low and in some cases mild nausea, headaches, dizziness, fatigue and a rise in body temperature or shivering/chills have been reported. These cases are however rare, and reports of even more adverse reactions such as anaphylaxis occur in **less than 1% of cases. As intralipid contains soybean oil and egg phospholipids, proper screening of any <u>known allergies</u> will be performed.**

Finally, while all strenuous physical exertion involves some possible risk of injury or complication, the exercise sessions in this study will not present any risk other than those experienced during a typical exercise session and will be supervised by experienced members of the research team.

What will I be asked to do?

If you are interested in participating in the study and meet the selection criteria, you will be required to complete and sign a consent form, a cardiovascular risk factor questionnaire and a muscle biopsy information form (all attached) for clearance for preliminary testing and the subsequent experimental trial day. It is desirable that your local doctor be advised of your decision to participate in this research project.

The factors that may exclude you from taking part in this study include: not meeting selection study inclusion criteria [male, aged 35-55 years, BMI of 27-32 (we can help you calculate this), sedentary lifestyle, non-insulin resistant] or an inability to consent to the study.

What does participation in this research involve?

Consent Form:

If you decide to participate in this study, a consent form will be signed prior to any study assessments being performed.

Muscle Biopsy Form:

A muscle biopsy form will be signed prior to the experimental trial to ensure this research technique is clearly explained and that you are comfortable and aware of all associated risks.

Cardiovascular Risk Factor Questionnaire:

A cardiovascular risk factor questionnaire will be completed prior to any study assessments.

Leg Strength Test

Maximum muscular strength will be determined during a series of one repetition maximum (1RM) leg extensions. Following a warm-up, a single exercise repetition will be performed with appropriate recovery between each attempt until the load that can be lifted once but not a second time is determined.

Cardiovascular Fitness ($\dot{V}O_{2peak}$) Test

The $\dot{V}O_{2peak}$ test is a safe and commonly performed test on a stationary bicycle. The test lasts between 8 to 15 minutes and involves cycling against a resistance (which increases gradually) until the required pedalling rate cannot be maintained.

DXA Body-Composition Scans

Whole-body DXA scans of total body mass, fat mass and lean (i.e., muscle) mass will be measured. Subjects will be scanned wearing light clothing at the same time of day (early morning) and will be instructed to follow a standardised (provided) diet and exercise control before each scan.

Diet/Exercise Control

Prior to the experimental trial day (described subsequently), you will be required to refrain from any form of exercise training and/or other vigorous physical activity for a minimum of 48 hours. **As caffeine and alcohol can affect test results, you will also be required to abstain from caffeine and alcohol for 24 hours prior to the experimental trial days.** Additionally, you will be provided with a standardised carbohydrate-based meal the evening prior to undertaking the experimental trial day. This meal will be based on chosen food preferences and takes into account any personal allergies and will be consumed no later than 10pm the night before the trial day. You may take any regular medication provided it does not contravene any medications listed in the consent form.

Experimental Trial Day

Eligible participants will arrive via taxi to the Australian Catholic University between 7am and 8am following an overnight fast. After resting for approximately 15 minutes, a catheter will be inserted into a forearm vein of the arm. A constant infusion of either a lipid emulsion, a commonly used research technique to increase fat in the blood, or Saline (control) will be administered (Figure 1). This infusion will continue for a period of five hours. Two hours into this infusion, another catheter will be inserted into the forearm vein of the opposite arm. A constant infusion of a phenylalanine amino acid tracer to measure muscle growth responses will then be administered. At the end of the five hour lipid or saline infusion, a resting skeletal muscle biopsy (described subsequently) will be obtained.

Intravenous infusion of L-[ring-$^{13}C_6$] phenylalanine

- 5 hours - 3 hours - 0 hours + 0 hours + 1 hour + 2 hours + 3 hours + 4 hours

Standardised Meal | Overnight Fast

Exercise OR Rest

Intravenous infusion of Saline or Intralipid Emulsion

= Blood Sample (5 mL)

= Muscle Biopsy

= Whey Protein Drink

Exercise
Cycling: 30 min @ 65% VO$_{2max}$
+ Resistance Exercise: 8 x 5 Leg Extension @ 80% 1-RM
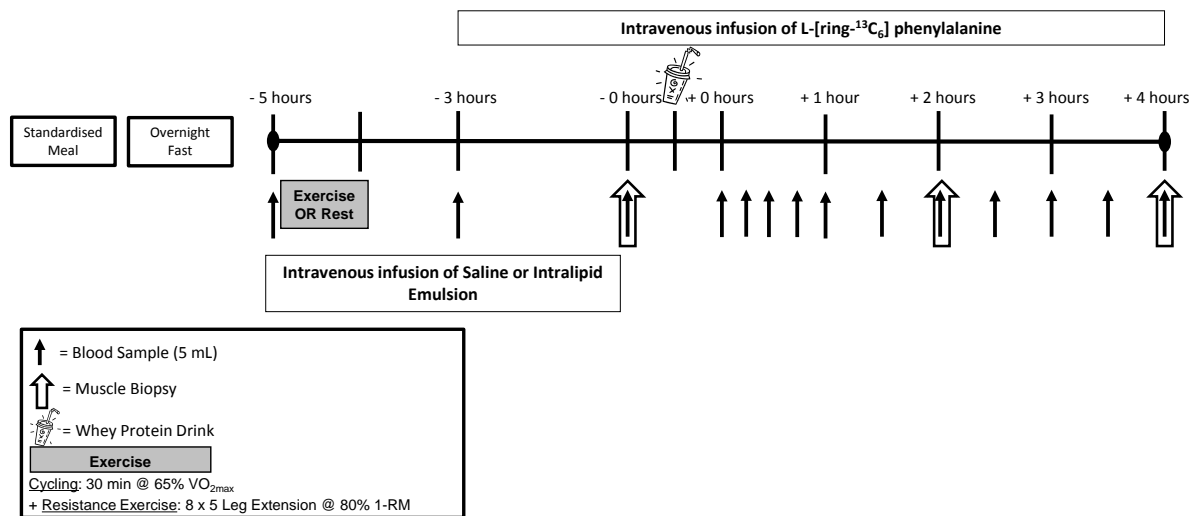
A total of 36 participants will be recruited for this study. There will be 3 experimental groups to which participants will be randomly and equally assigned:

Figure 1. Overview of experimental trials

Group 1: Control Trial

The control group will receive a constant infusion of saline for 5 hours before consuming a 500 mL (total) of a protein beverage. These proteins ingested are found in whole foods in a normal diet and have been shown previously to increase muscle growth.

Group 2: Lipid (Fat) Emulsion Trial

The lipid emulsion group will receive a constant infusion of lipid (fat) with heparin sodium for 5 hours prior to consuming a 500 mL (total) of a protein beverage. The lipid emulsion infusion (fat) is a commonly used research method to increase the amount of fat in the blood and resembles the consumption of high-fat foods. The protein beverage to be consumed is as exactly the same as in the control trial.

Group 3: Exercise Trial

Participants in the exercise group will be administered a constant infusion of lipid (fat) with heparin sodium for 5 hours. During the first hour of this infusion, participants will undertake an exercise session consisting of 30 minutes of moderate cycling. Participants will then complete a resistance exercise session consisting of an appropriate warm-up and then 8 sets of 5 repetitions of heavy, lower body (leg

205

extensions) resistance exercise. At the end of the 5 hour infusion, participants will consume the 500 mL (total) protein beverage described above.

After consuming protein beverages, participants in each of the three groups will rest throughout a 4 h recovery period during which additional skeletal muscle biopsies will be obtained at 2 hours and 4 hours. Blood samples (described subsequently) from the inserted catheter will also be obtained at selected time points during the experimental trial day. At the end of the experimental trial day and following clearance from medical staff, all participants will receive a meal and taxi voucher for travel home.

Muscle Tissue Biopsy

A total number of three biopsies will be obtained during the experimental trial day. The muscle biopsy will be obtained from the outer thigh by Dr. Andrew Garnham. The muscle biopsy is a rapid procedure (~5-10 seconds) performed to obtain small samples of muscle tissue about the size of a corn kernel. In preparation for a biopsy, a small amount of local anaesthetic is injected under the skin which may result in a mild burning sensation while the fluid is injected. A small, 4-5 mm incision will then be made into your skin to create an opening for the biopsy needle. There is often a small amount of bleeding from the incision, but this bleeding is generally minimal. The biopsy needle will then be inserted through the incision site and you may feel the sensation of deep pressure in the biopsy site, and on some occasions this can be moderately painful. However, **the discomfort very quickly passes and you are capable of performing exercise and daily activities within minutes.** There may also be some minimal bleeding when the needle is removed which may require the application of pressure for a few minutes.

At the completion of the trial day after the final biopsy, the incision will be closed with sterile tape and wrapped with a bandage. You are advised to refrain from excessive muscle use for the remainder of the day. Once the anaesthetic wears off, your leg may feel tight and often there is the sensation of a deep bruise. Pain killers such as paracetamol (e.g. Panadol) or Ibuprofen (e.g. Advil) are acceptable to use if you experience pain associated with the biopsy. Periodically applying an ice pack to the biopsy site the following day will reduce any local swelling and/or remaining soreness. The following day your leg may feel uncomfortable when walking down stairs and to a lesser extent, activities that involve forceful movements of the abdomen. Tightness usually disappears within 2 days and participants generally recommence exercising within 48 hours. To allow the incisions to heal properly and minimise any risk of infection, avoid prolonged submersion in water for 4 days. Daily showers are acceptable, but baths, swimming, saunas etc. should be avoided for at least 4 days following the biopsy.

Infusions

Phenylalanine (Amino Acid) Tracer Infusion

The administration of a small amount of a phenylalanine isotope tracer is a common technique used in nutrition research to determine muscle growth. This technique has been previously performed multiple times by the researchers and there are no known risks involved. These stable isotopes are tested for sterility and for the potential to cause fever before the study commences and lastly, the isotope is naturally occurring in the body and is non-radioactive.

Lipid (fat) Emulsion or Saline (Control) Infusion

The infusion of a lipid (fat) emulsion with heparin sodium or saline (control) is another commonly used technique in nutrition research to increase fat levels in the bloodstream. You will receive an infusion of ~400-500 mL (approximately 1.5 mL per minute) of intralipid with heparin sodium (totalling ~10000 U) which temporarily causes "insulin resistance", but the effects of this infusion should be completely absent by 24-48 h after the intralipid is terminated. The risks associated with this procedure have been stipulated on page 2 of this document.

Tracer and Infusion Procedures

All instruments involved in preparation for the phenylalanine and lipid emulsion infusions will be maintained under strict sterile conditions. For both infusion procedures you will be lying down and Dr. Andrew Garnham will insert a plastic sterile tubing (catheter) into each forearm vein. Catheterisation is slightly discomforting and may lead to bruising and a minimal chance of infection. The use of sterile, disposable catheters, syringes, swabs, etc. will reduce the possibility of infection caused by the catheterisation procedure. This procedure allows for the separate infusion of the phenylalanine and lipid emulsion into each arm as well as taking blood samples (explained below). The phenylalanine and lipid infusions will be performed continuously but through separate catheters (i.e., separate arms) by the use of an automatic pump. During this time, you will rest in a reclined position.

Blood Sampling

Multiple blood samples will be obtained during the experimental trial, however the total volume of blood to be taken (approximately 90 mL) does not exceed one quarter of a standard Red Cross donation. Each blood sample is approximately 5 mL, which is a volume close to a level teaspoon and will be taken via the catheter placed into a forearm vein.

Exercise

All physical exertion involves some possible risk of injury however all precautions, included a proper 'warm up' and 'cool down,' will be implemented to reduce any chance of injury. Nonetheless, you may experience some mild-onset of delayed muscle soreness.

How much time will the project take?

Time commitments:

•   You will be required to make a total of 2 visits

•   **1 preliminary visit** at ACU for the preliminary DXA scan and exercise testing lasting approximately 90-120 minutes.

•   **1 experimental trial day** at ACU lasting approximately 8-10 hours depending on the group you are in.

•   Experimental trials will take place between two and three weeks following all preliminary testing. Therefore, the total duration of the study is ~2-3 weeks.

Reimbursement and Costs

There are no costs associated with participating in this research project, nor will you be paid. However, you will be provided with a gift voucher to Coles Myer to the value of $600 for your participation and time commitment in the study. You will also be provided with a cab-charge card for travel to and from the Australian Catholic University on the experimental trial day.

Local Doctor Notification

It is desirable that your local doctor is advised of your decision to participate in this research project. If you have a local doctor, we strongly recommend that you inform them of your participation in this research project. If you decide to participate in this research project, the study doctor will inform your local doctor.

What are the benefits of the research project?

We cannot guarantee or promise that you will receive any benefits from this research; however possible benefits may include an improved understanding of personal muscular strength, cardiovascular fitness and important body composition measures.

There will be no clear benefit to you from your participation in this research.

Can I withdraw from the study?

If you decide to withdraw from this research project, please notify a member of the research team before you withdraw. A member of the research team will inform you if there are any special requirements linked to withdrawing.

If you do withdraw your consent during the research project, the study doctor and relevant study staff will not collect additional personal information from you, although personal information already collected will be retained to ensure that the results of the research project can be measured properly and to comply with law. Please be aware that data collected up to the time you withdraw will form part of the research project results. If you do not want them to do this, please advise them upon your withdrawal from the research project.

Will anyone else know the results of the project?

Information for each volunteer will be identified by a code so that your name does not appear on any data sheets. Individual information will be kept for 5 years in a locked cabinet at ACU and only the principal investigators Mr. William Smiles, Dr. Donny Camera, and Prof. John Hawley will have access to this information. At the conclusion of this five-year period, all material containing confidential information will be destroyed. If you wish to gain access to your data, contact one of the principal researchers and it will be provided to you. Please note that no material that could personally identify you will be used in any reports or presentations of this project.

Any information that you provide can be disclosed only if (1) it is to protect you or others from harm, (2) a court order is produced, or (3) you provide the researchers with written permission. The results from this study will be presented at scientific conferences and published in peer-reviewed scientific journals.

Will I be able to find out the results of the project?

You will be provided with a report detailing results of preliminary testing. If you wish to gain access to any additional data from the study please contact the principal research and it will be provided to you.

Who do I contact if I have questions about the project?

Please contact Mr. William Smiles (will.smiles@acu.edu.au **ph: 0415 377 469**), Dr. Donny Camera (donny.camera@acu.edu.au) or Professor John Hawley (email: john.hawley@acu.edu.au) if you have any question relating to participation in this study.

What if I have a complaint or any concerns?

The study has been reviewed by the Human Research Ethics Committee at Australian Catholic University (approval number 2016-53H). If you have any complaints or concerns about the conduct of the project, you may write to the Manager of the Human Research Ethics Committee care of the Office of the Deputy Vice Chancellor (Research).

Manager, Ethics

c/o Office of the Deputy Vice Chancellor (Research)

Australian Catholic University

North Sydney Campus

PO Box 968

NORTH SYDNEY, NSW 2059

Ph.: 02 9739 2519

Fax: 02 9739 2870

Email: res.ethics@acu.edu.au

Any complaint or concern will be treated in confidence and fully investigated. You will be informed of the outcome.

I want to participate! How do I sign up?

Please contact William Smiles via phone or email if you are interested or have further questions relating to this research project. Consent forms and questionnaires can be returned either in person or by email.

Please note, both copies of the consent form must be signed by the participant and returned to the investigators.

Yours sincerely,

PRINCIPAL INVESTIGATORS

Prof. John Hawley PhD

_____

Dr. Donny Camera PhD

_____

STUDENT RESEARCHER

Mr. William Smiles

_____

CONSENT FORM

TITLE OF PROJECT: Increased Fat Availability and Effects on Muscle Growth Responses after Exercise

PRINCIPAL INVESTIGATORS: Dr. Donny Camera, Prof. John Hawley

STUDENT RESEARCHER: Mr. William Smiles

I .... have read and understood the information provided in the Letter to Participants. Any questions I have asked have been answered to my satisfaction. By signing this consent form I agree to participate in the study outlined in the Letter to the Participants:

1. I have received a statement explaining the test/procedures involved in this project.

2. I consent to participating in the above project and have had the details of the tests and procedures explained to me.

3. I agree to strictly follow the diet and exercise schedule provided by the investigators of this project and to notify the investigators should an unforeseeable event prevent my adherence to the outlined procedures.

4. I acknowledge that:
   a) The potential benefits of the tests or procedures have been explained to my satisfaction.
   b) I am able to withdraw from this study at any time and to withdraw any unprocessed data previously supplied (unless follow-up is required for safety reasons).
   c) The project is for the purpose of research and/or teaching. It may not be of direct benefit to me.
   d) The security of the research data is assured during and after the completion of the study. The data collected during the study may be published, and a report of the project outcomes will be provided. Any information that may appear in publications or provided to other researchers will not result in my identification.

By signing this consent form I agree to participate in the following tests and procedures:

- Preliminary testing comprised of lower body maximum strength and cardiovascular fitness and DXA body composition scanning.

- A standardised pre-exercise carbohydrate-based meal to be consumed the evening prior to reporting to the laboratory for an experimental trial day

- Administration of two separate catheters for intravenous infusions of:
  - Amino acid (non-radioactive) tracers (lasting 7 hours)
  - 5 hours of 20% intralipid plus heparin sodium or, a saline (0.9%) control

- 3 skeletal muscle biopsies during the trial day taken from the lateral thigh under local anaesthetic
- 14-16 blood samples (~5 mL per sample) taken throughout an experimental trial day

- Completion of an acute exercise bout consisting of resistance (lower body weights) and endurance exercise (cycling)

- A time commitment of approximately 10 hours (depending on the group you are allocated to) on the day of an experimental trial
- Consumption of a single 500 mL protein beverage after the intralipid or saline infusion

NAME OF PARTICIPANT:

SIGNATURE .... DATE ....

SIGNATURE OF PRINCIPAL INVESTIGATOR
DATE:………………………..

SIGNATURE OF STUDENT RESEARCHER:
DATE:.........................………

BIOPSY SCREENING FORM – Intralipid Anabolic Resistance

To help us ensure your safety and wellbeing please answer the following questions.

1. Have you ever had a negative or allergic reaction to local anaesthesia (e.g. during dental procedures)? (Please circle the appropriate response)

No / Yes

2. Do you have any tendency toward easy bleeding or bruising (e.g with minor cuts or shaving)?

No / Yes

3. Are you currently taking any medications that may increase the chance of bleeding or bruising (e.g. Aspirin, Coumadin, Anti-inflammatories, Plavix)?

No / Yes

If yes, please list the medication used (including herbal medication) _____

_____

4. Have you ever fainted or do you have a tendency to faint when undergoing or watching medical procedures?

No / Yes

5. Will you contact the physician who did the biopsy directly if you have any concerns about the biopsy site including: excessive redness, swelling, infection, pain or stiffness of the leg?

No / Yes

6. Are you willing to visit the physician who did the biopsy 7-10 days following the biopsy for an assessment of the biopsy site?

No / Yes

Subject Name (print): _____

Subject Signature: _____ Date: _____

Signature of Person Conducting Assessment: _____ Date: _____

CARDIOVASCULAR RISK FACTOR AND SCREENING QUESTIONNAIRE

To be eligible to participate in the experiment you are required to complete the following questionnaire which is designed to assess the risk of you experiencing a harmful cardiovascular event during the course of the trial. A full and honest disclosure of your medical history is vital.

Name:

Date of Birth:

Age: _____ years   Weight: _____ kg   Height: _____cm

Give a brief description of your average weekly activity pattern:

_____

_____

Circle the appropriate responses for the following questions:

1.	Are you overweight?	Yes	No	Don't Know
2.	Aged between 35-55 yrs	Yes	No	
3.	BMI between 25-32	Yes	No	Don't Know

Calculate below if unsure [BM (kg) / Height (m^2)]:

_____

_____

4.	Do you smoke?	Yes	No	Don't Know
5.	Does your family have a history of premature (<70 years) cardiovascular problems (e.g., heart attack, stroke)?	Yes	No	Don't Know
6.	Are you asthmatic?	Yes	No	Don't Know
7.	Are you diabetic?	Yes	No	Don't Know
8.	Do you have high blood cholesterol levels?	Yes	No	Don't Know
9.	Do you have high blood pressure?	Yes	No	Don't Know

10.	Do you have low blood pressure?	Yes	No	Don't Know
11.	Do you have a heart murmur?	Yes	No	Don't Know

12. Do you have, or have you ever had, any   Yes   No   Don't Know

blood-clots in any of your blood vessels

(e.g. deep-vein thrombosis)?

13. Do you have, or have you ever had, any disease or   Yes   No   Don't Know

condition that resulted in reduced or slower than

normal blood-clotting?

14. Do you have, or have you ever had, any tendency to   Yes   No   Don't Know

bleed for long periods after cutting yourself?

15.	Do you have varicose veins?	Yes	No	Don't know
16.	Are you currently using any medication?	Yes	No	

        If so, what is the medication? _____

17. Have you ever experienced any of the following during exertion (exercise or physical

labour) or at rest? (Please circle).

        a. Light headedness or dizziness

        b. Pain in the chest, neck, jaw or arm

        c. Numbness or pins-and-needles in any part of your body

        d. Loss of consciousness

18. Have you suffered any known adverse effects to caffeine ingestion?   Y / N

19. Do you have any known allergies?   Y / N

20. If you answered yes above please specify if you are allergic to the

        following (Please circle).

        a. Eggs or any egg-containing product

        b. Soy or any soy-containing product

        c. Olive oil

        d. Peanuts

        e. Heparin (this is a commonly used anti-coagulant drug)

        f. If none of the above please specify _____

21. Do you think you have any medical complaint, allergy or any other reason which you

        know of which you think may prevent you from participating in this trial?   Y / N

If yes, please elaborate. _____

I, _____, believe that the answers to these questions are true and correct.

Signed: Date: _____

BIOPSY INFORMATION SHEET

Exercise Metabolism Laboratory

School of Exercise Science, ACU

Melbourne Campus

You have volunteered to take part in a research study that requires you to undergo a <u>muscle tissue biopsy</u>. This is a commonly performed procedure in research studies and for the medical diagnosis of muscle disease. The procedure will be performed by a medical doctor trained to perform muscle and fat biopsies or a specially trained researcher directly supervised by a medical doctor.

The biopsy involves the removal of a small piece of muscle tissue from one of the muscles in your leg and fat tissue from the abdominal region using a sterile hollow needle. The area over the outside of your lower thigh muscle (vastus lateralis muscle) will be carefully cleaned. A small amount of local anaesthetic will be injected into and under the skin. You will likely experience a mild burning sensation while the fluid is injected. Then a small, 4-5 mm incision will be made in your skin in order to create an opening for the biopsy needle. There is often a small amount of bleeding from the incision, but this is usually minimal.

The biopsy needle will then be inserted through the incision site and a small piece of muscle (100-200 mg; about the size of a kernel of corn) and fat (40-50 mg), will be quickly removed and the needle taken out. While the muscle sample is being taken (about 5 seconds) you may feel the sensation of deep pressure in your thigh and, on some occasions, this is moderately painful. However, the discomfort very quickly passes and you are quite capable of performing exercise and daily activities. There may be some minimal bleeding when the needle is removed which may require application of pressure for a few minutes.

Following the biopsy, the incision will be closed with sterile tape (steri-strips) and wrapped with a tensor bandage. You should refrain from excessive muscle use for the remainder of the day.

Once the anaesthetic freezing wears off, your leg may feel tight and often there is the sensation of a deep bruise, "corked-thigh" or "Charlie-horse". Pain killers such as paracetamol (e.g. Panadol) or Ibuprofen (e.g. Advil) are acceptable to use if you experience pain associated with the biopsy. It is also beneficial to periodically apply an ice pack to the biopsy site the following day, as this will help to reduce any local swelling and/or residual soreness. The following day your leg may feel uncomfortable when going down stairs and to a lesser extent activities that involve forceful movements of the abdomen. The tightness (particularly in the muscle) usually disappears within 2 days and participants routinely begin exercising at normal capacity within 48 hours. In order to allow the incisions to heal properly and minimise any risk of infection, you should avoid prolonged submersion in water for 4 days. Daily showers are acceptable, but baths, swimming, saunas etc. should be avoided for at least 4 days following the biopsy procedure.