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MECHANISMS AND NUTRITIONAL COUNTERMEASURES TO MUSCULOSKELETAL DISUSE ATROPHY

By

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ABSTRACT

Musculoskeletal disuse negatively impacts muscle mass and physiological functioning. Mechanistically, disuse-induced alterations in muscle mass are underpinned by alterations in myofibrillar protein turnover. However, the implications of disuse on mitochondrial functioning remains to be fully eluded. Accordingly, Chapter 2 explored whether a 7d period of reduced ambulation (<1,500 steps.d^{-1}) promoted alterations in skeletal muscle oxidative metabolism in young males. We report that 7d of reduced ambulation significantly reduced citrate synthase activity, without altering the abundance of proteins involved in mitochondrial functioning, mitochondrial dynamics, oxidative metabolism or skeletal muscle insulin signalling. Based on the importance of skeletal muscle anabolic sensitivity to prevent disuse-induced atrophy, Chapter 3 assessed whether a high-dose leucine supplementation intervention could prevent alterations in fat free mass and strength during a 7d period of lower limb immobilization in young males. Here we report that there was a significant reduction in fat free mass (3.6 ± 0.5%) and strength (27.9 ± 4.4%) following 7d immobilization, with no effect of high-dose leucine supplementation compared with placebo. Mechanistically, disuse-induced declines in fat free mass occurred in parallel to impaired MyoPS and mitochondrial protein synthesis (MitoPS) rates in the immobilised leg *vs.* control leg. Chapter 4 assessed the mechanistic ability of (-)-epicatechin (EPI) and its metabolite hippuric acid (HA) upon muscle morphology and metabolism within an *in vitro* model of atrophy. Under atrophy like conditions (24h dexamethasone (DEX)) C2C12 myotube diameter was significantly greater following co-incubation with either HA or EPI compared to the vehicle control (VC). Mechanistically, the co-incubation with EPI or HA abrogated the DEX-induced reductions in MPS rates and partially attenuated proteolysis, preventing DEX-induced alterations in autophagic signalling. In conclusion, this thesis improved our understanding of the

mechanisms underpinning disuse-induced muscle atrophy and the effectiveness of two nutritional countermeasures to prevent the negative physiological consequences of musculoskeletal disuse.

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Edwards SJ, Smeuninx B, Mckendry J, Nishimura Y, Luo D, Marshall RN, Perkins M, Ramsay J, Joanisse S, Philp A, Breen L. High-dose leucine supplementation does not prevent muscle atrophy or strength loss over 7 days of immobilization in healthy young males. Am J Clin Nutr. 2020 Nov 11;112(5):1368-1381. doi: 10.1093/ajcn/nqaa229. PMID: 32910813.

Luo D*, Edwards S*, Smeuninx B, McKendry J, Nishimura Y, Perkins M, Philp A, Joanisse S, Breen L. Immobilization Leads to Alterations in Intracellular Phosphagen and Creatine Transporter Content in Human Skeletal Muscle. Am J Physiol Cell Physiol. 2020 May 6. doi: 10.1152/ajpcell.00072.2020.

Allen SL, Marshall RN, Edwards SJ, Lord JM, Lavery GG, Breen L. The effect of young and old ex vivo human serum on cellular protein synthesis and growth in an in vitro model of aging. Am J Physiol Cell Physiol. 2021 Jul 1;321(1):C26-C37. doi: 10.1152/ajpcell.00093.2021. Epub 2021 Apr 28. PMID: 33909501.

Morgan PT, Harris DO, Marshall RN, Quinlan JI, Edwards SJ, Allen SL, Breen L. Protein Source and Quality for Skeletal Muscle Anabolism in Young and Older Adults: A Systematic Review and Meta-Analysis. J Nutr. 2021 Jul 1;151(7):1901-1920. doi: 10.1093/jn/nxab055. PMID: 33851213; PMCID: PMC8245874

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Data obtained during postgraduate study resulted in the following international conference communications:

European Sports Science Conference (ECSS), online conference, September 2021 (Yong Investigator Award (YIA) shortlist) Poster communication. Short-term step reduction does not alter skeletal muscle markers of oxidative metabolism or insulin-mediated signalling in young males despite reductions in whole-body insulin sensitivity.

Physiology, online conference, July 2021- Poster communication. (-)-Epicatechin and it's colonic metabolite Hippuric acid protect against dexamethasone-induced atrophy in skeletal muscle cells.

American College of Sports Medicine (ACSM), online conference, May 2020- Poster communication. Leucine supplementation does not attenuate metabolic or functional declines following 7-days of unilateral lower-limb immobilisation

ECSS, Prague, July 2019- Oral communication. Investigating the effectiveness of supplemental leucine on skeletal muscle metabolism and function during 7-days of unilateral lower-limb immobilisation.

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4EBP1;	Eukaryotic initiation factor 4E binding protein 1
ACC;	Acetyl-CoA carboxylase
ADP;	Adenosine 5'-diphosphate
ALS;	Autophagy lysosome system
AMPKα;	5' AMP-activated protein kinase
Akt;	Total protein kinase B
ATP;	Adenosine triphosphate
CAMKII;	Calcium/calmodulin-dependent protein kinase type II
CS;	Citrate synthase
CSA;	Cross sectional area
CTL;	Control limb
DEX;	Dexamethasone
DRP1;	Dynamin-related protein 1
E;	Maximal electron transport capacity
eEF2;	Eukaryotic elongation factor 2
EPI;	(-)-Epicatechin
EPI-CTL;	24h, 25μM epicatechin supplementation
EPI-DEX;	24h, 25μM epicatechin supplementation and 100μM dexamethasone treatment
FAK;	Focal adhesion kinase
FIS1;	Mitochondrial fission 1
GLUT4;	Glucose transporter type 4
GS;	Glycogen synthase
GSK3αβ;	Glycogen synthase kinase-3
HA;	Hippuric Acid

HA-CTL; 24h, 25μM hippuric acid supplementation

HA-DEX; 24h, 25μM hippuric acid supplementation and 100μM dexamethasone treatment

HMB; Beta-hydroxy-beta-methylbutyrate

IGF1; Insulin growth factor 1

IMB; Immobilized limb

IR; Insulin receptor B

IRS; Insulin receptor substrate

LC3 α/β; Microtubule-associated proteins 1A/1B light chain 3B

LEU; 15g·d^{-1} leucine supplementation during immobilization

L_i; Complex I linked leak respiration

MAFbx; Muscle atrophy f-box

MFF; Mitochondrial fission factor

MFN2; Mitofusin 2

MitoPS; Mitochondrial protein synthesis

mnSOD; Manganese superoxide dismutase

MPS; Myotube protein synthesis

mTOR; Mechanistic target of rapamycin

mTORC1; Mechanistic target of rapamycin complex I

mTORC2; Mechanistic target of rapamycin complex II

MURF-1; Muscle ring finger protein 1

MyoPB; Myofibrillar protein breakdown

MyoPS; Myofibrillar protein synthesis

NBAL; Net protein balance

NOS; Nitric oxide synthase

NRF1; Nuclear respiratory factor 1

OPA1; Optic atrophy protein 1

OXPHOS; Oxidative phosphorylation protein

P_i; Complex I linked phosphorylating respiration

P_{i+ii}; Maximal phosphorylating respiration through complex I and II

PERM1; PGC-1 and ERR-induced regulator in muscle protein 1

PGC1α; Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PI3K; Phosphoinositide 3-kinase

PIP2; Phosphorylate phosphatidylinositol 4,5-bisphosphate

PIP3; PI 3,4,5-triphosphate

PLA; $15 g \cdot d^{-1}$ placebo (non-essential amino acid) supplementation during immobilization

PRAS40; 40kDa Akt substrate

Rag; Rheb and ras-related GTP-binding proteins

Raptor; Regulatory associated protein of mTOR

Rheb; Ras homolog enriched in brain

RPS6; Ribosomal protein S6

S6K1; Ribosomal protein S6 kinase beta-1

SR; Step reduction

TFAM; Mitochondrial transcription factor A

TSC2; Tuberous sclerosis 2

ULK1; Unc-51 like autophagy activating kinase

UPS; Ubiquitin proteasome system

VC-CTL; 24h control (no atrophic stimulus or treatment)

VC-DEX; 24h, 100μM dexamethasone treatment

VO_{2max}; Maximal oxygen uptake

VPS34; Vacuolar protein sorting 34

1. GENERAL INTRODUCTION

1.1. The importance of skeletal muscle for health and locomotion

Skeletal muscle accounts for ~40% of total body mass (1) and is essential for human locomotion (2). Beyond this primary role, skeletal muscle also plays a critical role in whole-body lipid, carbohydrate and protein metabolism (2-4), ensuring the constant stabilisation of metabolic homeostasis (2, 5, 6). As a dynamic tissue, skeletal muscle undergoes constant metabolic remodelling to maintain homeostasis, aiding glucose disposal and lipid oxidation in the fed state, whilst being responsible for the excretion of amino acids and glucose into the plasma pool during periods of fasting (2, 4, 7). Therefore, it is perhaps unsurprising that the maintenance of skeletal muscle mass, and thus ability of skeletal muscle to adapt to metabolic stimuli is inversely associated with the development of type II diabetes (7), cardiovascular disease (7, 8) and mortality risk (9). As a consequence, the maintenance of skeletal muscle across the lifespan is critical to extend the health span (10).

With increasing age, skeletal muscle mass slowly diminishes (termed sarcopenia) and without intervention ~30% of an individual's muscle mass is lost before they reach the eighth decade of life (1). Even in 'healthy' aging, changes to skeletal muscle structure can occur, and from the age of 25 years there is a reduction in muscle cross sectional area, predominantly through a loss of type II fibres (11) and a shortening of sarcomere length (12). Since type II fibres produce more force than their type I counterparts, the disproportionate loss of type II fibres leads to loss of strength that is 2-5 times greater than the loss of muscle mass by the eighth decade of life (13). Disturbingly, these reductions in skeletal muscle quality also lead to a decline in mitochondrial volume density and a subsequent ~50% reduction in skeletal muscle oxidative capacity (14). Combined, this results in a significant decline in whole body maximal oxygen uptake, at a rate of ~1% per year from the age of 25 (15) predisposing individuals to frailty and ultimately leading to an increased risk of mortality (16). Consequently, interventions that promote the maintenance of skeletal muscle

mass and quality is vital to support healthy ageing, promote physical independence and reduce the ever-increasing dependence on healthcare services (17).

Age-related declines in skeletal muscle quality are associated with alterations in metabolic homeostasis. Mitochondria also play an important role in fuel utilisation and insulin signalling (18), with an increased skeletal muscle mitochondrial volume associated with improved insulin sensitivity in older individuals (19). Thus reductions in aerobic respiration capacity result in a change to fuel utilisation, leading to an age related increased risk of developing type II diabetes (2). In 2021, 20% of individuals aged 75 – 79 years globally are estimated to have impaired glucose tolerance; fourfold higher prevalence than in individuals aged 20 – 24 years (~5%) (20). However, chronic endurance and/or high-intensity exercise training programs have displayed beneficial effects on insulin sensitivity (21) demonstrating the importance of maintaining adequate skeletal muscle functioning (22) across the lifespan.

Beyond the importance of skeletal muscle mass to prolong the health span, the maintenance of adequate skeletal muscle mass is crucial to the prognosis of many diseases (e.g., cancer). The reduced skeletal muscle mass that often accompanies cancer not only limits functional independency but is also associated with an impaired responsiveness to chemo-/radiotherapy and provides an increased risk of complications associated with surgery (23, 24). Therefore, it is perhaps unsurprising that skeletal muscle mass is correlated with survival outcomes in these patients (25). Similar findings are also noted across multiple chronic health conditions (i.e. chronic liver disease, IBD, arthritis), where skeletal muscle wasting is associated with mortality (26). Collectively, the maintance of skeletal muscle mass across the lifespan is vital not only to improve an individuals preparedness to deal with injury/illness but to utlimately prolong optimal physiological functioning and functional independence.

1.2. Skeletal muscle protein turnover

Skeletal muscle proteins are in a constant state of turnover. The intricate balance of myofibrillar protein synthesis (MyoPS) and breakdown (MyoPB) rates determines the net deposition of myofibrillar proteins in skeletal muscle, with changes in the myofibrillar net protein balance (NBAL) ultimately underpinning changes in skeletal muscle mass. Skeletal muscle is a highly plastic tissue with diurnal alterations in protein metabolism integral to muscle remodelling (27). For healthy, younger adults who have not partaken in demanding exercise training, these diurnal alterations in MyoPS and MyoPB generally result in a neutral NBAL, where NBAL is zero and skeletal muscle mass is effectively in a state of maintenance. As displayed in Figure 1.1, in the postabsorptive state skeletal muscle enters a catabolic state where rates of MyoPB exceed those of MyoPS resulting in a negative NBAL (28). However, this is counteracted following protein feeding or acute exercise, where rates of MyoPS significantly increase and skeletal muscle enters a state of positive NBAL or accretion (29-31). Beyond diurnal proteostasis rhythms, changes to MyoPS rates with ageing, inactivity or disease have the greatest influence on the net loss of skeletal muscle mass (32). Typically, alterations in skeletal muscle mass are underpinned by a blunting of MyoPS in response to nutritional and/or exercise stimuli, a phenomenon termed anabolic resistance (33-36) (Figure 1.1). Overtime, muscle anabolic resistance in combination with a blunted MyoPS in the postabsorptive state results in a chronic state of catabolism and ultimately underpins the net loss of muscle in situations characterized by atrophy (33). Historically, the influence of MyoPB on skeletal muscle remodelling is poorly understood due to the difficulties in obtaining measurements *in vivo*. In catabolic conditions, the protein content of several key catabolic signalling targets are altered (for a comprehensive review see (37)). However, since protein signalling and turnover rates do not always reciprocate one another, further understanding is required to elucidate the role of MyoPB in skeletal muscle remodelling.

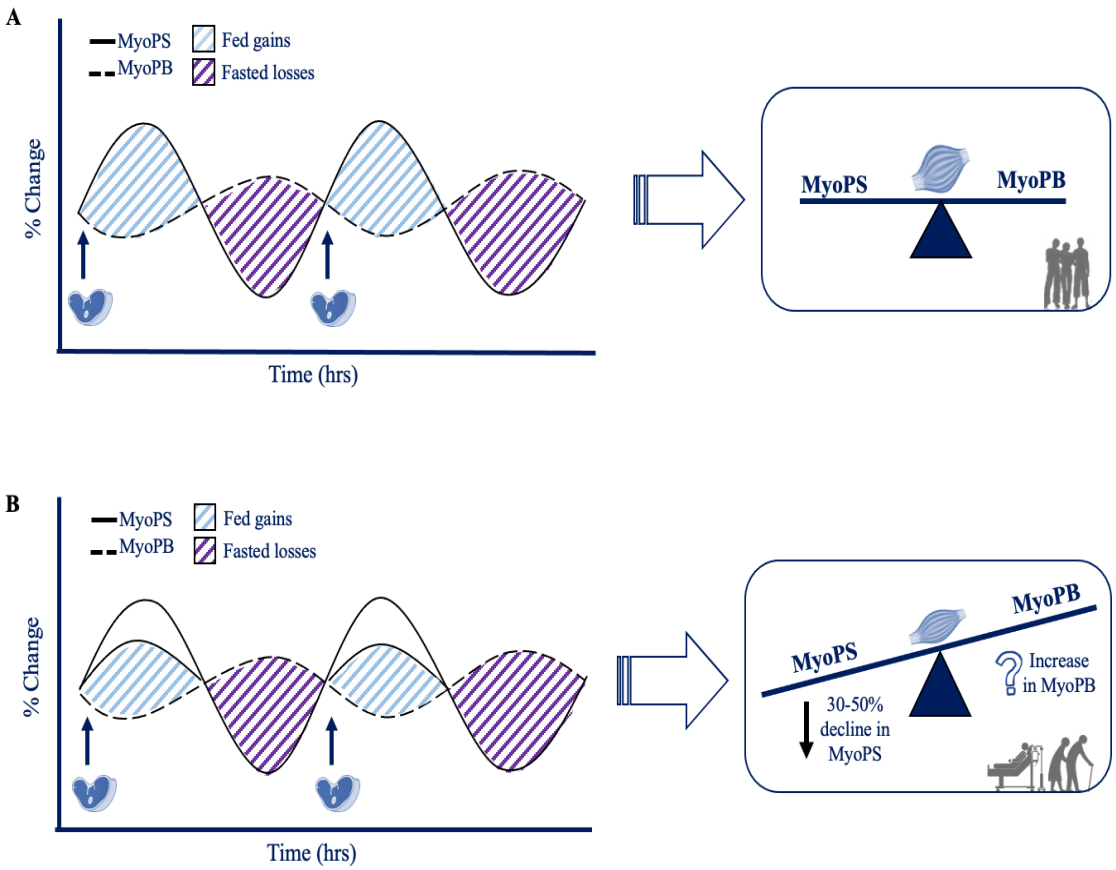

Figure 1.1 Daily alterations in skeletal muscle protein turnover.

(A) represents diurnal protein turnover in 'normal' conditions, (B) represents diurnal protein turnover in periods of disuse/with ageing, leading to an imbalance of MyoPS and MyoPB rates

1.2.1. Signalling mechanisms underpinning alterations in MyoPS

Historically, anabolic signalling cascades are suggested to centre around the mechanistic target of rapamycin (mTOR) which is a serine/threonine kinase that is central to sensing changes in energy status and nutrient availability (38). The signalling hub protein consists of two distinct complexes (mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2)), which act through distinct mechanisms to control cell growth, metabolism and survival (39). The two complexes both contain mTOR as the catalytic component and consist of a G protein

beta-subunit like protein (40). However, they both have unique components. mTORC1 consists of the 40 kDa protein kinase b (Akt) substrate (PRAS40), the regulatory associated protein of mTOR (raptor), the mammalian lethal with SEX13 protein and the DEP domain-containing mTOR-interacting protein (40). Whereas mTORC2 contains a rapamycin-insensitive companion of mTOR and the mammalian stress-activated map kinase-interacting protein 1 (40, 41). Of the two mTOR complexes, mTORC1 has been consistently reported to regulate anabolic processes in response to resistance exercise and/or protein feeding, whilst inhibiting autophagic processes (42, 43).

The activation of mTORC1 is dependent on the GTPase protein, ras homolog enriched in brain (Rheb), which binds to mTORC1 catalytic domain (44). In order for Rheb to activate mTORC1, the activity of its inhibitor, tuberous sclerosis 2 (TSC2), must be reduced. TSC2 acts to induce GTP hydrolysis, which in turn inhibits Rheb activity (44, 45). The importance of TSC2/Rheb in the activation of mTORC1 is widely noted in *in vitro* cell lines (46), with Rheb inactivation significantly reducing ribibosomal protein S6 kinase beta-1 (S6K1) expression (a downstream signalling target of mTORC1) in HEK293 cells (a kidney cell line), confirming the importance of Rheb to mTORC1 activation and subsequent anabolism (44). Therefore, in order for mTORC1 activation to occur, upstream regulating factors must either inhibit TSC2 activity, or promote the binding of Rheb to the catalytic domain on mTORC1.

Numerous mechanical, hormonal and nutritional factors provoke an upstream signalling cascade that cumulates to result in the activation of mTORC1 through this pathway. Firstly, a spike in growth factor concentrations (i.e., growth hormone or insulin growth factor 1 (IGF1)) is noted to enhance mTORC1 activity. *In vitro* work using C2C12 myotubes exposed to IGF1 for 2h lead to a significant increase in mTORC1 activity (47). Further studies have also suggested that a 48h exposure to IGF1 led to a significant increase

in the phosphorylation of S6K1 and this response was blocked by the mTORC1 inhibitor, rapamycin (48). Mechanistically, growth factors are suggested to stimulate mTOR activation through a signalling cascade that begins from the binding of growth factors to their skeletal muscle receptor (48, 49). In turn, their specific membrane bound receptors act to phosphorylate phosphatidylinositol 4,5-bisphosphate (PIP2) on the cell membrane at the 3 position of inositol through the enzyme class I phosphatidylinositol-3-kinase (PI3K) (49, 50). Upon phosphorylation, PIP2 yields a second messenger, phosphatidylinositol to PI 3,4,5-triphosphate (PIP3). This interaction recruits protein kinase b (Akt) to the inner face of the plasma membrane where PIP3 permits a conformational change that exposes Akt for phosphorylation at Akt^{S473} and Akt^{T308} (51, 52). The activation of Akt then acts to inhibit TSC2 , promoting the binding of Rheb to mTORC1's catalytic domain and through phosphorylating $PRAS40^{T246}$, promoting its disassociation from mTORC1 (46, 53, 54) and allowing downstream signalling targets (i.e. eukaryotic translation initiation factor 4E binding protein 1 (4EBP1) and S6K1) to dock and be phosphorylated by mTOR (45). Although it is apparent that growth factors activate mTORC1 through this pathway, it is not a prerequisite for mTORC1 activation following mechanical stimuli (55). Although growth hormone concentrations peak following a bout of resistance exercise (56-58), several studies demonstrate that the inhibition or complete depletion of IGF1 does not prevent the activation of mTORC1 following a mechanical stimulus (59-61), suggesting that growth factors do not play a significant role in promoting mTORC1 activation following load bearing activity (55).

Instead, research suggests the involvement of mechanotransducive proteins that surround the extracellular matrix. Although the exact mechanisms linking these proteins to mTORC1 activation remain to be fully eluded, the focal adhesion kinase (FAK) is suggested to be a principle mediator of mechanically induced mTORC1 stimulation (62). It has been noted the phosphorylation of FAK^{T293} results in the binding of FAK to PI3K at the SH2

domain (63), increasing PI3K activity and ultimately activating mTORC1 (47). However, FAK is also suggested to modulate mTORC1 activity directly through the destabilisation of TSC2. This interaction has widely been confirmed *in vitro* in C2C12 muscle cells, where a knockdown of FAK utilising FAK-specific siRNA led to a decrease in TSC2 phosphorylation and a subsequent reduction in the protein expression of targets downstream of mTORC1 (47).

Amino acids (AAs) are also potent activators of mTORC1 (42, 53, 64, 65), which in turn triggers a signalling response that elevates MyoPS rates. Indeed, there is significant evidence to demonstrate AAs are both an independent and synergistic (+ resistance exercise) activator of MyoPS (66). Mechanistically, AAs are purported to activate mTORC1 through an IRS1/Akt independent cascade (67-69). Despite this difference, the enzyme PI3K is believed to be central to the AA activation of mTORC1 due to the vacuolar protein sorting 34 (VPS34; (67)). In HEK293 cells (a kidney cell line), the removal of AAs dampened the activity of VPS34 and the overexpression of VPS34 led to a 2-fold increase in S6K1 activity (70), suggesting its central role in AA stimulated mTORC1 activation. Another purported route of AA induced mTORC1 activation is through the translocation of mTORC1 to the lysosomal membrane, where Rheb and ras-related GTP-binding (Rag) GTPases reside. Within skeletal muscle there are four Rag GTAPases (RagA, RagB, RagC and RagD) are suggested to be critical for mTORC1 activation (64, 71). Although these rag proteins are functionally redundant individually, they bind to form a functionally relevant Rag complex that is nutrient sensitive (72). When AA are scarce, RagA/B is GDP-bound and RagC/D is GTP-bound rendering an inactive Rag complex (73). However, under AA sufficient conditions RagA/B becomes GTP-bound and RagC/D is GDP-bound rendering the Rag complex active (71). Following AA provision, mTORC1 translocates to the lysosomal membrane, where the Rag complex acts to anchor mTORC1 to the lysosome, bringing mTORC1 in contact with Rheb thus allowing its subsequent activation (44, 53).

Following activation of mTORC1 by mechanical, hormonal or nutritional stimuli a series of signalling cascade events occur to promote translation initiation. Translation initiation is a requirement for MyoPS (74, 75) and involves the formation of an eukaryotic initiation factor complex, which consists of the translation initiation factors; eIF4a, eIF4E and eIF4G (76). The initiation of translation is mediated by the activation of two key downstream signalling proteins S6K1 and 4EBP1 (74, 77). When 4EBP1 is in a hypophosphorylated form it binds to eIF4E (78). Since the binding of eIF4E at the 5'cap end is a necessity to promote the binding of the additional two translation initiation factors to form the complex, mTOR acts to phosphorylate 4EBP1, which in turn promotes its disassociation from eIF4E, allowing the complex to form and protein translation to be initiated (78). The activation of S6K1, in response to phosphorylation at $S6K^{T389}$ initiates several downstream factors that aid translation efficiency through promoting the phosphorylation of RPS6 (79). The phosphorylation of RPS6 improves the recruitment of 5'STOP mRNAs to the ribosomes, a rate limiting step of translation initiation (79). Secondly, S6K1 promotes the phosphorylation of $eIF4B^{S422}$, which acts to increase helicase activity of the eukaryotic initiation complex, promoting mRNA translation initiation (76). In addition to the increase in helicase activity, S6K1 can also enhance translation elongation efficiency through the phosphorylation of the eukaryotic elongation factor 2 kinase, which governs the phosphorylation of the eukaryotic elongation factor 2 (eEF2) at an inhibitory site (80). Upon phosphorylation of $eEF2^{T56}$ there is a reduced inhibition, allowing eEF2 to bind with ribosomes and ultimately increase translation elongation (80). The integral role of S6K1 signalling to promote translation initiation and thus MyoPS rates has been demonstrated in models of aging (81, 82). In rodent models, S6K1 phosphorylation is deemed less sensitive to leucine stimulation in older rat muscle compared to younger counterparts (81). In older humans, both the relative protein content and EAA-induced phosphorylation of

mTORC1/S6K1 is reported to significantly decline compared to younger counterparts (82). Cumulatively, older individuals have a 16% lower increase in EAA-induced increases in MyoPS rates compared to younger individuals (83), suggesting the key role of mTORC1 signalling in anabolic sensitivity to feeding (see figure 1.2 for more information). Further information detailing the importance of mTORC1 signalling to MyoPS in response is detailed in section 1.5.3.

1.2.2. Signalling mechanisms underpinning alterations in MyoPB

Three main catabolic systems contribute towards skeletal muscle protein turnover; the ubiquitin-proteasomal, the autophagy- lysosome and the calpain-Ca^{2+} pathway. The signalling cascades within these pathways that result in protein breakdown are discussed in more detail below.

1.2.2.1. Ubiquitin-proteasome system

The ubiquitin-proteasome system (UPS) is perhaps the most well researched of the catabolic signalling pathways and is responsible for the degradation of defected and misfolded proteins (84). Degradation of proteins in the UPS occurs through the covalent attachment of a multi-ubiquitin chain to the 26S proteasome (85, 86), which acts to degrade proteins attached to the protein ubiquitin (87). The ubiquitination of proteins begins with an E1 ubiquitin-activating enzyme, which captures ubiquitin and catalyse the subsequent thioesterification (88). Subsequently, ubiquitin is transferred to an E2 ubiquitin-conjugating enzyme via transthioesterification before an E3 ubiquitin ligase transfers the activated ubiquitin to a lysine group of the target protein (87). The two 19S regulatory subunits on the 26S proteasome recognise and bind to these ubiquitinated proteins, initiating the ATP-dependent degradation process that occurs within the 20S core (85). Within skeletal muscle, the E3

ligases, atrogin 1 (MAFbx) and muscle specific ring finger protein 1 (MuRF1) are believed to play a crucial role in UPS mediated protein degradation. Seminal work identified MuRF1 and MAFbx as a common trigger for atrophy utilising a rodent model of ankle immobilisation. This approach detailed that the expression of these two E3 ligases were relatively low in baseline conditions, but significantly increased in the 24h following inactivity/decreased loading (89). Following this seminal work, a plethora of research has confirmed the integral role of the UPS in atrophy resulting from musculoskeletal disuse, with the two aforementioned E3 ligases now believed to be a requirement of skeletal muscle atrophy (89, 90). However, the UPS is not able to degrade intact myofibrils without the aid of one or both of the additional protein degradation systems (91, 92).

1.2.2.2. Autophagy-lysosome system

The autophagy-lysosome system (ALS) is involved in the recycling and degradation of bulk cytoplasm, organelles and long-lived proteins (93). Despite the original belief that the ALS was a purely unselective pathway, it is also able to selectively remove specific organelles, including mitochondria through mitophagy (28). The ALS works though a double-membrane vesicle encapsulating a portion of the cytosol and fusing with lysosomes, upon which their content is degraded by lytic enzymes. The autophagic flux is monitored through several proteins. The first of which is the Microtubule-associated protein 1A/1B-light chain 3 (LC3αβ), a mammalian homolog of the Atg8 gene. During the formation of the autophagosomal membranes, cytosolic LC3αβ (LC3αβ I) undergoes lipidation to LC3αβ II through two ubiquitylation-like reactions catalysed by an E1-like and E2-like enzyme (94). During lysosomal degradation, intra-autophagosomal LC3αβ II is also degraded, thus providing a readout of autophagic activity. A second monitoring protein is p62, which

undergoes degradation by lytic enzymes following the fusion of the autophagosome to the lysosome (95), providing an additional measure of autophagic activity.

Over the past decade, research has suggested that the ALS acts to recover amino acids and thus meet skeletal muscle energetic demands, promoting cell survival (96, 97). Beyond the crucial role in maintaining an energetic homeostasis, the ALS has been suggested to modulate protein degradation in numerous physiological conditions, including; disuse (98), ageing (99, 100) and critical illness (101). Furthermore, the ALS is demonstrated to be activated following endurance exercise (102), detailing the importance of the ALS for the removal of load-induced damage to myofibrillar proteins and thus its role in muscle remodelling processes.

1.2.2.3. The calpain-Ca^{2+} system

The calpain-Ca^{2+} degradation system is perhaps the most under researched of the three catabolic systems (103). Calpains are Ca^{2+} dependent non-lysosomal proteases that target cytoskeletal, sarcolemmal and myofibrillar proteins (37). Within skeletal muscle, calpain-1, calpain-2 and the muscle specific calpain-3 are expressed (104). Unlike calpain-1 and calpain-2, calpain-3 is bound to myofibrillar proteins, primarily the structural protein titin in an inactive manner to prevent degradation (105). Calpain-3 renders itself inactive and acts to stabilise the sarcomere until sarcomeres cross a load-bearing threshold, where calpain-3 partakes in the remodelling process via the activation of proteins such as myosin heavy chain 1 (105-107). Upon activation, calpains increase total protein degradation by ~65% (108) by targeting titin and nebulin and destabilising the sarcomere (109). Interestingly, a lack of calpain-3 is also suggested to result in muscular dystrophy (110) due to the reduction in stability of sarcomere proteins, which is exacerbated by exercise (105) and thus it is

suggested that the maintenance of calpain-3 protease content is a necessity to protect muscle from degradation (105).

1.3. Mitochondrial function across the lifespan

Of the physiological changes that occur with increasing age, the changes in cardiovascular functionality are perhaps the most important. Maximal oxygen uptake ($VO2_{max}$) is a measurement used to assess the functional capacity of the body to meet its energetic demands (111). Low cardiorespiratory fitness (measured using $VO2_{max}$) is associated with the onset of cardiovascular disease (112-114) and is an independent risk factor for mortality (112, 113). Worryingly, an individual's $VO2_{max}$ is suggested to decline at a rate of 5% per decade (115) and by the age of 75yrs, $VO2_{max}$ is suggested to have declined by over 50% in comparison to its peak (116). As a result of this drastic limitation in functional capacity, cardiovascular fitness drops below the levels required for the completion of daily living activities (117). However, physical activity is a confounding factor for cardiorespiratory fitness, with chronic exercise training demonstrated to increase $VO2_{max}$ (118). Therefore, it is suggested that age-related alterations in $VO2_{max}$ are actually a consequence of age-related reductions in physical activity, rather than ageing perse. For example, when corrected for lean mass, master athletes have been demonstrated to have a 60% higher in $VO2_{max}$ compared to their inactive middle-aged counterparts (119), suggesting that age-related declines in functional capacity can be attenuated, or even reversed with lifelong physical activity.

Mitochondria are mechanically (120) and nutrient sensitive (121, 122) organelles that are responsible for the majority of ATP synthesis (123). The number and functionality of mitochondria positively correlates with an increase in functional capacity in healthy humans (124-126). Conversely, reductions in $VO2_{max}$ are accompanied by a decline in skeletal muscle oxidative capacity (127, 128).

Similar to myofibrillar proteins, mitochondrial proteins are dynamic and undergo periods of synthesis (MitoPS) and breakdown (through mitophagy). The maintenance of mitochondrial homeostasis across the lifespan and thus optimal mitochondrial functioning is crucial to the maintenance of whole-body functional capacity. Unfortunately, a reduction in mitochondrial function is a hallmark of ageing (123, 129), with a reduction in mitochondrial coupling efficiency and respiratory capacity resulting in a reduction in ATP synthesis noted in older adults (130). However, when individuals are matched for cardiorespiratory fitness or physical activity levels there is no differences in mitochondrial respiration noted between young and old adults (131, 132), suggesting that alterations in mitochondrial function are in fact a consequence of inactivity (133). Consequently, it is important to understand the mechanisms underpinning alterations in mitochondrial function in order to preserve functional capacity in response to inactivity and/or ageing and these are discussed in more detail below.

1.3.1. The regulation of mitochondrial biogenesis

Mitochondrial biogenesis is a term used to describe the growth and division of pre-existing mitochondria (134), resulting in variations in mitochondria size, mass and number and ultimately dictating mitochondrial functional capacity. Mitochondrial biogenesis is dependent on the replication of mtDNA, a circular DNA molecule that has its own transcriptional/translational system (135). This genomic system contains 37 genes that encode subunits of the oxidation phosphorylation (OXPHOS) complexes I, III, IV and V, 22 transfer RNAs as well as 2 ribosomal RNAs that are a necessity for translation (134). The transcription of mtDNA is performed by the mitochondrial RNA polymerase POLRMT (136), which itself is regulated by the mitochondrial transcription factor A (TFAM), a factor that enhances the binding of POLRMT to mtDNA promoters. For mitochondrial biogenesis

to occur, ~1000 proteins encoded by the nuclear genome need to be correctly synthesized and imported (134). Furthermore, a portion of these proteins also need to be assembled with proteins encoded by mtDNA within the inner and outer mitochondrial membranes (134). Therefore, it is perhaps unsurprising that these processes need to be tightly regulated in order to meet the energetic demands of skeletal muscle. The signalling cascades relating to mitochondrial biogenesis are triggered by various nutritional and contractile stimuli and the proteins regulating these processes are described in more detail below.

PGC1α is considered an important regulator of mitochondrial biogenesis and the responsiveness of this protein to nutritional and exercise stimuli cannot be ignored. Indeed, the activation of PGC1α in skeletal muscle has been demonstrated to promote an increase in mitochondrial biogenesis (137, 138) as well as an improvement in endurance exercise capacity (138, 139). The coordination of mitochondrial biogenesis through PGC1α occurs via a convergence of many cellular signals. Although PGC1α is unable to bind to DNA, it interacts and co-activates mitochondrial transcription factors, including TFAM and the nuclear respiratory factor 1 (NRF1) (140), which plays an important role in the regulation of TFAM expression (141).

The regulation of PGC1a following exercise occurs through its responsiveness to alterations in intracellular [Ca^{2+}]. Following a sustained period of contractile activity, the prolonged intracellular rise in [Ca^{2+}] promotes the phosphorylation of calcium-calmodulin dependent kinases (CAMK) (142, 143). In skeletal muscle, CAMKII is the predominant CAMK isoform and it appears to be activated in an intensity dependent manner (143). Mechanistically, the activation of CAMKII leads to an increase in mitochondrial content (144) through the indirect regulation of PGC1a. Once activated CAMKII acts to enhance the phosphorylation of the activating factor 2 or the myocyte enhancer factor-2, which in turn augment PGC1a transcription (145). Indeed, following endurance exercise (which constitutes

a prolonged period of contractile activity) the abundance of mitochondrial PGC1a increases (146). In turn, these increases in mitochondrial PGC1a abundance coincide with the upregulation of the downstream target genes that are involved in the transcription and translation of mtDNA (147-150).

The regulation of PGC1a is also suggested to be governed through the energy sensing protein, 5' AMP-activated protein kinase (AMPK). AMPK plays an important role in intracellular energy homeostasis and the subsequent promotion of mitochondrial biogenesis. The increase in cellular [AMP:ATP] that occurs following exercise or a period of fasting results in the activation of AMPK, which in turn is noted to enhance skeletal muscle oxidative capacity through the promotion of mitochondrial biogenesis (151, 152). The activation of AMPK occurs by the phosphorylation at AMPKα ^{T172} at the catalytic (α) subunit (153). Despite the ability of CAMKII to augment the activation of PGC1a independently of AMPK, AMPK can also be directly phosphorylated at AMPKα ^{T172} by CAMKII (154-156) following periods of AA starvation (157) and in hypoxic conditions (158). Following activation, AMPK leads to an increased activation of PGC1a and NRF1, resulting in mitochondrial biogenesis (151, 159, 160).

1.3.2. The regulation of mitochondrial morphology

Beyond the promotion of mitochondrial biogenesis, the regulation of mitochondrial morphology is critical to maintain optimal mitochondrial function across the lifespan. Mitochondria morphology is reliant upon the balance between mitochondrial fission and fusion. In ageing there is a loss of mitochondria proteostasis, which coincides with a dysregulation in mitochondrial dynamics where fission rates exceed fusion (161, 162). This in turn contributes to the reduction in respiratory capacity and coupling efficiency that occurs with increasing age (130).

The fusion of smaller mitochondria allows the expansion of the mitochondrial reticulum and facilitates the sharing of mitochondrial material. This leads to an increased bioenergetics efficiency and thus adequate ATP production(163, 164). Mechanistically, mitochondrial fusion is initiated by two proteins within skeletal muscle, Mitofusion 1 and 2 (Mfn 1 and Mfn2) act by anchoring the outer membrane of adjacent mitochondria and optical atrophy 1 and 2 (OPA1 and OPA2) acts to anchor the inner membranes of adjacent mitochondria, aiding fusion (165). On the other hand, mitochondrial fission promotes the degradation of fragmented organelles, the first step in the removal of dysfunctional mitochondria from the mitochondrial pool through mitophagy. Increased rates of mitochondrial fission accompany ageing and disease, resulting in a reduction in ATP production and increased rates of oxidative stress (166). Mechanistically, the process of fission is reliant on three main proteins. Firstly, the dynamin-related protein 1 (DRP1) resides on the outer membrane of mitochondria and interacts with the fission protein 1 (FIS1) and the mitochondrial fission factor (MFF) to constrict mitochondria and promote their separation (167).

1.3.3. Mitochondrial regulation of myofibrillar protein turnover

Myofibrillar protein turnover is a dynamic process that requires an adequate ATP production. Alterations in mitochondrial function often accompany atrophy, but the involvement of mitochondria in the pathology of human atrophy is often debated. Here, we examine the proposed mechanisms of cross talk between altered mitochondrial function and muscle atrophy (Figure 1.2).

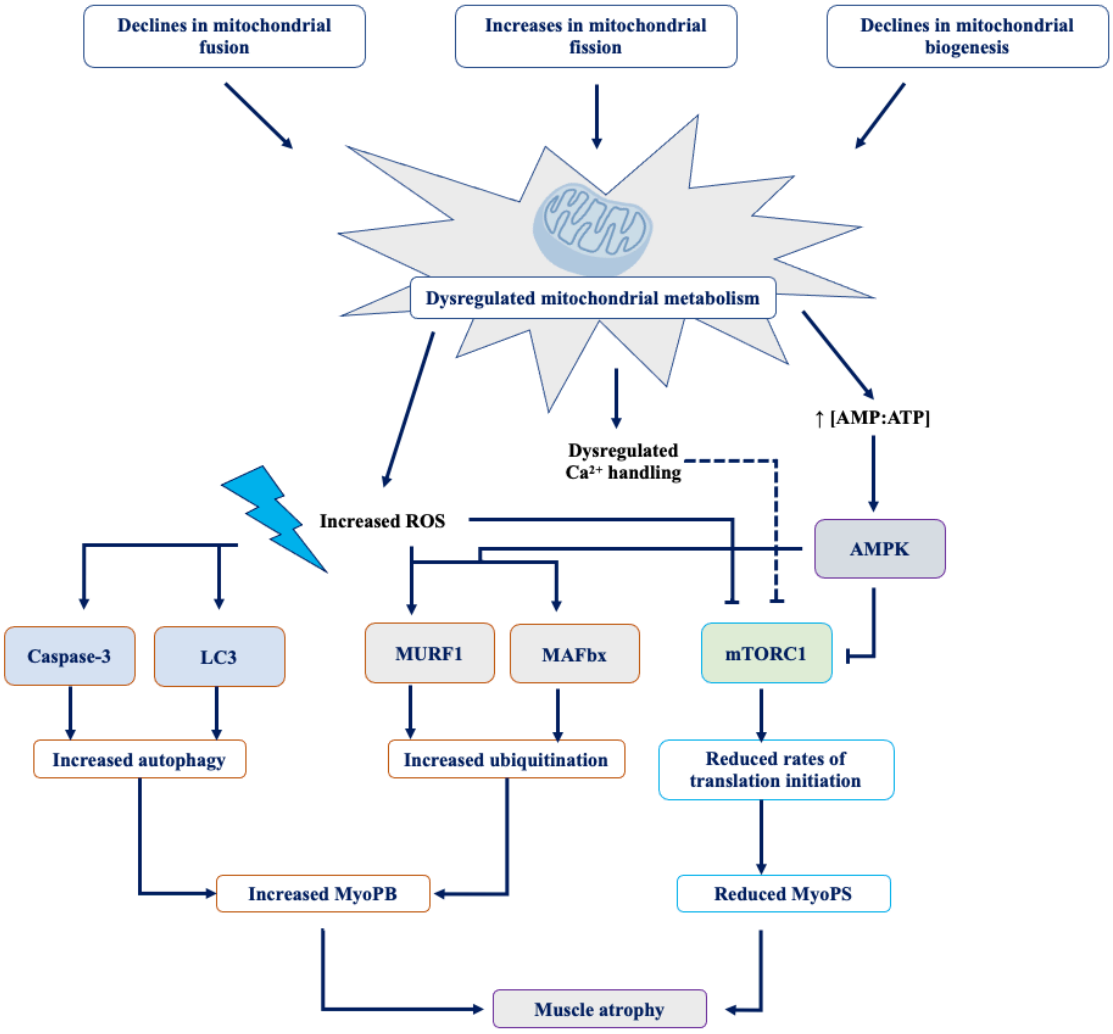

Figure 1.2 Mitochondrial regulation of myofibrillar protein turnover during periods of disuse

LC3: Microtubule-associated protein 1A/1B-light chain 3, MURF1: muscle ring finger 1, MAFBx: Muscle Atrophy F-box gene, mTORC1: mechanistic target of rapamycin complex 1, AMPK: AMP-activated protein kinase

Dysfunctional mitochondria result in an excessive production of reactive oxygen species (ROS), through the stimulation of the citric acid cycle or electron leakage from the ETC; subsequently activating nitric oxide synthase (NOS) and additional ROS generating enzymes (168, 169). Although ROS production is deemed necessary for musculoskeletal adaptation to exercise (170), the chronic generation of ROS has putatively been linked to an acceleration in muscle proteolysis (171). In fact, an increase in ROS are suggested to be a

required step in the induction of atrophy (172, 173). Although, due to the heterogeneity of ROS signalling molecules, this has yet to be consistently shown in a human model. Chronically high levels of ROS production, that exceed musculoskeletal antioxidative defence mechanisms is a common characteristic of musculoskeletal disuse (174) and in ageing (129, 175).

Mechanistically, ROS are proposed to induce atrophy through alterations in several mechanistic pathways. An increased ROS production is suggested to enhance rates of MyoPB through an increased in the gene expression of key components (e.g., the atrogenes) involved proteolysis (96, 98). Furthermore, the increase in oxidative muscle proteins increases the susceptibility of these proteins to proteolytic processing (176) and promotes an increase in caspase-3 and calpain expression, key catabolic signalling intermediates (176).

An increase in ROS production may also impede MyoPS, with evidence suggesting that ROS can obstruct mRNA translation initiation (177). The reduction in translation initiation has been suggested to occur through the reduction of 4EBP1 and S6K1 phosphorylation (177). However, these findings have only been reported in cell models and thus further research is warranted to determine interactions between ROS production and MyoPS in a human model.

Muscle atrophy in response to musculoskeletal disuse is often accompanied by a shift in mitochondrial dynamics towards fission. The overexpression of the fission machinery is associated with an increase in oxidative stress and AMPK activation, due to a decline in bioenergetic efficiency and thus ATP production. The reduction in ATP production promotes a state of energy starvation within the cell. To overcome this, the activation of AMPK prevents myofibrillar anabolism simultaneously through the activation of UPS signalling intermediates and through blocking mTORC1 activation, in order to prevent further energy wastage (178). Mechanistically, the reduction in mTORC1 activation occurs through the

phosphorylation of $TSC2^{S1387}$ (the mTORC1 inhibitor (179) and raptor, suppressing mTORC1 activity (180) and subsequently reducing the rate of translation initiation. The inhibition of fission machineries has been suggested to protect against atrophy (178) suggesting the importance of this mechanism in the pathology of atrophy.

Mechanistically, for fission rates to exceed rates of fusion, there must also be a decline in the expression of fusion regulatory proteins (i.e., OPA1 and MFN2). The downregulation in expression of these proteins may also contribute to atrophy. Firstly, a rection in MFN2 protein content has been noted in various atrophy-inducing models (181) and is suggested to promote a remodelling of the mitochondrial network, resulting in mitochondria dysfunction and an increase in reactive oxygen species production (182), which may contribute to muscle proteolysis through the aforementioned mechanisms. Furthermore, a downregulation of MFN2 has been demonstrated to induce endoplasmic reticulum stress, which alters Ca^{2+} handling within skeletal muscle (182, 183). Since optimal intracellular $[Ca^{2+}]$ results in the activation of the mitogen-activated protein kinase (MAPK) pathway and mTORC1 (184), it is plausible that a dysregulation in Ca^{2+} handling could downregulate anabolic signalling towards translation initiation and may even blunt MyoPS. The second primary regulating protein of fusion, OPA1 may also be involved in the pathology of atrophy through promoting alterations in the UPS and ALS. The overexpression of OPA1 has been noted to promote a reduction in MURF1 and LC3 expression (185). Therefore, it is plausible that a reduction in OPA1 expression triggers an increase in catabolism through the activation of catabolic signalling pathways contributing to the pathophysiology of atrophy (186).

1.4. Plasticity of skeletal muscle to exercise and nutrition

Skeletal muscle is a highly adaptable tissue, responding to small changes in contractile activity as well as nutritional stimuli (187). Resistance exercise is the most potent stimulus

for muscle hypertrophy, inducing a ~2-3 fold increase in MyoPS following a single bout of resistance exercise (188, 189). Moreover, when resistance exercise is combined with protein consumption, the increases in MyoPS can be extended for ~24h (190). The cumulative effect of resistance exercise bouts in combination with adequate protein intake results in a sustained increase in muscle mass (191). During periods of musculoskeletal disuse, the neuromuscular electrical stimulation of the disused muscle prevented the loss of muscle mass (192). Furthermore, cross-sectional interventions detail a superior muscle mass and strength in strength trained master athletes in comparison to their sedentary controls (193, 194), detailing the superiority of contractile stimuli to promote optimal skeletal muscle functioning. Despite the potency of resistance exercise to promote optimal functioning in suboptimal conditions (i.e. with ageing or musculoskeletal disuse), in periods of injury and/or illness the use of contraction, through resistance exercise or synthetically through neuromuscular stimulation may not be appropriate, and thus optimising nutritional interventions (an important mediator of muscle mass) should be a priority. Therefore, for the purpose of this thesis we will focus on the negative adaptations of skeletal muscle to periods of musculoskeletal disuse and evaluating potential nutritional counter-measures to offset these negative adaptations to periods of disuse.

1.5. Factors affecting skeletal muscle mass: musculoskeletal disuse

Musculoskeletal disuse is typically studied in healthy humans across the lifespan, utilising lab based models that aim to represent clinically relevant scenarios of illness and injury (represented in Figure 1.3). Since the average length of hospitalisation in the United Kingdom is 5-6d (195), the majority of disuse interventions are between 5d and 14d in length. A model of step-reduction (SR) is the least severe of the disuse models, involving a reduction in daily step count to <2000 steps.d^{-1} over a 7-14d period (e.g. (33, 196)).

Typically, this model is utilised to represent a period of acute illness at home (i.e. sofa/bed bound) or in hospitalisation (i.e. illness or elective surgery), where daily step count is typically around 750 steps.d^{-1} (197). A second model of musculoskeletal disuse is unilateral limb immobilisation, where a single limb is placed in a brace/cast over a 5-14d period (e.g. (36, 198, 199). This model provides a practical model of disuse, as participants are asked to continue their normal daily living activities at minimum inconvenience. Furthermore, unilateral immobilisation is unique because it provides a localised model of inactivity, where musculoskeletal disuse is localised to a single muscle group, while the contralateral limb serves as a control. Unlike the other models of disuse, this also provides the opportunity to study any musculoskeletal changes associated with disuse independent to systemic factors. Finally, the most severe model of disuse is bed-rest, which is representative of short-term hospital stays, and even can be used to mimic spaceflight when combined with a 6° head tilt (e.g.(200)). This is potentially the most clinically relevant model of disuse in an older population, due to their increased risk of hospitalisation. This model also takes into consideration the increased metabolic stress response that is consistent with a removal of contractile function, combining musculoskeletal disuse with a plethora of systemic alterations. Utilising a combination of these models over the past decade has significantly increased our understanding of the metabolic implications of short-term periods of illness/injury and can be utilised to better understand whether novel nutritional interventions could improve clinical relevant outcomes.

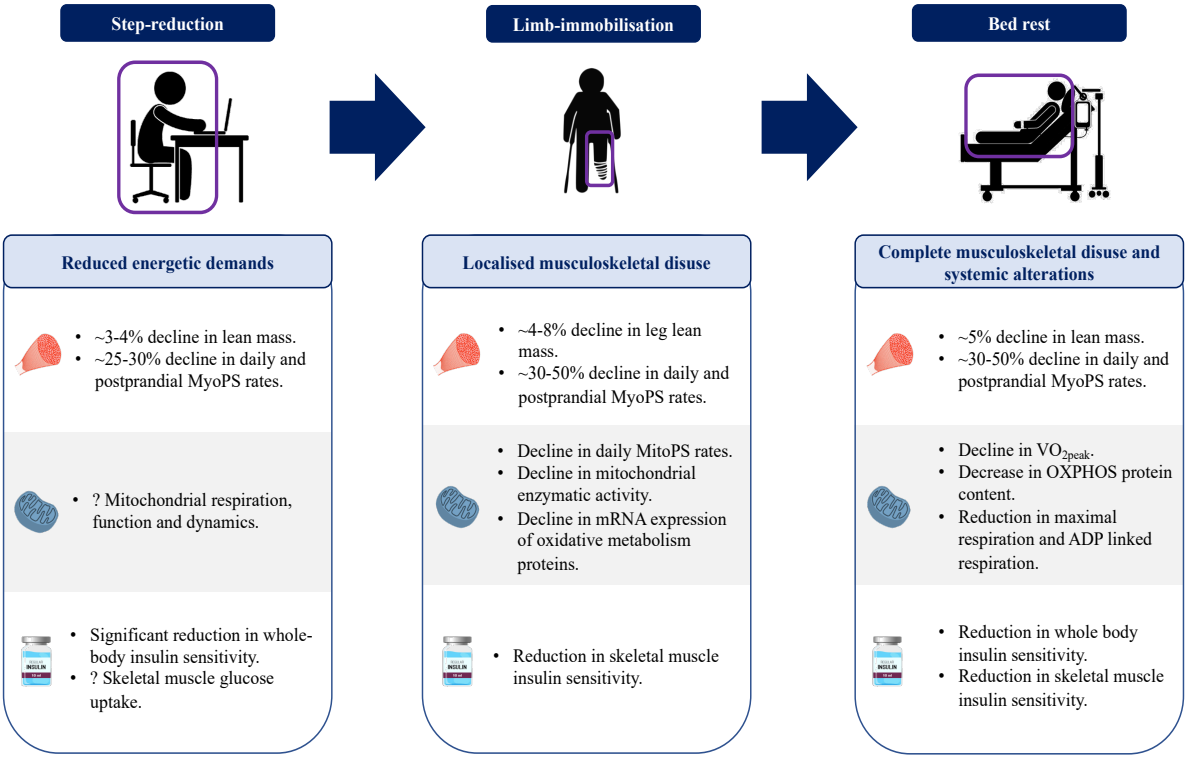

Figure 1.3 Impact of 7-14d of musculoskeletal disuse on skeletal muscle metabolism.

The impact disuse models, of increasing severity on muscle atrophy, mitochondrial function and insulin sensitivity.

1.5.1. Skeletal muscle disuse atrophy

Disuse atrophy is a term utilised to describe the loss of lean mass that occurs in response to disuse events. The rate of disuse atrophy is typically determined through the use of magnetic response imaging (MRI), computerised tomography (CT) or dual energy x-ray absorptiometry (DXA). The choice of measurement technique depends on access and funding availability, with MRI and CT the most sensitive measures of muscle volume, but DXA providing a more easily accessible alternative that is highly correlated to both MRI (r=0.88) and CT (r=0.77-0.95, (201-209)). Marked reductions in muscle mass are noted across all three experimental disuse models. The rates of muscle loss in response to SR are lower than in models of complete musculoskeletal disuse, with the 7-14d reduction in contractile activity

equating to a ~3% decline in muscle cross sectional area (CSA (33)). The observed rate of decline in models of lower limb immobilisation is ~3.5% in the first 5d of disuse (210, 211), progressing to a ~8% decline in quadriceps CSA following 14d of unilateral immobilisation (199). Interestingly, in these models, no significant alterations in muscle CSA are evident in the contralateral control limb, demonstrating the localisation of disuse atrophy. Although there is a lack of evidence directly comparing rates of muscle loss between models of limb immobilisation and bed rest, due to the influence of systemic factors, the observed rate of muscle loss is around 0.6% per day, in comparison to the 0.5% loss noted in models of immobilisation (212). Similarly to immobilisation, atrophy occurs most rapidly at the onset of disuse, with a ~5% loss of mass occurring in the first 5-7d days of bed rest (34, 213).

Beyond the models of immobilisation, different rates of atrophy are noted across different fibre types. Historically, alterations in fibre CSA are determined utilising immunofluorescent approaches, requiring a muscle biopsy and subsequent biochemical analysis and as a result the measurement of fibre CSA is utilised as a secondary outcome measure to changes in lean mass. In response to prolonged models of disuse (i.e. 180d of space flight) greater rates of atrophy have been noted muscles composing of mainly type I fibres (i.e. soleus) in comparison to muscles composed of predominantly type II fibres (i.e. gastrocnemius, (214)). This trend is also noted within an individual muscle following a prolonged (35d) bed rest intervention, with the rate of loss in type I fibres greater than their type II counterparts within the vastus lateralis (98). Whether this alteration in fibre CSA holds in a shorter model of disuse (i.e. ≤14d) remains undetermined, with contrasting research reporting either no changes in fibre CSA following 14d of disuse (215) or a significant decline in both type I and II fibre CSA following 14d, but not 5d of disuse (199). Differences between studies may be due to the lack of uniform change in atrophy across the entire length of a muscle (216),

suggesting that the site of biopsy may influence alterations in fibre atrophy following short-term disuse events.

1.5.2. The impact of disuse on muscle protein turnover

As outlined previously, skeletal muscle mass is regulated by the fine balance between MyoPS and MyoPB rates. Acute periods of disuse have been shown to alter muscle metabolism, dysregulating the muscle NBAL. The measurement of MyoPS *in vivo* is invasive and technically challenging, requiring the use of stable isotope tracers (intravenously administered labelled amino acid tracers or orally ingested deuterium water) in combination with serial blood/muscle biopsy sampling (see (217) for an in depth review of stable isotope tracers). Founding work by Gibson and colleagues (218) demonstrated a ~30% reduction in postabsorptive MyoPS rates in comparison to the control limb following a period of unilateral limb immobilisation. Subsequently, numerous interventions have examined alterations in MyoPS across disuse interventions, with more recent research utilising deuterium water (D_2O) to measure daily alterations in MyoPS. In response to as little as 7d of unilateral immobilisation, or bed rest a similar ~30% decline in daily MyoPS has been noted (211, 213). Interestingly, this ~30% decline in daily MyoPS rates is also similar following a 7d of SR (196), meaning that the complete removal of contractile stimuli is not necessarily required to elicit declines in MyoPS rates.

Despite the importance of understanding daily alterations in protein turnover, the measurement of daily MyoPS encompasses both postabsorptive and postprandial MyoPS rates and as a result doesn't provide clarification on whether the alterations in protein turnover rates occur in the basal state, or due to an anabolic resistance to protein intake. To determine this, researchers utilise intravenously administered stable isotope tracers. In contrast to founding work, whether declines in postabsorptive MyoPS following

musculoskeletal disuse underpin the loss of muscle CSA remains debated, with some research observing a ~30% decline in response to disuse (210), whereas others have reported no impairments in postabsorptive MyoPS rates in response to disuse (36, 198). Instead, a blunted MyoPS response to protein intake is suggested to drive disuse induced muscle atrophy (34-36). Across all disuse models, a 30-50% decline in postprandial MyoPS has been noted in the first 14d of the disuse event (34-36). Collectively, this suggests that mitigating impairments in postprandial MyoPS should be the focal point of any targeted interventions.

1.5.3. The impact of disuse on anabolic signalling pathways

Alterations in MyoPS rates are historically believed to originate from changes in mTORC1 signalling. However, whether mTORC1 signalling is altered following short-term disuse remains to be fully established. In the postabsorptive state, there are increasing suggestions that the protein content, or mRNA expression of mTORC1 and its downstream target S6K1 is not altered following short term disuse events (34, 35, 219-221). However, in response to nutrition, there are some suggestions of a blunted mTORC1 response (36, 222), which may underpin the blunted postprandial MyoPS. For example, following 14d of immobilisation, there was a delayed responsiveness of both Akt and S6K1 to an amino acid infusion (34). However, this finding is certainly not uniform, with a multitude of research also suggesting that there are no disenable changes in the expression of mTORC1, S6K1, RPS6 or 4EBP1 in the postprandial state following disuse, despite reductions in MyoPS (35, 199, 221). This discord between alterations in anabolic signalling process may appear as a result of missing the peak activation of these proteins, which commonly occurs in the 1-2h following protein ingestion. Many studies investigating alterations in MyoPS, would have acquired muscle tissue in the 3-6h following the anabolic stimulus (34, 36), so it could be suggested that the anabolic signalling response at this time point would be blunted and perhaps not

representative of peak changes. As a result, further research is required to underpin the exact mechanisms resulting in an attenuated postprandial MyoPS following disuse.

1.5.4. The impact of disuse of catabolic signalling alterations

Of the three molecular regulating systems of MyoPB, the UPS appears to be the most involved in the regulation of disuse atrophy. The mRNA expression of the two muscle E3 ligases (MURF1 and MAFbx) have been suggested to be upregulated in response to unloading (196, 199, 219, 223). Although there are some inconsistencies in literature surrounding the upregulation of MURF1 following disuse in a human model (e.g. (211, 222)), MAFbx is almost always shown to be upregulated. While the expression of these E3 ligases is suggested to be regulated by the transcription factor FOXO3, there is no suggestion of an increased FOXO3 mRNA expression following disuse events (219). The upregulation of the UPS pathway is suggested to occur within the first 48h of unloading events (224) remaining elevated throughout the 7-14d period of disuse (e.g. (221)). The effect of unloading on the calpain and ALS remains less established, with some evidence to suggest an upregulation of the LC3II:I ratio in younger and older adults (222). However, additional research suggests no alterations in the LC3II:I ratio or P62 (an additional marker of autophagy) following 24d of bed rest (98), suggesting that in longer periods of disuse, the role of autophagy may be limited. Similarly, varied results have been noted with calpain-3 expression, with studies noting both an increase (223) and no change (219) in response to short-term disuse. Collectively, the UPS appears to drive alterations in MyoPB pathways following a disuse event, however further work is warranted to confirm these findings and confirm the role of MyoPB in disuse atrophy.

1.5.5. The impact of disuse on mitochondrial metabolism

In whole-body models of disuse (i.e. SR or bed rest) a reduction in aerobic capacity is present following 7d of disuse (225, 226). Similarly, alterations in mitochondrial respiration appear dependent on the time-frame of disuse. Intrinsic mitochondrial capacity has been reported to increase in the first 5d of disuse (211, 227), whereas declines in maximal respiratory function have been noted following 7-14d of disuse (10). These time-frame dependent alterations in mitochondrial respiration and aerobic capacity is further supported by the reduction in the protein content of key markers of mitochondrial function (i.e. OXPHOS complex proteins (225, 228)) as well as alterations to the activation and protein content of key signalling targets involved in oxidative metabolism (e.g. PGC1α, citrate synthases, SIRT1) in the first 14d of disuse (229, 230). Decrements in the protein content of these markers likely precede any declines in mitochondrial respiratory capacity that have been noted in previous literature. Interestingly, alterations in submaximal respiratory (measured through ADP titrations) have been reported to occur following as little as 3d of immobilisation (231), suggesting that alterations in submaximal respiration precede those in maximal respiration and indicating a lack of efficiency in ATP generating pathways following the onset of disuse.

More recently, tracer methodology has been utilized to assess the impact of disuse on MitoPS rates, with a significant decline in daily MitoPS noted following 10d of immobilization (232). However, it is currently unclear whether there is a change in responsiveness of mitochondrial proteins to anabolic stimuli following disuse events. Mitochondria are nutritionally sensitive organelles and increases in MitoPS have been noted in response to a continuous amino acid infusion (29) and protein-carbohydrate co-ingestion (233). Therefore, it would be interesting to determine the impact of disuse on the

mitochondrial responsiveness to nutrition, which may provide an indication as to whether mitochondria play a role in the onset of disuse atrophy.

The disuse induced alterations in mitochondrial function result in a decline in ATP synthesis and potentially a dysregulation in calcium handling (234-236). Such disturbances are suggested to cause an increase in ROS production. However, in models of disuse there is little suggestion of an increase in nitric oxide synthase (NOS) or manganese superoxide dismutase (mnSOD; key markers of oxidative stress) in skeletal muscle, despite some evidence of an increased H_2O_2 production (226). In models of disuse, both NOS and mnSOD are typically utilised as markers of oxidative stress, due to their putative link to alterations in MyoPS and disuse atrophy, through the mechanisms outlined in Figure 1.2. However, research has yet to confirm this association in a human model, with the only study conducted thus far suggesting that an increased H_2O_2 production cannot explain alterations in glucose or lipid metabolism following disuse (226). Therefore, we are unable to speculate whether these alterations in mitochondrial metabolism remain a side-effect of the reduced requirement for ATP production, or whether they are indeed involved in the onset of disuse atrophy. Furthermore, despite the overarching evidence of a compromised mitochondrial functioning in severe models of disuse, there is no evidence to determine whether a simple reduction in ambulation (i.e. SR interventions) would promote similar decrements in mitochondrial function over a 7-14d period. Collectively, future research is required to determine whether (a) similar alterations to mitochondrial metabolism exist following a reduction in ambulation, (b) whether these alterations in mitochondrial metabolism underpin changes in disuse atrophy and (c) whether the nutritional sensitivity of mitochondria is impacted by a period of disuse.

1.6. Counter-measures to optimise skeltal muscle metabolism: leucine supplementation

Adequate dietary protein intake is a requirement for optimal skeletal muscle mass, providing the essential amino acids to replace those lost in catabolism and stimulating MyoPS and growth (237). The importance of adequate dietary protein is confirmed by a low protein intake being associated with an accelerated muscle loss in older adults, the majority of whom do not meet the Recommended Daily Allowance for protein intake (238). However, the role of dietary protein intake to prevent muscle wasting is still unresolved. In a model of bed-rest, adequate protein intake alone (1.0g.kg.d^{-1}) failed to attenuate disuse atrophy (239) and in a model of step-reduction, a high protein diet (1.6 g.kg^{-1}.d^{-1}) still failed to prevent reductions in MyoPS (240). The essential amino acid, leucine, is suggested to potentiate the muscle metabolic response to a sub-optimal protein meal (241) and may provide a more optimal intervention than dietary protein to offset the negative physiological alterations occurring in response to musculoskeletal disuse. The mechanisms through which leucine may act to attenuate the muscle metabolic maladaptation's to disuse are described below.

1.6.1. Effectiveness of leucine to alter MyoPS in periods of musculoskeletal disuse

Leucine is a key mediator of postprandial MyoPS (242-246), in an older individual 3-4g.meal^{-1} of leucine is suggested to provide a near optimal MyoPS response (238). Indeed, it is suggested that leucine can restore the anabolic sensitivity of skeletal muscle to dietary amino acids in ageing (247). Mechanistically, leucine acts to enhance MyoPS through the robust enhancement of translation initiation (248), through mTORC1 activation (249). For example, treatment with leucine in L6 myoblasts increased 4EBP1, S6K1 and RPS6 phosphorylation and treatment with Rapamycin (an mTORC1 inhibitor) abolished this effect (250), detailing the importance of mTORC1 to the leucine mediated increase in MyoPS.

Translating this to a human model, an infusion of leucine increased the phosphorylation of S6K1 and 4EBP1, leading to the increase in postprandial MyoPS (251, 252). Interestingly, the oral ingestion of essential amino acids (including leucine) provides a similar muscle anabolic response to the intravenous infusion, with significant increases in mTORC1 signalling intermediates and MyoPS evident in the initial 3h postprandial period (82). The increase in anabolism following leucine ingestion is also evident following a bout of resistance exercise, where a mixture of leucine-enriched essential amino acid and carbohydrates potentiated the mTORC1 signalling response in the 2h post exercise in young adults (253). Indeed, this led to a 145% increase in MyoPS compared to a 40% increase with resistance exercise alone (253).

In models of disuse, the potency of leucine to protect against disuse induced atrophy is more debated, with leucine supplementation of 2.5 $g \cdot meal^{-1}$ unable to attenuate the loss of muscle and function following short-term disuse (254), whereas much higher leucine doses (0.06g/kg/meal) partially spared disuse-induced muscle and strength loss in middle-aged and older adults (255, 256). Interestingly, it has been suggested that 3-5g of leucine is required to elicit an optimal MyoPS response in younger adults. Since $5g \cdot meal^{-1}$ of leucine supplementation has been demonstrated to enhance integrated MyoPS rates in free-living conditions independent of contractile activity (29) further research is required to detail the effectiveness of this higher dosing strategy to offset disuse atrophy in young adults.

1.6.2. Effectiveness of leucine to offset disuse induced alterations in mitochondrial metabolism

Supplementation with amino acids is also suggested to provide substrate for oxidative metabolism (257, 258), with increased amino acid plasma concentrations suggested to increase the maximal adenosine triphosphate (ATP) production rate (259) and adenosine 5'-

diphosphate (ADP) stimulated respiration in young, lean individuals (260) as well as reducing the phosphorylation of the energy sensing protein AMPKα (261). In response to a continuous amino acid IV infusion (29) or protein-carbohydrate co-ingestion (233), rates of MitoPS are reported to increase. Interestingly, in a model of disuse, supplementation with the leucine metabolite, beta-hydroxy-beta-methylbutyrate (HMB) has also been demonstrated to mitigate impairments in oxidative metabolism and mitochondrial function (262). Similarly, leucine supplementation during bed rest in older adults improved resistance to oxidative stress, with significantly lower levels of muscle bound 4HNE compared with placebo supplementation (263). Since declines in mitochondrial functioning and increases in oxidative stress not only lead to reductions in aerobic capacity following disuse, but also may be implicated in disuse atrophy, further research is required to understand the effectiveness of leucine supplementation to offset mitochondrial alterations in disuse.

1.7. Counter-measures to optimise skeltal muscle metabolism: a focus on polyphenol supplementation

Beyond protein nutrition, over the past decade there has been increase in research into the ability of small bioactive compounds to improve skeletal muscle health. Polyphenols are naturally occurring biologically active compounds found in a variety of fruits and vegetables. In plants, polyphenols are secondary metabolites, typically involved in defence against pathogens and ultraviolet radiation (264). Over 8,000 polyphenolic compounds have been identified (265) and classified into four main groups dependent on their structure of their phenolic ring (265, 266).

Flavonoids, a diverse group of polyphenolic compounds categorised by their 15-carbon skeleton containing 2 benzene rings, are regularly consumed in the human diet (267) and are the focus of experimental work in this thesis. They are of particular interest to human

metabolic health due to the habitual intake of flavanols (3-hydroxy flavonoid derivatives (267)) promoting a reduction in coronary artery disease risk and mortality (268, 269). Cocoa contains more flavanols than most foods (270) and has been associated with a range of anti-inflammatory and anti-oxidative capabilities (267). Specifically, cocoa supplementation has been suggested to promote clinically relevant improvements in cardiovascular related outcomes (271) and offer protection against cognitive aging (272). Furthermore, within skeletal muscle, cocoa polyphenols exert anti-oxidant effects, reducing the accumulation of protein oxidation products and strengthening skeletal muscle antioxidant capacities (273).

1.7.1. Mechanisms of cocoa metabolism

Cocoa is composed of numerous polyphenol metabolites. Specifically, (-)-epicatechin (EPI) is the most abundant flavanol within cocoa (274), representing ~35% of total polyphenols in cocoa powder and chocolate (275). As a result it is widely suggested that many of the health promoting effects of cocoa, originate directly from EPI (276). However, in humans it is well reported that EPI has a poor oral bioavailability and as a consequence is only present in systemic circulation at extremely low concentrations (277). Unabsorbed EPI phytochemicals are metabolised by colonic microbiota to form smaller structurally related metabolites (277). In total, 70% of EPI is absorbed into circulation in the colon, suggesting the importance of the colonic microbiome in the absorption and thus metabolic effects of EPI within the human peripheries. The smaller, bioactive colonic metabolites are often categorised into three distinct sub-groups, dependent on their appearance in circulation (277). Firstly, the structurally related epicatechin metabolites (SREMs) peak in concentration in the first 4h following EPI ingestion, but remain present in circulation for the 24h following EPI ingestion. The next class of metabolites to peak in concentration is the 5-carbon ring fission metabolites (5C-RFM), which peak in circulation from 4-12h following EPI ingestion (277). In comparison to the other two classes of metabolites, the 5C-RFM's are the most abundant

in concentration, with a urinal concentration of ~87μmol in the 48h following EPI ingestion (277). Finally, the smallest of the colonic metabolites are the 3/1-carbon ring fission metabolites (3/1C-RFMs), which peak in concentration in the 12-48h following EPI ingestion, rending themselves the longest acting EPI metabolites in circulation (277). Of particular interest here is the most abundant 3/1C RFM metabolite, hippuric acid (HA). HA is present in circulation throughout the 48h period following EPI ingestion and has the second highest urinal concentration (~26μmol) of all EPI metabolites (277). Taken together, it seems likely that these aforementioned colonic metabolites are ultimately responsible for the *in vivo* short-to-long term changes associated with native flavanol supplementation in skeletal muscle. Thus ,any *in vitro* research directly treating cultures with flavanols, should consider the use of colonic metabolites in order to more closely represent complex human physiology.

1.7.2. Effectiveness of flavanol supplementation to offset disuse induced alterations in skeletal muscle metabolism

In *in vitro* models, EPI has been demonstrated to attenuate insulin resistance (278) as well as enhance mitochondrial function through increasing citrate synthase and cytochrome C oxidase activity (279), important metabolic improvements that may mitigate muscle atrophy. Furthermore, *in vitro* EPI supplementation at physiologically relevant concentrations was able to promote myogenesis and increase C2C12 myotube width (280). In fact, anti-atrophic effects have also been noted following EPI supplementation *in vitro*, with EPI downregulating key atrogenes, muscle ring finger protein 1 (MURF-1) and atrogin-1 (MAFbx) in C2C12 myotubes subjected to clinorotation (281). These data have led to the suggestion that EPI supplementation may be able to combat muscle atrophy through the maintenance of mitochondrial function, insulin sensitivity and the suppression of MyoPB *in vitro*. However, in human models there is contrasting evidence on the effectiveness of EPI to

promote favourable changes in skeletal muscle metabolism with evidence suggesting that acute supplementation with cocoa flavanols was unable to improve muscle anabolic responsiveness in older males, despite improvements in the muscle vasculature (282). In addition, another (pilot) study suggested that 7d of EPI supplementation can improve hand grip strength in middle-aged adults (283). Furthermore, 3-months of cocoa supplementation improved skeletal muscle mitochondrial function (via increases in AMPKα and PGC1α phosphorylation and citrate synthase activity), reduced markers of oxidative stress and increased exercise capacity in middle-aged sedentary subjects (284).

A plausible explanation for the discrepancies between human models as well as the potential lack of translation from the promising *in vitro* data is the aforementioned poor bioavailability of EPI (277). Therefore, *in vitro* research stimulating skeletal muscle with EPI may not be not representative of the *in vivo* skeletal muscle periphery following natural flavanol supplementation, limiting the translatability of current research to clinically meaningful endpoints (e.g., muscle mass retention). Furthermore, acute human studies (i.e. (282)) displaying data over a duration of ~4hrs post-cocoa supplementation would have likely reflected the effectiveness of SREM metabolites to influence muscle anabolism, since they are the primary metabolite in circulation in the first 4-6hrs post cocoa supplementation (277). Interestingly, the predominant 3/1C-RFM metabolite HA has been suggested to stimulate glucose metabolism and preserve mitochondrial function following insult in C2C12 myotubes (285), actions that are in line with those witnessed following supplementation with a native flavanol (e.g., (278, 279)). Since a reduction in insulin sensitivity and mitochondrial dysfunction are negative metabolic consequences of disuse and potential mechanisms resulting in the onset of muscle atrophy, further research is warranted to determine the role of long-acting EPI metabolites to offset muscle atrophy.

1.8. *In vitro* experimental methods: increasing applicability to human research

The measurement of protein metabolism *in vivo* is invasive and technically challenging, requiring complex methodology involving the use of stable isotope tracers (labelled amino acid tracers or deuterium water) in combination with serial blood and muscle biopsy sampling (see (217) for review). Despite being the 'gold standard' to determine the effectiveness of nutritional, contractile and/or pharmacological interventions, the *in vivo* measurement of MyoPS and muscle CSA is not always feasible due to financial constraints, ethical obligations and a lack of specialist resources required to undertake such projects (217). Due to this investigators rely on *in vitro* methodologies that utilise skeletal muscle cells, isolated from human skeletal muscle or derived from immortalised cell lines (i.e. C2C12 or L6), to assess the effectiveness of interventions prior to the completion of *in vivo* clinical trials. Despite extensive research and encouraging findings *in vitro*, these findings often do not translate into a human *in vivo* model. As such, we are not yet able to determine optimal interventions to attenuate muscle atrophy in response to aging, inactivity or in disease. The discord in results between *in vitro* and *in vivo* methodologies likely centers in the use of supra-physiological *in vitro* conditions. Improving the translatability between models and utilizing more physiological *in vitro* conditions not only increases the cost-effectiveness of conducting human research, but will aid a faster and more reliable transition between *in vitro* and *in vivo* research. Accordingly, the secondary aim of the *in vitro* methodology applied in this thesis was to utilize methods that would more accurately represent our aforementioned *in vivo* analysis techniques and thus improve the translatability of treatment interventions to future human experimental interventions.

1.8.1. Considerations for growth and differentiation protocols for the measurement and analysis of myotube protein metabolism.

Immortalised cell lines (i.e. C2C12) are commonly cultured in pre-supplemented Dulbecco's Modified Eagles Medium (DMEM) media containing high glucose concentrations (i.e. 25mM). During differentiation, the use of high-glucose media ensures that the high energetic demands are met for 48hrs, reducing the number of media changes in comparison to lower glucose cultured cells. Furthermore, there is an increased rate of myotube formation and thus greater myotube diameter when C2C12 were cultured in 25mM *vs.* 3.3mM glucose concentrations (286, 287). However, culturing in these supra-physiological conditions may also influence experimental outputs by inducing a state of hyperglycemia (288, 289) and promoting a reliance upon anaerobic glycolysis (290). For example, following exposure to chronic high-glucose conditions, myotube protein synthesis (MPS) rates were 2-fold greater than in cells exposed to low-glucose media (291). When stimulated with insulin (100 nmol/L) the high-glucose treated cells also displayed an impaired relative insulin stimulated MPS response *vs.* the low-glucose treated cells (291), indicating a basal level of 'anabolic resistance' in high-glucose cultured cells. Since MPS rates as well as the basal gene and protein expression of key anabolic signaling intermediates are altered in high-glucose cultured cells (292, 293), the results from experimental treatments applied to these cultures will likely not readily translate to *in vivo* research, due to a lack of representation of the proposed model of interest. Therefore, in in vitro utilizing more physiological glucose conditions (e.g. 5mM) are not only are more responsive to anabolic stimuli (291), but also hold a more oxidative phenotype (294). In comparison to high-glucose *in vitro* cultures, low-glucose cultured cells have been suggested to be more responsive to adenosine triphosphate provision (295) and have a higher maximum respiratory capacity (296), providing a more accurate representation of a 'healthy' cell model.

The second important component of culture media in the measurement of protein metabolism is the amino acid composition, which is vital to aid the development of myotubes (297, 298). Specifically, the non-essential amino acid, l-glutamine is vital for the growth and survival of cells in culture (299, 300), due to its important role in energy metabolism and ability to act as a precursor for protein and nucleic acid synthesis (301, 302). However, l-glutamine is unstable and can degrade quickly overtime, resulting in a build-up of ammonia (303, 304). The concentration of ammonia is an important mediator of MPS rates, since ammonia induces myostatin-mediated atrophy in mammalian myotubes (305), suggesting that the fast-degradation of l-glutamine could negatively impact MPS rates *in vitro*. Therefore, it may be important to reduce the degradation of l-glutamine and prevent basal pre-treatment cellular atrophy (independent of external treatment protocols). This approach can be achieved through the utilization of GlutaMAX^{TM}, which provides a more stable source of l-glutamine (as alanyl-glutamine and glycyl-glutamine) and results in a lower-amount of glutamine usage and ammonia production (306).

1.8.2. Considerations for the measurement and analysis of myotube protein metabolism.

The measurement of acute (i.e. MPS rates) and chronic (i.e. muscle morphology) alterations in skeletal muscle metabolism are crucial to the study of skeletal muscle atrophy. In human research, muscle fibre morphology is commonly determined through optimised immunofluorescence techniques (as in (307, 308)), whilst acute and chronic alterations in MyoPS rates are detected using stable isotope methodologies (217).

In vitro, chronic alterations in myotube growth and development are determined through the measurement of myotube diameter (309), an important consideration when determining the efficacy of a treatment intervention (310). Traditionally, myotube size has been calculated using measurements of average myotube diameter derived from multiple measurements along

the length of a myotube and acquired using grey-scale microscopic images (311). Whilst this technique is feasible, the irregular conformation of myotubes and the lack of cytoskeletal definition limits the ability of this technique to detect small, but potentially significant differences in myotube diameter (311). To overcome these limitations, immunofluorscene techniques can be utilized, with a fluorescent tagged cytoskeletal marker (e.g. desmin) and a nuclei identification marker (e.g. 4′,6-diamidino-2-phenylindole; DAPI (312, 313), demonstrated to provide a more accurate identification of myotubes and a greater definition of the cell boarder (311). These techniques provide an increased similarity to fibre CSA measurements *in vivo* and may enhance the translatability of *in vitro* experements.

In vivo, the use of deuterium water (D_2O) allows the quantification of integrated MyoPS rates in a range of chronic physiological states (219, 314, 315). Whereas, labelled amino acid tracers detect acute alterations in basal *vs.* stimulated (i.e. contractile or nutrient stimulus) MyoPS rates in response to ageing (316, 317) and musculoskeletal disuse (33-36). The distinction between basal, stimulated and/or integrated MPS rates is yet to be applied *in vitro*, where labelled amino acid tracers are typically used to detect alterations in the basal MPS response to pharmacological (e.g. (318-321)) and nutritional interventions (e.g. (319, 321, 322)). In contrast to human clinical research, where labelled amino acid tracers are capable of detecting very minor alterations in MPS responsiveness (217), questions remain regarding the specificity and sensitivity of these measurements *in vitro*. Unlike *in vivo* methodologies, the lack of perfusion *in vitro* requires the application of a flooding dose, leading to alterations in basal MPS rates dependent on the duration of incubation. For example, basal MPS rates measured using methyl[D_3]-^{13}C-methionine, significantly increased following a 6hrs incubation at 15% atom percent excess (320). However, following a 48hr incubation, FSR rates were significantly reduced (320). Interestingly, it was only at the 48hr time point where differences in the MPS rates in insulin growth factor 1 (IGF-1; a potent stimulator of MPS) and

dexamethasone (DEX; a potent MPS inhibitor) were noted (320), suggesting that labelled amino acid tracers may not be sensitive enough (following a flooding dose) to detect alterations in MPS rates without independently affecting myotube metabolism.

A feasible alternative to stable isotope tracers is the use of the Surface Sensing of Translation (SUnSET) Technique. The SUnSET technique, which utilises puromycin to determine protein synthesis rates, was originally developed from *in vivo* rodent research that a ^{3}H-puromycin tracer to demonstrated a low-protein diet significantly decreased the formation of peptidyl-puromycin, a marker of protein synthesis rates in various tissues including skeletal muscle (323). Since these initial findings, further validation experiments concluded that puromycin not only exclusively measures rates of translation initiation in skeletal muscle through labelling nascent polypeptides but also showed comparable results to [^{35}S]methionine and [^{35}S]cysteine in the sensitivity and range of MPS detection (324). Subsequently, the application an anti-puromycin antibody for the detection of puromycin-labelled proteins (the SUnSET technique) is widely utilised as a cost-effective, technically feasible technique to measure MPS rates *in vitro* (325) . Indeed, in fully differentiated myotubes, acute incubations in puromycin (typically 30mins to 1hr at 1μM) can be used to determine alterations in MPS rates in response to nutritional (e.g. (321, 326-330)) and pharmacological interventions (e.g. (321, 331-333)) that are comparable to those measured via labelled amino acid tracers (321). To further enhance the translatability of this measurement technique to *in vivo* physiology, where atrophy is underpinned by a resistance to anabolic stimuli (27, 35), the SUnSET technique can be utilized to measure both basal and acutely stimulated postprandial MPS rates. Previously, 1hr of leucine exposure (at 5,10 and 15mmol/L) has been suggested to enhance MPS rates in an *in vitro* model of atrophy (334). Since it is crucial to understand whether an intervention alters basal or postprandial MPS rates, this technique could provide further

information to aid translation *in vivo*, since anabolic resistance is a crucial factor underpinning muscle atrophy.

1.9. Specific thesis objectives and hypotheses

The overarching aims of the thesis were to enhance our understanding of (1) whether SR provides a great enough stimulus to promote alterations in skeletal muscle mitochondrial function (2) whether a high-dose leucine supplementation protocol can prevent disuse induced muscle atrophy in young healthy subjects during a short-term period of disuse, and (3) the mechanistic underpinnings as to whether EPI and it's colonic metabolite HA can offset metabolic alterations in a model of atrophy. **Chapter 2** describes a study where young healthy males underwent a 7d SR intervention. Given the importance of mitochondria to the negative physiological adaptations to disuse, the protein content and enzymatic activity of key markers of mitochondrial function and insulin signalling were measured. We hypothesised that 7d of SR in young males would (1) reduce the protein expression and enzymatic activity of key signalling intermediates involved in oxidative metabolism, (2) promote mitochondrial fission and (3) dampen the protein expression of markers involved in the maintenance of skeletal muscle insulin-sensitivity. **Chapter 3** describes an intervention where young healthy males underwent 7d of unilateral lower limb immobilisation, with or without 15g.day^{-1} leucine supplementation. Due to the current knowledge gaps surrounding the impact of disuse on mitochondrial function, MitoPS and rates of mitochondrial respiration were also measured. We hypothesized that applying a higher-dose leucine supplementation (15g.day^{-1}) than previous studies during 7d of lower-limb immobilization would effectively prevent disuse-induced muscle atrophy in young healthy males. Mechanistically, disuse-induced muscle atrophy in the absence of leucine supplementation would be underscored by impairments in MyoPS, MitoPS, associated signaling intermediates and respiratory function.

Chapter 4 describes an investigation in which we utilised physiologically relevant *in vitro* techniques to determine the effects of EPI and HA on skeletal muscle morphology, muscle protein turnover and mitochondrial metabolism within an *in vitro* model of skeletal muscle atrophy. Our hypotheses were twofold; (1) under atrophy-inducing conditions, concomitant treatment with EPI would prevent declines in myotube diameter through the preservation of MPS rates, a reduction in catabolic signalling and the preservation of mitochondrial metabolism and (2) under atrophy-inducing conditions, the colonic metabolite HA would mimic the actions of the natural flavanol EPI to preserve myotube diameter through similar regulatory mechanisms. **Chapter 5** provides a critical discussion and detailed insights of the findings described in Chapters 2-4 and provides an overview of the conclusions. Furthermore, the practical 'real world' implications of the three experimental chapters is discussed.

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2. SHORT-TERM STEP REDUCTION REDUCES CITRATE SYNTHASE ACTIVITY WITHOUT ALTERING SKELETAL MUSCLE MARKERS OF OXIDATIVE METABOLISM OR INSULIN-MEDIATED SIGNALLING IN YOUNG MALES.

Disclaimers

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Author declarations

B.J.S conducted the sample acquisition. S.J.E was involved in all aspects of the biochemical analysis of this research, Throughout the research, S.J.E was supported by; B.J.S, R.N.M, P.T.M, G.A.W and L.B.

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2.1. Abstract

Background: Mitochondria are critical to skeletal muscle contractile function and metabolic health. Short-term periods of step-reduction (SR) are associated with alterations in muscle protein turnover and mass. However, the effects of SR on muscle oxidative metabolism and insulin-mediated signalling are unclear.

Aim: We tested the hypothesis that the protein expression of key skeletal muscle markers of mitochondrial/oxidative metabolism and insulin-mediated signalling would be altered over 7d of SR in young healthy males.

Methods: Eleven, healthy, recreationally active males (Mean±SEM, age: 22±1yrs, BMI: 23.4±0.7 $kg.m^2$) underwent a 7d period of SR. Immediately prior to and following SR, fasted-state muscle biopsy samples were acquired and analysed for the protein expression of key markers of mitochondrial/oxidative metabolism and insulin-mediated signalling.

Results: Daily step count was significantly reduced during the SR intervention (13054±833 to 1192±99 $steps.d^{-1}$, $P<0.001$). Following SR there was a significant decline in citrate synthase activity (Fold change: 0.94±0.08, $P<0.05$) and a significant increase in the protein expression of p-glycogen synthase ($P\text{-}GS^{S641}$; Fold change: 1.47±0.14, $P<0.05$). No significant differences were observed in the protein expression of other key markers of insulin-mediated signalling, oxidative metabolism, mitochondrial function or mitochondrial dynamics (all $P>0.05$).

Conclusions: These results suggest that short-term SR reduces the activity of citrate synthase without altering the protein expression of key markers of skeletal muscle mitochondrial metabolism and insulin signalling in young healthy males.

2.2. Introduction

Musculoskeletal disuse occurs during illness (i.e., bed rest) and injury (i.e., limb immobilisation). In addition to these periods of severe disuse, periods of reduced ambulation also occur throughout the human lifespan in times of illness and injury. Periods of physical inactivity are accompanied by skeletal muscle atrophy (1-3), a decline in aerobic capacity (4) and a reduction in whole-body insulin sensitivity (5-7). However, the mechanisms underpinning these responses remain to be fully elucidated and this is having a meaningful impact on the development of therapeutic interventions to improve patient treatment and outcome.

Disuse atrophy is underpinned by alterations to muscle protein turnover, primarily attributed to reductions in myofibrillar protein synthesis rates (8-11). Recent evidence has also suggested that disuse atrophy is accompanied by alterations to mitochondrial metabolism and impaired aerobic capacity (12-16). Mitochondria are mechanically sensitive organelles (17) that are critical to contractile function (18), fuel utilisation and metabolic health (19, 20), which dictate aerobic capacity (21-23). Therefore, it is plausible that alterations in mitochondrial function during disuse may not only underpin reductions in aerobic capacity, but also contribute to muscle atrophy and impaired insulin-sensitivity. In models of more severe musculoskeletal disuse (e.g., bed rest/immobilisation), reductions in mitochondrial respiratory capacity (12), protein synthesis rates (11, 24) and oxidative phosphorylation (OXPHOS) complexes proteins (7, 12) have been noted in the first 14d of disuse (25, 26). Furthermore, mitochondria morphology is ultimately dependent upon the fine balance between rates of mitochondrial fission and fusion. Pre-clinical models suggest that disuse events are accompanied by alterations to mitochondrial dynamics (27), with the balance tilting towards mitochondrial fission (28) resulting in an increase of fragmented mitochondria (29-31). Although significant alterations in mitochondrial gene expression (e.g. COX7A2,

ATP5E, MRPS36) has been noted following two weeks of step reduction (SR) in overweight, older adults (32), whether short-term SR in young adults results in similar alterations to muscle mitochondrial metabolism as more severe, longer-term models of disuse has not yet been explored. Examining alterations in mitochondrial/oxidative metabolism across a short-term period of disuse is of increasing importance due to the average length hospital stay (and thus a period of significant step-reduction) in the UK is 5-6 days (33).

Disuse induced alterations in mitochondrial fragmentation and, thus, functioning have been implicated in the development of impaired insulin sensitivity, which is dampened during periods of severe disuse (5-7). Mitochondrial abnormalities are commonly observed in metabolically compromised patients (34) with an accompanying increase in reactive oxygen species (ROS) production, alterations in fuel utilisation and increases in mitochondrial fission often noted (35, 36). In pre-clinical models of severe disuse (i.e. hind limb unloading), mitochondrial dysfunction has been linked to alterations in fuel utilisation through a shift towards glycolysis (37), which may underpin changes in whole-body insulin sensitivity. Importantly, whole-body insulin sensitivity appears to be preserved in insulin resistant models following a decline in ROS generation (35). Although muscle mitochondrial dysfunction may precede reductions in insulin sensitivity during periods of severe disuse (i.e., bed rest/ immobilisation), this has yet to be investigated in the context of SR. Therefore, we aimed to determine the impact of 7d of SR on the expression of key skeletal muscle markers of mitochondrial/oxidative metabolism and insulin-mediated signalling in young, healthy males. Our hypothesis was that 7d SR in young males would (1) reduce the activity of oxidative enzymes (i.e., citrate synthase) (2) reduce the expression of key signalling intermediates involved in oxidative metabolism (e.g. OXPHOS complex proteins, AMPK, PGC1α), (3) promote mitochondrial fission (e.g., DRP1, FIS1, MFN2) and (4) dampen the

expression of markers involved in the maintenance of skeletal muscle insulin-sensitivity (e.g., GS, Akt).

2.3. Methods

2.3.1. Participants

The current study represents an extended retrospective analysis of a previously published study from our collective group (38). Eleven healthy, young males (Mean±SEM age: 22±1yrs; BMI: 23.4±0.7kg.m^2) completed 7d of SR. Prior to obtaining written informed consent, participants received oral and written information regarding the nature of the intervention and the possible risks of participation. All participants were deemed in good general health based on their responses to a general health questionnaire and were only excluded if they were diagnosed with existing health conditions (e.g., hypertension, diabetes), were a current smoker and/or were suffering from musculoskeletal injury. If deemed eligible, participants were provided with an ActivePAL3 ^{TM} (PAL Technologies Ltd., Glasgow, UK) to assess step count for the 7d prior to the step-reduction intervention. Participants that averaged <7,000 steps.d^{-1} were excluded from participation. Study approval was granted by the Research Ethics Service Committee West Midlands, Edgbaston, United Kingdom (Reference: 16/WM/0011) and the study was conducted in accordance to the Declaration of Helsinki.

2.3.2. Experimental design

The experimental design is outlined in Figure 2.1. Participants were included if they were non-competitive athletes who partook in 3x exercise sessions per week for the past 6 months. During the period of habitual physical activity and SR, participants were asked to complete 4d diet dairy and a standardized meal, containing 20% protein (~700kcal), was provided to participants on the evening before each experimental trial. Prior to the week of SR

participants were instructed to maintain their habitual physical activity levels for 7d and any participant walking <7,000 steps.day^{-1} was excluded from the SR intervention. Thereafter, participants were instructed to refrain from any structured physical activity and reduce their step count to ~1,500 steps.d^{-1} for the 7d period of SR. Activity was measured throughout the intervention using an ActivePAL3 ^{TM} accelerometer. During SR participants were provided with visual feedback on daily step count through a hip worn pedometer (Yamax Digi-Walker SW-200). Following the 7d period of habitual physical activity and again following the 7d period of SR, participants reported to the laboratory at 08:00hrs in an overnight fasted state, where a muscle biopsy was obtained from the middle portion of the vastus lateralis using a suction-adapted percutaneous needle biopsy technique under local anesthesia (1% lidocaine). Muscle samples were freed from any visible non-muscular material and rapidly frozen in liquid nitrogen before being stored at −80◦C for future analysis. A more compressive description of the experimental protocol can be found in our previous publication (38).

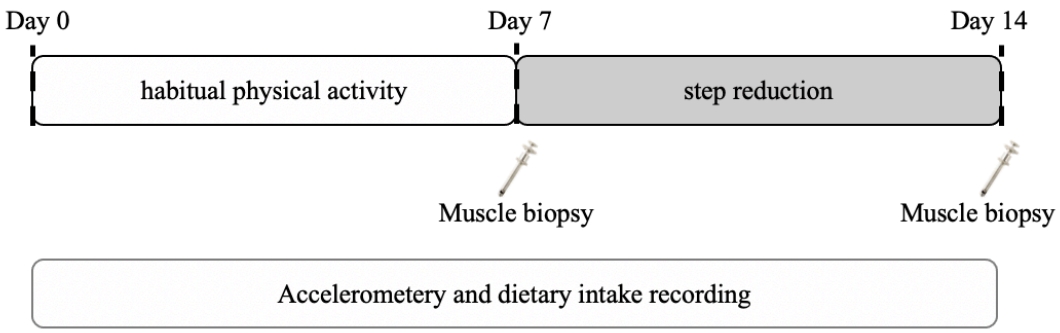

Figure 2.1 Experimental design schematic.

2.3.3. Western blotting

Snap-frozen muscle samples (~50mg) were manually homogenised on ice using a pestle in 10 µL of a standard extraction buffer per 1mg tissue. Samples underwent centrifugation at 2500g, 4°C for 5mins and the supernatant was removed for western blot analysis. Gels were loaded according to the protein concentration assessed by the DC protein assay (Bio-Rad. CA, USA), before western blot aliquots of 2µg/1µL were prepared in 4x laemmli sample buffer and ddH_2O. Prior to analysis, samples were left at room-temperature overnight to denature in order to maintain membrane integrity. Equal amounts of protein (18-30µg) were loaded onto Criterion™ TGX™ Precast Midi protein gels (Bio-rad) or homemade 12.5% protein gels and separated by SDS-PAGE at a constant voltage of 100V for 10mins and then 150V for 1hr. Protein samples were then transferred at a constant voltage (100V for 1hr) to a polyvinylidene difluoride (PVDF) or protran nitrocellulose membrane. The membranes were then incubated overnight at 4°C with a validated primary antibody; total OXPHOS human antibody cocktail (ab110411; 1:1000 in 5% BSA:TBST), citrate synthase (CS; CST143095 1:1000 in TBST), total acetyl-CoA carboxylase (ACC; CST3676 in TBST), p-ACCS79 (CST36615 in TBST), total 5' AMP-activated protein kinase (AMPKα; CST2757, 1:1000 in TBST), phospho-AMPKαT172 (CST2535, 1:1000 in TBST), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α; MM3248419, 1:1000 in 5% BSA:TBST), Calcium/calmodulin-dependent protein kinase type II (CAMKII; CST3362 1:500 in 5% BSA:TBST), PGC-1 and ERR-induced regulator in muscle protein 1 (PERM1; HPA031712, 1:500 in 5% BSA:TBST), mitochondrial transcription factor A (TFAM; SAB1401383 1:1000 in 5%BSA: TBST), nitric oxide synthase (NOS; AB76198, 1:1000 in 5%BSA: TBST), manganese superoxide dismutase (mnSOD; AB214675, 1:1000 in 5%BSA: TBST), p-DRP1S616 (CST4494, 1:1000 in TBST), total dynamin-related protein 1 (DRP1; CST5391, 1:1000 in TBST), mitofusin 2 (MFN2; CST143095, 1:1000 in TBST),

mitochondrial fission factor (MFF; CST84580, 1:1000 in TBST), p-MFFS176 (CST49281, 1:1,000 in TBST), total unc-51 like autophagy activating kinase (ULK1 CST4773, 1:1000 in 5% BSA: TBST), p-ULK1S555 (CST5869 1:1000 in 5% BSA: TBST), optic atrophy protein 1 (OPA1; BD Bioscience, 612607, 1:1000 in TBST),), mitochondrial fission 1 (FIS1; Atlas Antibodies, HPA017430, 1:1000 in TBST),), insulin receptor B (IR; CST23413, 1:1000 in 3%BSA:TBST), total insulin receptor substrate (IRS; CST2390, 1:1000 in 5% BSA: TBST), phosphoinositide 3-kinase (PI3K; CST4257, 1:1000 in 5% BSA: TBST), total protein kinase B (Akt; CST9272, 1:1000 in TBST), p-AktS473 (CST4060,1:1000 5% BSA in TBST), p-AktT308 (CST9275, 1:5000 in TBST), glucose transporter type 4 (GLUT4; CST2213, 1:1000 in TBST), total glycogen synthase kinase-3 (GSK3αβ; CST5676, 1:1000 in TBST), p-GSK3αβS21/9 (CST9331, 1:1000 in TBST), total glycogen synthase (GS; CST3886, 1:1000 in TBST), p-GSs641 (CST3886, 1:1000 in TBST). Samples were then washed 3X 5min in TBST before undergoing a 1hr incubation with a previously validated horseradish peroxidase (HRP)-linked anti-rabbit (CST7074, 1:10 000 in TBST) or anti-mouse (CST7076, 1:10 000 in 5% BSA:TBST) IgG. Thereafter, immobilon western chemiluminescent HRP substrate (Millipore) was used quantify protein content, visualised using a:BOX Chemi XT4 imager with GeneSys capture software (Syngene UK, Cambridge, UK). Quantification of bands was achieved using Chemi Genius Bioimaging Gel Doc System (Syngene, Cambridge, UK) and values were corrected to a loading control (ponceau). Where appropriate, the phosphorylation of proteins, as a proxy of their activation was expressed relative to the total amount of each protein. Data are presented as fold changes from the pre-SR condition.

2.3.4. Citrate synthase (CS) activity assay

CS enzyme activity was determined as previously described and adapted to 96-well microplate format for spectrophotometric analysis (39). Prior to measurement, sarcoplasmic homogenates

were prepped at a concentration of 2µg/µL ddH_2O. CS reaction buffer (50mM KPI buffer pH 7.4, 100µM DTNB and 115µM Acetyl CoA in ddH_2O) and spectrophotometer were warmed to 30°C for optimal enzymatic reactions. For baseline measurements, 10µL (20µg protein) of sample and 186µL of warm reaction buffer was pipetted into a 96-well microplate, with a single participant measured at a time in triplicate. Baseline absorbance was read every 15s for 3mins at 412nm in a microplate reader (FLUOstar Omega, BMG Labtech, Aylesbury, UK). Immediately following this baseline measurement, 4µL of oxaloacetate (100µM final concentration) was added to each well to initiate the reaction before the plate was returned to the spectrophotometer and read again every 15s for 3min at 412nm, to measure the rate of thionitrobenzoate anion (TNB) appearance. The protocol has previously been validated (39) and enzyme activity was calculated as: The Δ Absorbance/min × 1,000)/[(extinction coefficient × volume of sample used in ml) × (sample protein concentration in $mg.ml^{-1}$)]. The average enzyme activity across 3 replicates was taken forward for analysis. The within-plate coefficient variation of the three technical replicates was 3.51 ± 2.51%, and within the assay's acceptable range, as previously reported (40).

2.3.5. Statistics

Data are presented as mean± SEM. Statistical assumptions were checked prior to analysis and analysis was performed using SPSS statistics version 25 (IBM corp.). Measures of protein expression and enzymatic activity were assessed using a paired samples T-Test (pre-SR *vs.* post-SR). Missing data was not imputed and n numbers for each analysis are reported in figure legends. The level of significance was considered $P \leq 0.05$.

2.4. Results

2.4.1. Mitochondrial function

The expression of key proteins of mitochondrial function following 7d of SR can be viewed in Figure 2.2A. No significant alterations were noted following SR in OXPHOS CI (fold change, pre *vs.* post: 0.87±0.19, P=0.492), OXPHOS CII (1.00±0.08, P=0.938), OXPHOS CIII (fold change, pre *vs.* post: 0.87±0.21, P=0.534), OXPHOS CIV (fold change, pre *vs.* post: 1.01±0.16, P=0.935), OXPHOS CV (fold change, pre *vs.* post: 1.01±0.08, P=0.873), TOTAL OXPHOS (fold change, pre *vs.* post: 0.98±0.09, P=0.790) and CS (fold change, pre *vs.* post: 0.91±0.08, P=0.267). In contrast, CS activity significantly reduced following a 7d period of SR (Figure 2.2C; fold change, pre *vs.* post: 0.94±0.08, P=0.012).

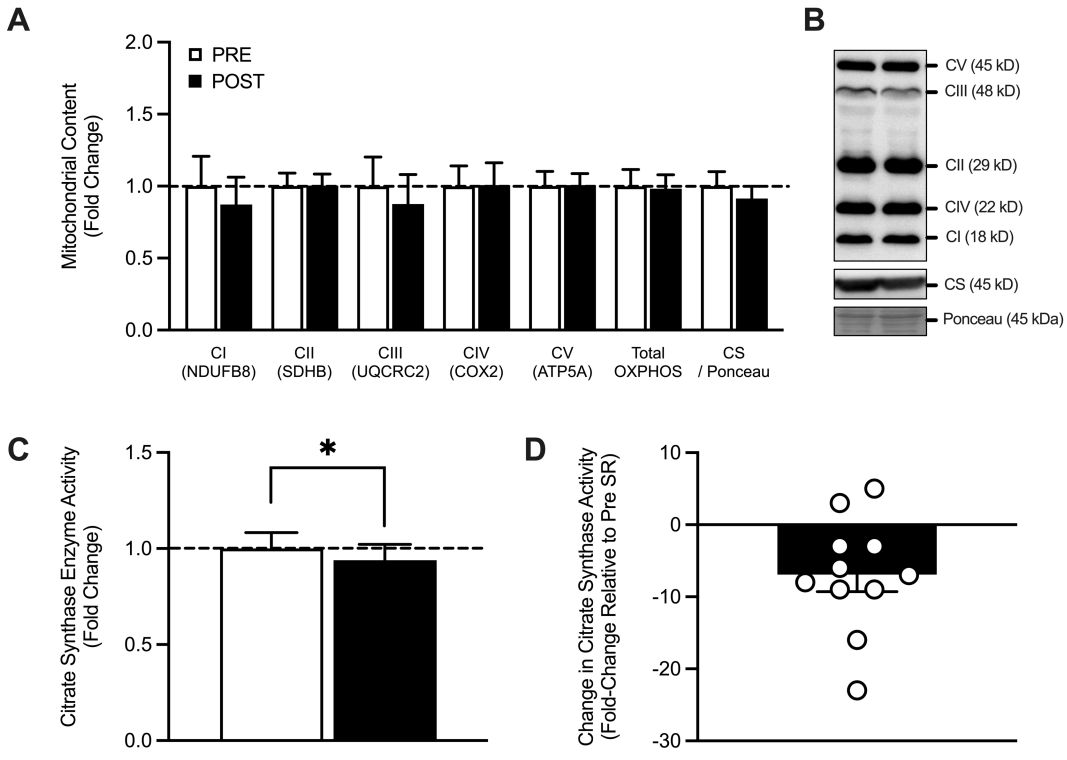

Figure 2.2 Mitochondrial function in response to 7d SR in young adults.

(A) OXPHOS CI, OXPHOS CII, OXPHOS CIII, OXPHOS CIV, OXPHOS CV, total OXPHOS (*n*=11) and Citrate Synthase (*n*=11), (B) western blot representative image, (C-D) Citrate synthase activity assay (*n*=11),. Data are presented as mean ± SEM and were analysed

using a repeated measures T-TEST. * Represents post-SR was significantly different from pre-SR at the $P<0.05$ level. Pre-SR values are noted by white bars; Post-SR values are noted by black bars

2.4.2. Oxidative metabolism

Expression of key markers of oxidative metabolism and oxidative stress can be seen in figure 2.3A and 2.3B, respectively. There were no significant differences in the expression of PCG1α (fold change, pre *vs.* post: 0.92±0.17, P=0.514), PERM1 (fold change, pre *vs.* post: 0.83±0.19, P=0.074), CAMKII (fold change, pre *vs.* post: 1.00±0.08, P=0.845) or TFAM (fold change, pre *vs.* post: 0.96±0.07, p=0.265) following 7d SR. Furthermore, the activation of AMPKα^{T172} (fold change, pre *vs.* post: 0.92±0.18, P=0.597) and ACC^{S79} (fold change, pre *vs.* post: 0.93±0.14, P=0.523) was not significantly different following the SR intervention. Finally, there were no significant differences in the expression of mnSOD (fold change, pre *vs.* post: 0.97±0.22, P=0.840) or NOS (fold change, pre *vs.* post: 1.01±0.09, P=0.942) following 7d of SR.

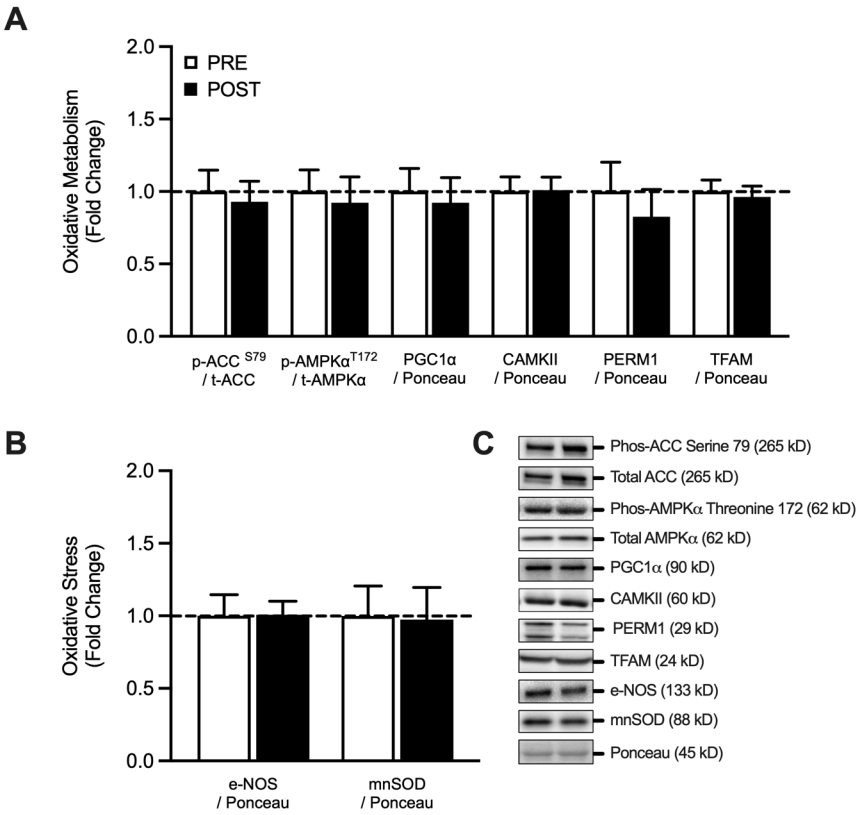

Figure 2.3 Oxidative metabolism in response to 7d SR in young adults.

(A) Oxidative metabolism protein expression (*n*=11), (B) protein expression for markers of muscular oxidative stress (*n*=11) and, (C) western blot representative image of oxidative metabolism and oxidative stress markers. Data are presented as mean ± SEM and were analysed using a repeated measures T-TEST. * Represents post-SR was significantly different from pre-SR at the $P<0.05$ level. ACC; acetyl-CoA carboxylase, AMPKα; 5' AMP-activated protein kinase, PGC1α; peroxisome proliferator-activated receptor gamma coactivator 1-alpha, NOS; nitric oxide synthase, mnSOD; manganese superoxide dismutase. Pre-SR values are noted by white bars; Post-SR values are noted by black bars

2.4.3. Mitochondrial dynamics

In response to 7d of SR, no significant differences were noted in the expression or activation of proteins involved in mitochondrial fission or fusion (Figure 2.4A**)**. Specifically, the

expression of FIS1 (fold change: pre *vs.* post: 1.04±0.19, P=0.516), MFF (fold change: pre *vs.* post, 1.42±0.34, P=0.152), MFN2 (fold change, pre *vs.* post: 0.98± 0.28, P=0.923) and OPA1 (fold change, pre *vs.* post: 0.92±0.15, P=0.329) remained unchanged following SR. Likewise, the phosphorylation of DRP^{S616} (fold change, pre *vs.* post: 1.19±0.16, P=0.223) and ULK1^{S555} (fold change, pre *vs.* post: 0.96±0.13, P=0.829) were not significantly different following 7d of SR. Finally, the ratio of MFN to t-DRP1 (fold change, pre *vs.* post:1.16±0.22, P=0.141) remained unchanged.

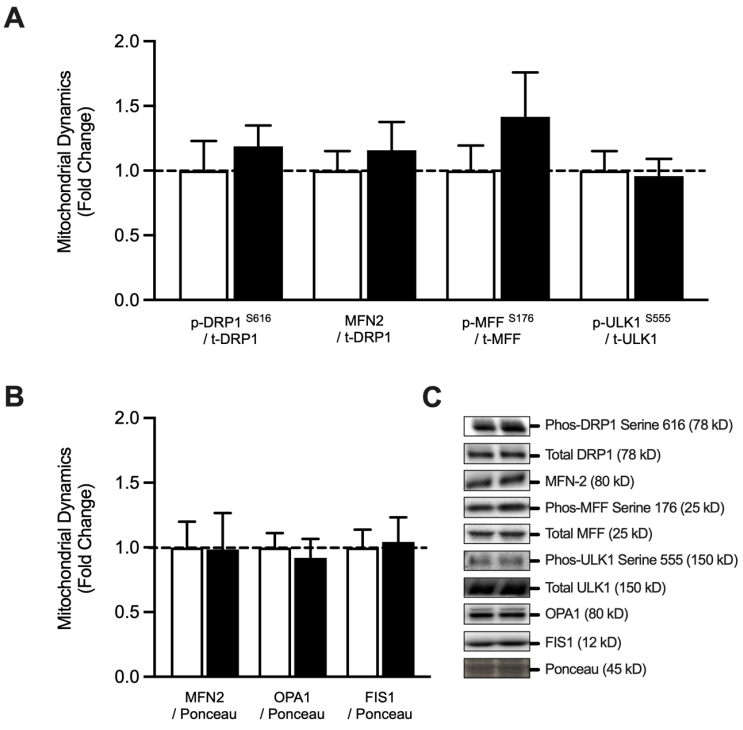

Figure 2.4 . Mitochondrial dynamics in response to 7d SR in young adults.

(A-B) protein expression of protein involved in mitochondrial dynamics (n=11), (C) western blot representative image of mitochondrial dynamic markers. Data are presented as mean ± SEM and were analysed using a repeated measures T-TEST. * Represents post-SR was significantly different from pre-SR at the P<0.05 level. DRP1; dynamin-related protein 1, FIS1; Mitochondrial fission 1, MFF; Mitochondrial fission factor, MFN2; Mitofusin 2, OPA1; dynamin-like 120 kDa protein, ULK1; unc-51 like autophagy activating kinase. Pre-SR values are noted by white bars; Post-SR values are noted by black bars

2.4.4. Glucose metabolism

Alterations of key markers of skeletal muscle glucose metabolism are presented in **Figure 5.** In response to 7d of SR, there was a significant increase in the phosphorylation of GS^{S641} (fold change, pre *vs.* post: 1.47±0.14, P=0.012). There were no further significant differences noted in the phosphorylation of $GSK3\alpha\beta^{S21/9}$ (fold change, pre *vs.* post: 0.97±0.04, P=0.486), AKT^{S473} (fold change, pre *vs.* post: 0.92±0.13, P=0.520) or AKT^{T308} (fold change, pre *vs.* post: 0.84±0.06, P=0.161) following the 7d intervention. Similarly, the expression of IR (fold change, pre *vs.* post: 0.97±0.17, P=0.882), IRS (fold change, pre *vs.* post: 0.96±0.17, P=0.738), PI3K (fold change, pre *vs.* post: 0.94±0.13, P=0.278) and GLUT4 (fold change, pre *vs.* post: 0.93±0.23, P=0.558) remained unchanged following 7d of SR.

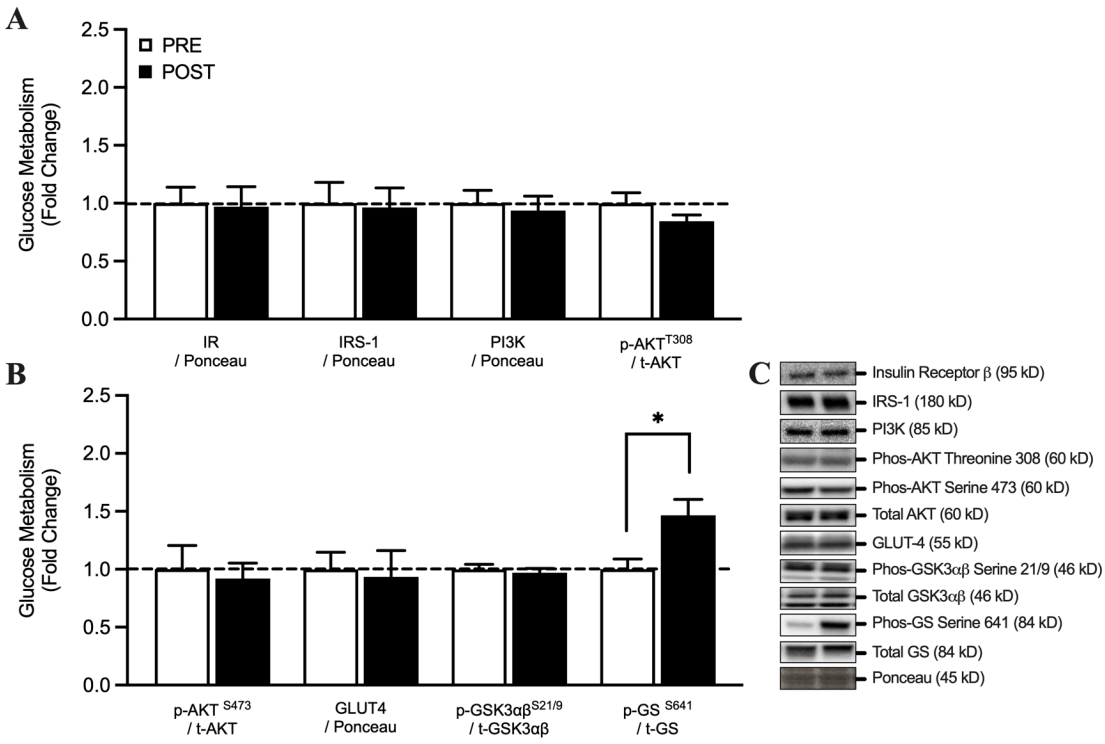

Figure 2.5 Skeletal muscle glucose metabolism in response to 7d SR in young males.

(A-B) protein expression of markers of skeletal muscle glucose metabolism (*n*=11), (C) western blot representative image of skeletal muscle glucose metabolism markers. Data are presented as mean ± SEM and were analysed using a repeated measures T-TEST. * Represents post-SR was significantly different from pre-SR at the $P<0.05$ level. AKT; protein kinase B, GLUT4; glucose transporter 4, GS; glycogen synthase, GSK3αβ; glycogen synthase kinase-3αβ, IR; insulin receptor, IRS; insulin receptor substrate, PI3K; phosphoinositide 3-kinase. Pre-SR values are noted by white bars; Post-SR values are noted by black bars

2.5. Discussion

We report that in response to 7d of SR in young, healthy males there was a significant decline in citrate synthase (CS) activity. Despite this alteration in CS activity, no significant declines in the expression of markers of mitochondrial function (e.g., OXPHOS complex I-V), oxidative metabolism (e.g., PGC1α, AMPKα) or mitochondrial dynamics (e.g., FIS1, DRP1, MFN2) were noted in response to 7d SR. Due to the purported link between

alterations in mitochondrial metabolism and fuel utilisation during musculoskeletal disuse, we also examined the expression of proteins involved in skeletal muscle insulin sensitivity. We found a significant increase in p-GS^{S641}/t-GS in response to 7d SR. However, no additional changes in markers of insulin sensitivity (e.g., IR, AKT, GLUT4) were noted.

Previous work in severe models of musculoskeletal disuse (i.e., bed rest/limb immobilisation) in young healthy individuals has demonstrated a significant decline in CS activity (7, 25, 41, 42), which is accompanied by declines in the expression of CS and the OXPHOS complex proteins (7, 11, 25, 43). Despite previous evidence of compromised mitochondrial function following a short-term period (7-14d) of severe musculoskeletal disuse, whether the reduced loading and energetic demand of 7d of SR would alter mitochondrial functioning was unknown. Here, we report for the first time that a significant decline in CS activity occurred following 7d of SR, which was not accompanied by alterations in the expression of CS or OXPHOS CI-V. The reduction in CS activity is perhaps unsurprising as CS is an important regulator of the citric acid cycle and is inhibited under conditions of a high-energy supply (44). During periods of SR, there is a reduction in contractile activity and likely a subsequent reduction in the requirement for ATP synthesis. Indeed, it is likely that energy intake may exceed skeletal muscle energetic demand throughout the period of reduced ambulation, resulting in the 'underutilisation' of ATP. Since high [ATP] allosterically inhibits CS (45), it is plausible that the reduction in contractile activity (and thus ATP usage) would reduce the saturation of this enzyme with acetyl CoA and subsequently dampen its activity. Interestingly, and in contrast to our hypotheses, any shift in energy utilisation and enzymatic activity was not severe enough to promote alterations in the expression of CS or OXHPOS CI-V over 7d of SR. This finding is also in contrast to previous literature in models of bed rest/limb immobilisation (11, 25, 43) and suggests that the complete removal of contractile stimuli and potentially more drastic

alterations in physical activity may be required to alter protein content in a young population over a 7d period. Due to the retrospective sample analysis of a larger experimental trial, and thus lack of fresh tissue for analysis, we were unable to determine the OXPHOS respiratory capacity, which remains a limitation of these data and provides rationale for further research to measure intramuscular ATP and general energy utilization across disuse models to fully resolve this question. Nevertheless, our data does provide novel insights into the compensatory declines in CS enzymatic activity following as little as 7d SR in young adults, without associated alterations in the expression of key markers of mitochondrial function.

The complete removal of contractile stimuli (e.g., bed rest) has been shown to trigger a cascade of alterations in the expression of key signalling intermediates of oxidative metabolism (11, 16, 46, 47), ultimately leading to a reduced rate of mitochondrial synthesis (11, 24). Here, we hypothesised that SR would adversely affect the expression of key signalling proteins involved in oxidative metabolism (e.g., AMPKα, PGC1α, ACC, TFAM) and Ca^{2+} handling (e.g., CAMKII, PERM1). In contrast to previous studies (11, 16, 46, 47), we reported no alterations in the total expression or phosphorylation of proteins involved in oxidative metabolism. Similarly, there was no significant difference in the CAMKII or PERM1 protein expression, which contrasted with previous reports in catabolic conditions (48, 49). Taken together, these novel data suggest that any alterations in [Ca^{2+}] and [AMP:ATP] that may occur during 7d of SR may not be severe enough to potentiate alterations in mitochondrial biogenesis, whereas complete removal of contractile activity may instigate such adverse metabolic responses, at least in young healthy individuals.

Alterations in oxidative metabolism and Ca^{2+} handling as a result of musculoskeletal disuse (50-52) are linked to increases in ROS production, through the stimulation of the citric acid cycle and subsequent activation of ROS generating enzymes (53, 54). Our data may suggest that 7d of SR does not provide a robust enough ‘unloading stimulus’ to significantly

alter these parameters, and in combination with the reduction in CS activity, may explain why we did not detect any significant alterations in MnSOD or NOS expression following the 7d of SR. Furthermore, the generation of ROS following musculoskeletal disuse has been putatively linked to myofibrillar protein imbalance and the subsequent onset of muscle atrophy (27). However, following the 7d period of SR in the current intervention, declines in muscle protein synthesis in combination with increased gene expression of catabolic signalling targets (see (38) for these previously published data), occurred independently of alterations in markers of mitochondrial function and oxidative stress. Taken together, these data lead us to speculate that with 7d of SR in young individuals, alterations in myofibrillar protein turnover occur independently from alterations in the abundance of mitochondrial proteins.

Mitochondrial morphology is dependent on rates of mitochondrial fusion and fission (27). Pre-clinical models suggest a shift in mitochondrial dynamics towards fission (28) following a period of immobilisation, resulting in an increase of fragmented mitochondria (29-31). However, this is yet to be consistently demonstrated in human disuse studies. Here, we report no significant alterations in markers of mitochondrial fission or fusion following 7d of SR in young healthy males. Alterations in mitochondrial dynamics occur in response to cellular stress (55), thus the lack of significant changes noted in the expression of markers of mitochondrial dynamics herein, further suggests that 7d of SR does not significantly impact on cellular energy homeostasis. This finding is in line with previous work (46), in which no differences in the content of mitochondrial fission or fusion proteins was reported following 10d of best rest. These data shed further light on the discrepant alterations in mitochondrial dynamics between animal and human models of disuse atrophy.

The lack of alterations in mitochondrial metabolism and energy homeostasis may explain why we did not observe any alterations in the expression of signalling intermediates

of skeletal muscle glucose uptake (i.e., IR, IRS, Akt, GLUT4) following 7d of SR. However, we did note a significant increase in P-GS^{S641}/t-GS following the intervention, which is a marker of reduced GS activation. In response to muscular contraction, there is a reduction in GS phosphorylation, promoting an increase in GS activity (56), so it is perhaps unsurprising that a reduction in contractile activity promoted an increase in GS phosphorylation. This finding may also explain, in part, the mechanisms underpinning the significant decline in whole-body insulin sensitivity noted in this cohort of participants following the 7d SR intervention (see (38) for additional data). Importantly, a decline in glycogen content is key to maintaining skeletal muscle insulin sensitivity. Since a high-glycogen content reduces GS activity, it is possible that the increase in GS phosphorylation (which can reduce GS activity) noted here, may represent a protective mechanism to maintain insulin sensitivity towards homeostatic levels during a period of reduced ambulation (57). The regulation of GS activity is dependent on various kinases including GSK3, CAMKII and AMPK (58). However, we did not note any significant differences in the protein content or phosphorylation of these signalling targets, suggesting that, at least in the current cohort, alterations in GS activity occurred independently to alterations in oxidative metabolism and ATP synthesis.

Despite the novelty of the current research, there are limitations to the experimental protocol. Firstly, we were unable to assess mitochondrial respiratory capacity and the activity of OXPHOS complexes due to the requirement of fresh/permeabilised tissue (e.g. oroboros) and technical failures (e.g. additional enzymatic assays). Therefore, although this study provides significant mechanistic insight into mitochondrial functioning following short-term SR, whether alterations in ATP production accompany the reduction in citrate synthase activity (noted in Figure 2.2) remains to be determined. Secondly, this research was conducted utilizing males only, reducing the applicability of this research to a real world

population and further research should aim to determine whether gender discrepancies exist in mitochondrial metabolism in response to 7d SR.

In conclusion, 7d of SR in young males caused significant declines in CS activity, independent to alterations in the expression of key markers involved in mitochondrial function, oxidative metabolism and mitochondrial dynamics. Furthermore, following the 7d SR intervention, there was a significant increase in the phosphorylation of GS, which occurred independently to additional alterations in the expression of markers involved in glucose uptake. These data provide a further resolution to suggest declines in myofibrillar protein synthesis, demonstrated in our previous publication, in response to 7d SR occur independently to alterations to the expression of key markers involved in oxidative protein metabolism and glucose uptake in young healthy males.

2.6. References

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3. HIGH-DOSE LEUCINE SUPPLEMENTATION DOES NOT PREVENT ATROPHY OR STRENGTH LOSS OVER 7 DAYS OF IMMOBILISATION IN HEALTHY YOUNG MALES.

Disclaimers

The authors have no competing interests to declare related to this study. This data is previously published (DOI: https://doi.org/10.1093/ajcn/nqaa229) and taken in verbatim with the inclusion of the supplementary information.

Clinical trial registration number

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Authorship statement

S.J.E was involved in all aspects of this study, with the exception of the measurement of fibre CSA, where D.L lead the analysis. Throughout the research, S.J.E was supported by: Benoit Smeuninx, James Mckendry, Yusuke Nishimura, Dan Luo, Ryan N Marshall, Molly Perkins, Jill Ramsay, Sophie Joanisse, Andrew Philp, Leigh Breen.

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3.1. Abstract

BACKGROUND: Unavoidable periods of disuse lead to muscle atrophy and functional decline. Preventing such declines can reduce the risk of re-injury and improve recovery of normal physiological functioning.

OBJECTIVE: To determine the effectiveness of high-dose leucine supplementation on muscle morphology, strength and mechanisms of myofibrillar (MyoPS) and mitochondria (MitoPS) protein synthesis during 7d of unilateral lower-limb immobilization.

DESIGN: Sixteen, healthy males (23±1yrs) underwent 7d of unilateral lower-limb immobilization, with thrice daily leucine (LEU; *n*=8) or placebo (PLA; *n*=8) supplementation ($15g \cdot d^{-1}$). Prior to and following immobilization, muscle strength and compartmental tissue composition were assessed. A primed continuous infusion of L-[ring-$^{13}C_6$]-phenylalanine with serial muscle biopsies was used to determine postabsorptive and postprandial (20g milk protein) MyoPS and MitoPS, fibre morphology, markers of protein turnover and mitochondrial function between the control (CTL) and immobilized (IMB) leg.

RESULTS: Leg fat-free mass was reduced in IMB (-3.6±0.5%; *P*=0.030) but not CTL with no difference between supplementation groups. Isometric knee extensor strength declined to a greater extent in IMB (-27.9±4.4%) *vs.* CTL (-14.3±4.4%; *P*=0.043) with no difference between groups. In response to 20g milk protein, postprandial MyoPS rates were significantly lower in IMB *vs.* CTL (-22±4%; *P*<0.01) in both LEU and PLA. Postabsorptive MyoPS rates did not differ between legs or groups. Postabsorptive MitoPS rates were significantly lower in IMB *vs.* CTL (-14±5%; *P*<0.01) and postprandial MitoPS rates significantly declined in response to 20g milk protein ingestion (CTL; -10±8% and IMB; -15±10%, *P*=0.039), with no differences between legs or group. There were no significant differences in measures of mitochondrial respiration between legs, but PGC1α and OXPHOS

complex II and III were significantly lower in IMB *vs.* CTL ($P<0.05$), with no differences between groups.

CONCLUSIONS: High-dose leucine supplementation ($15g \cdot d^{-1}$) does not appear to attenuate any functional declines associated with 7d of limb immobilization in young, healthy males.

3.2. Introduction

Unavoidable periods of physical inactivity during injury (e.g. limb-immobilization) or illness (e.g. bed rest) result in a decline of skeletal muscle mass and strength, that appears to be most rapid during the early phase of unloading (1, 2). Eliminating this deconditioning is important to expedite recovery to normal physical functioning following injury/illness in otherwise healthy individuals.

Disuse-induced muscle atrophy is driven by declines in postabsorptive and postprandial (3-5) rates of myofibrillar protein synthesis (MyoPS) in response to amino acid/protein provision ('anabolic resistance'), which may stem from alterations in mammalian target of rapamycin (mTOR) regulatory signalling (6). Alterations in mitochondrial morphology are also suggested to accompany disuse-induced atrophy (7-10), with significant declines in integrated rates of mitochondrial protein synthesis (MitoPS) observed during 14d of unilateral immobilization (11). Since mitochondria are mechanically sensitive (12) and critical to contractile function (13), the development of nutritional strategies to offset the disuse-induced dysregulation of MyoPS and MitoPS is of fundamental importance.

The branched-chain amino acid, leucine, robustly enhances protein translation (14) and subsequently postprandial MyoPS (15-19) through mTOR signalling activation (20). Amino acid supplementation also provides some substrate for oxidative metabolism (21, 22), with increased amino acid plasma concentrations suggested to increase the maximal

adenosine triphosphate (ATP) production rate (23) and adenosine 5'-diphosphate (ADP) stimulated respiration in young, lean individuals (24). The leucine metabolite beta-hydroxy-beta-methylbutyrate (HMB) has also been demonstrated to mitigate impairments in oxidative metabolism and mitochondrial function during short-term disuse (25). Consequently, in the absence of contractile stimulus, leucine may provide a viable strategy to attenuate disuse-induced muscle atrophy, through acting as an anabolic trigger and substrate to maintain rates of postprandial MyoPS and oxidative metabolism.

In younger adults, leucine supplementation of 2.5 $g \cdot meal^{-1}$ was unable to attenuate the loss of muscle and function following short-term disuse (26), whereas much higher leucine doses (0.06g/kg/meal) partially spared disuse-induced muscle and strength loss in middle-aged and older adults (27, 28). Since $5g \cdot meal^{-1}$ of leucine supplementation has been demonstrated to enhance integrated MyoPS rates in free-living conditions, independent of contractile activity (29), we hypothesized that applying the same higher-dose leucine supplementation during 7d of lower-limb immobilization would effectively prevent disuse-induced muscle atrophy in young healthy males. Mechanistically, disuse-induced muscle atrophy in the absence of leucine supplementation would be underscored by impairments in MyoPS, MitoPS, associated signaling intermediates and respiratory function.

3.3. Methods

3.3.1. Participants

Sixteen healthy young males (23 ± 1yrs, 23.0 ± 0.6$kg \cdot m^{-2}$; values are mean ± SEM) participated in this double-blinded, parallel-group, randomised controlled intervention. Participants characteristics are presented in Table 3.1.

Table 3.1 Baseline characteristics of the sixteen young, healthy males who underwent 7d of unilateral leg immobilization with (LEU) or without (PLA) 15g.d^{-1} leucine supplementation

	PLA (n=8)	LEU (n=8)	*P* value
Age (yrs)	23±1	22±1	0.64
Height (m)	1.82±0.04	1.75±0.03	0.21
Weight (kg)	74.2±4.7	73.0±2.7	0.83
BMI (kg·m^{-2})	22.3±0.9	23.8±0.83	0.24
Whole body fat mass (kg)	13.74±1.36	14.05±2.08	0.90
Whole body fat free mass (kg)	56.20±3.43	53.56±17.40	0.51
Habitual step count (steps·d^{-1})	8203±1091	8461±1295	0.88
Isometric KE strength at 70° (N)	672±76	651±57	0.91
Isokinetic KE strength at 60°·s^{-1} (Nm)	147±18	149±11	0.83

Values are displayed as mean ± SEM. There was an n=8 for each group. Data were analysed using an independent t-test (LEU vs. PLA). LEU, 15g·d^{-1} leucine supplementation during 7d immobilization; PLA, 15g·d^{-1} of placebo supplementation (non-essential amino acid mix) during 7d immobilization; BMI, body mass index; Isometric and isokinetic knee extensor (KE) strength is presented from the non-immobilized limb. Significance was set at P<0.05.

The study was registered at clinicaltrails.gov (NCT03762278, ID RG_16-206). All participants were deemed in good general health and were only excluded if they had any known health problems (e.g. heart disease, rheumatoid arthritis, diabetes, poor lung function,

uncontrolled hypertension, lactose intolerance), a family history of thrombosis and/or were taking medications known to affect protein metabolism. Prior to obtaining written, informed consent, participants received oral and written information regarding the nature of the intervention and the possible risks of participation. Study approval was granted by the NHS East Midlands Derby Research Ethics Committee (REC: 17/EM/0086) and conducted in accordance with the Declaration of Helsinki 1975 as revised in 2013. For subject flow through the protocol, see the CONSORT diagram in Figure 3.1.

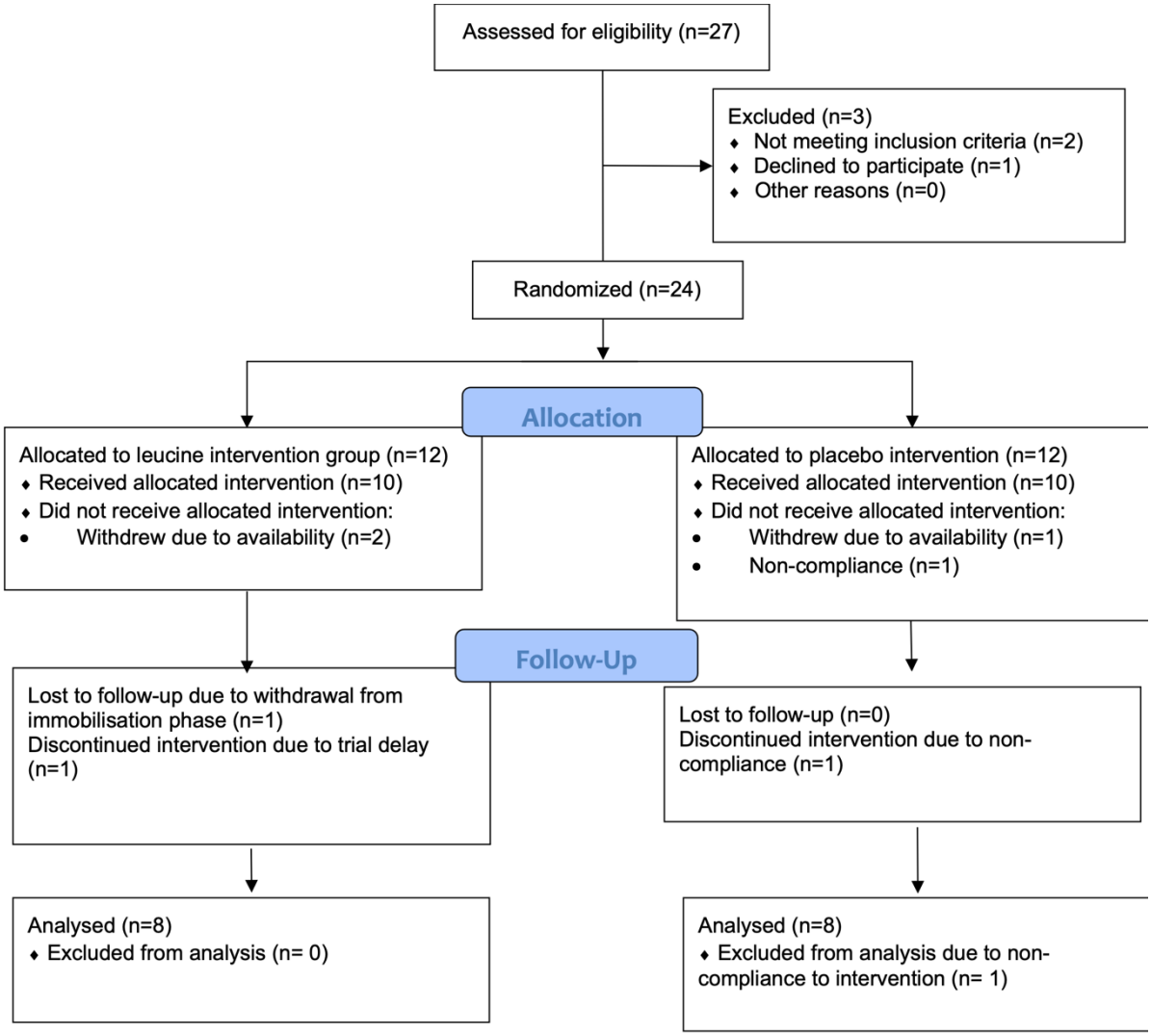

Figure 3.1 CONSORT flow diagram

3.3.2. Experimental design

The experimental protocol is outlined in Figure 3.2. Briefly, the experiment consisted of two pre-intervention and two post-intervention visits to the laboratory at the same time of day (±2hrs) for assessments of body composition and strength separated by 7d of lower-limb unilateral immobilization. During the 7d intervention, all food was provided by the research team. Dietary intake was controlled according to an estimation of the resting energy expenditure and energy requirements (activity factor of 1.3; (29)). This value was then checked against the habitual energy intake (measured via a 3d diet diary) to ensure no large deviations in energy intake occurred. The total protein intake was $1.0 g \cdot kg^{-1} \cdot d^{-1}$ provided in a skewed distribution in the ratio of 20:30:40:10% for breakfast, lunch, dinner, snacks, respectively. Participants were randomized on enrollment to receive either leucine (LEU; *n=8*) or placebo (PLA; *n*=8) supplementation ($15 g \cdot d^{-1}$). Participants were asked to consume their supplemental treatment with 250ml of water alongside each main meal (i.e. 3x/d). The LEU and PLA beverages were obtained from INFINIT nutrition (Ontario, Canada) and the composition of the supplementation beverages is noted in Table 3.2. Step-count was assessed for 3d prior to and throughout immobilization using a hip-worn pedometer (Omron Healthcare Co., Ltd, Kyoto, Japan) placed on the control (CTL) limb. Immediately following immobilization muscle biopsies and blood samples were obtained during a primed-continuous stable isotope amino acid infusion to determine muscle fibre morphology, postabsorptive and postprandial MyoPS and MitoPS, as well as key indicators of muscle anabolism, catabolism and mitochondrial function between the immobilized (IMB) and CTL limbs. For the experimental visit (infusion/biopsy trial), participants reported to the laboratory following a 10h overnight fast and having refrained from any vigorous physical activity and alcohol intake for the previous 72h.

Table 3.2 Contents of nutritional supplements consumed three times daily with 250ml water during the 7d immobilization period.

	PLA	LEU
Leucine (g)	0	5
Alanine (g)	4.5	0
Glycine (g)	1.5	0
Sucrose (g)	7.0	7.0
Maltodextrin (g)	3.0	3.0
Stevia/ Citric acid/ Flavour (g)	1.7	1.7

LEU, 15g·d^{-1} leucine supplementation during 7d immobilization; PLA, 15g·d^{-1} placebo (non-essential amino acids) during 7d immobilization.

3.3.3. Preliminary testing

Participants initially underwent measurements of height and body mass, using a stadiometer and digital scale. One day prior to immobilization, a dual-energy x-ray absorptiometry (DXA; Discovery DXA Systems, Hologic Inc., Bedford, MA, US) scan was also conducted to determine regional and whole-body composition (fat and fat-free mass) as previously described (30). Briefly, to limit the precision error associated with DXA (31), the scanner was calibrated prior to each scan. Participants were instructed to report to the laboratory following an overnight fast, having not completed any vigorous physical activity or consumed any alcohol for the previous 72 h and having refined from liquid intake for the 30

min prior to measurement. Levels of physical activity and dietary intake were assessed for the 3d prior to the first DXA measurement and throughout the intervention, increasing the confidence that these conditions were adhered to. Four-days prior to immobilization maximal knee extensor strength was assessed using isometric (70° flexion) and isokinetic (60°/s) dynamometry for both the CTL and IMB limb (KinCom, Chattex Corp., Chattanooga, TN). After familiarization using two sub-maximal voluntary contractions of increasing effort, participants performed two maximal voluntary contractions, the average of which was recorded. However, if values were >10% different, the maximum value was accepted.

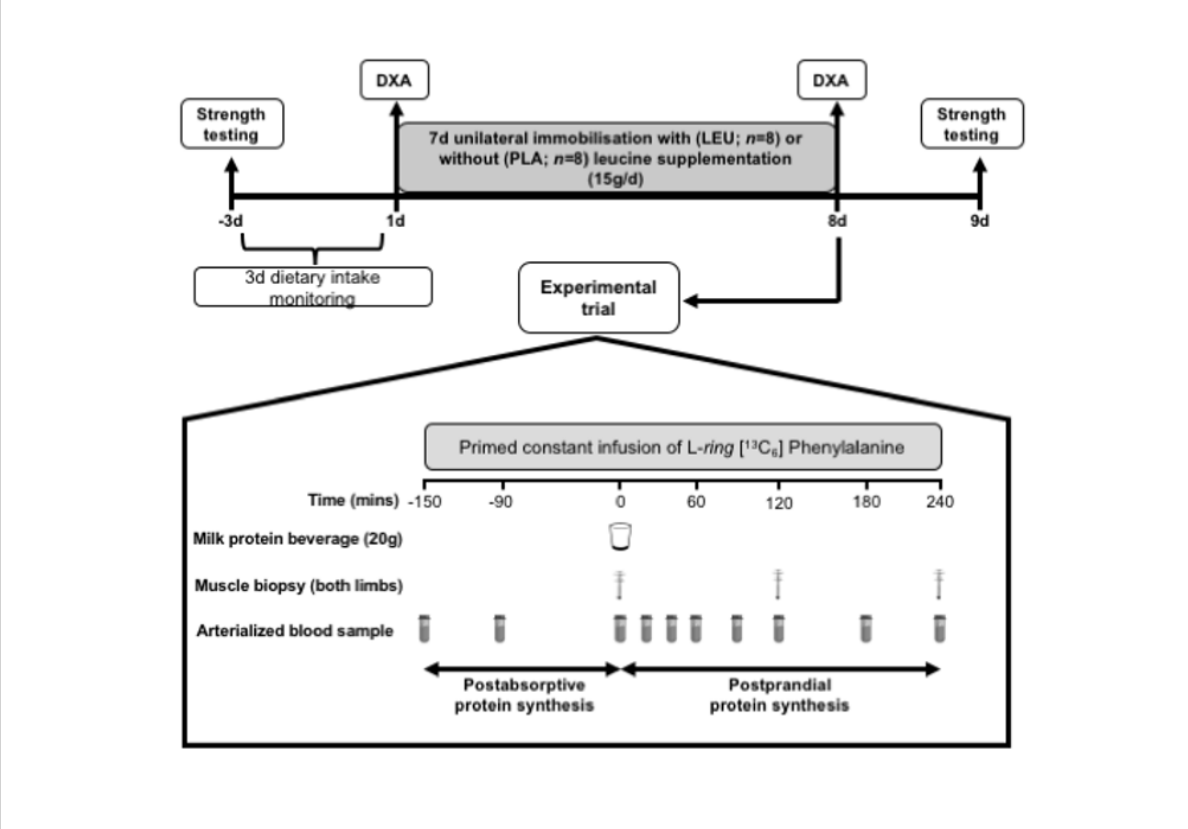

Figure 3.2 Schematic representation of the experimental design.

DXA; dual x ray absorptiometry.

3.3.4. Unilateral limb immobilization

Unilateral immobilization was conducted by means of a knee brace (DonJoy Telescoping IROM, DJO Global, California, US) set at a 30° angle of flexion and covered in anti-tamper tape. The leg to be immobilized was randomly selected and counterbalanced (left/right). During immobilization participants used crutches to ambulate freely and participants were encouraged to maintain their pre-intervention step-count, as best they could. In order to continue daily living activities, participants were provided with a waterproof shower cover and instructed to sleep with a pillow between their legs in order to ensure the brace was not removed for the entirety of the intervention. All participants were instructed to perform ankle exercises five times daily (including plantar/dorsiflexion and medial/lateral rotation) to minimize the risk of deep vein thrombosis.

3.3.5. Experimental trial

At 06:00h on the morning following immobilization (i.e. Day 8), participants arrived at the laboratory following an overnight fast. Upon arrival, DXA measurements were repeated as outlined above. Thereafter a 21-gauge cannula was inserted into a forearm vein of both arms for a continuous infusion of L-[ring-$^{13}C_6$]-phenylalanine (prime, 2μmol·kg^{-1}; infusion, 0.05μmol·kg^{-1}·min^{-1}; Cambridge Isotope Laboratories, Andover, MA, USA) and frequent arterialised blood sampling prior-to and following the consumption of milk protein (My Protein, NY, US) beverage (Figure 1). Arterialised blood samples were obtained by warming the contralateral arm to ~60°C, as described previously (32). At each sampling time-point, 10-ml of arterialized blood was collected in serum and EDTA-separator vacutainers, before being centrifuged and stored as previously described (33). Following ~150-mins of stable isotope tracer infusion, a muscle biopsy sample was acquired from the middle-portion of the

vastus lateralis of IMB and CTL legs using a suction-adapted percutaneous needle biopsy technique (34) under local anaesthesia (1% lidocaine). Muscle samples were freed from any visible non-muscular material and either rapidly frozen in liquid nitrogen and stored at -80°C for future analysis or placed in an ice-cold relaxation and biopsy preservation buffer (BIOPS) for high resolution respirometry. Immediately following the first biopsy, participants were given 20g of milk protein dissolved in 300mL of water and flavoured to the participant's preference (MyProtein Milk Isolate, Cheshire, UK). A small amount of L-[ring-$^{13}C_6$]-phenylalanine tracer was added to the drink (enriched to ~4%) to ensure constant enrichment following milk protein ingestion. Two- and four-hours following milk protein ingestion, further muscle biopsies were obtained from IMB and CTL. Muscle biopsies were obtained from separate incisions in a distal-to-proximal orientation, spaced ~3cm apart. Using basal fasted plasma values in combination with pre- and 4h post-feeding biopsies, both postabsorptive and postprandial MyoPS and MitoPS were determined. Before leaving the laboratory, participants were provided with a standardised lunch and were instructed to continue their standardized meal plans for the remainder of the day. For participant comfort, the following morning (08:00-09:00am), leg strength assessments were repeated in an identical manner to pre-intervention testing.

3.3.6. Blood analysis

Plasma leucine phenylalanine and plasma L- [ring-$^{13}C_6$] phenylalanine enrichment was determined by gas chromatography-mass spectrometry (GCMS; model 5973; Hewlett Packard, Palo Alto, CA, USA) by monitoring ions 234/240. Briefly, 100µL of plasma was diluted with 200µL 1:1 acetic acid and purified on cation-exchange columns before being dried down under nitrogen at 70°C. The purified amino acids were then converted to their N-*tert*-butyldimethyl-silyl-*N*-methyltrifluoracetamide (MTBSTFA) derivative. Phenylalanine

concentrations were determined by GCMS using an internal standard method. The internal standard U-[$^{13}C_9$-^{15}N] phenylalanine (ions 336/346) was added to the sample at a quantity of 6µL. Serum insulin concentrations were measured using commercially available enzyme-linked immunosorbent assay kits (R&D Systems, Inc. Abingdon, UK).

3.3.7. Myofibrillar and mitochondrial protein enrichment

Muscle samples collected from CTL and IMB were analyzed for L-[ring-$^{13}C_6$]-phenylalanine enrichment in both the myofibrillar and mitochondrial enriched fractions. To isolate the myofibrillar and mitochondrial enriched fractions, ~80-100mg of tissue was rapidly homogenised using clean, sharp scissors in 10µL.mg^{-1} of tissue of homogenisation buffer (50mM Tris-HCL, 1mM EDTA, 1mM EGTA, 10mM B-glycerophosphate, 50mM NaF, 0.5mM Sodium Orthovanadate and a single EDTA-free protease inhibitor per 10mL of buffer). Samples were placed on a Vibrax shaker for 10-minutes at room temperature at a speed of 1000rpm before being centrifuged at 11,000g, 4°C for 15mins to pellet the myofibrillar and mitochondrial fractions. The pellet was washed twice and supernatant (sarcoplasmic fraction) stored for western blot analysis. To separate the myofibrillar and mitochondrial fractions, the pellet was re-suspended in mitochondrial extraction buffer (MEB; 20mM MOPS, 110mM KCl, 1mM EGTA), transferred to a glass dounce homogenizer with a tight-fitting glass pestle and manually homogenised 12 times. The resulting homogenate was centrifuged at 1,000g, 4°C for 5mins to pellet the myofibrillar enriched fraction, before the supernatant was collected and centrifuged at 11,000g, 4°C for 15mins to pellet the mitochondrial enriched fraction. The myofibrillar enriched pellet was combined with 750µL of 0.3NaOH and incubated at 37°C for 30mins, then centrifuged at 13,000rpm, 4°C for 30mins before the solubilised myofibrillar proteins (the supernatant) was transferred to a fresh boiling tube. The pellet was then re-washed in 750µL of 0.3NaOH, centrifuged as

above and resultant supernatant combined in a fresh boiling tube. Myofibrillar proteins were precipitated through the addition of 1mL 1M PCA and centrifugation at 3200rpm, 4°C for 20mins. The resulting pellet was then washed twice with 2mL 70% ethanol and centrifuged at 3200rpm, 4°C for 20-mins. Both the resulting myofibrillar pellet as well as the mitochondrial pellet were then combined with 0.1M HCL and 1mL of Dowex slurry and left to hydrolyse at 110°C overnight. The next morning the amino acids were then purified on cation-exchange columns before being converted to their N-acetyl-n-propyl ester (NAP) derivative. The ^{13}C /^{12}C ratio of the phenylalanine NAP derivative was determined using a Thermo Delta V isotope ratio mass spectrometer (IRMS).

3.3.8. Intracellular protein signalling

Intracellular protein signalling was assessed by western blot analysis on the sarcoplasmic protein fraction obtained during myofibrillar and mitochondrial protein isolation. Gels were loaded according to the sarcoplasmic protein concentration assessed by the DC protein assay (Bio-Rad, Hertfordshire, UK), before western blot aliquots of 3µg/1µL were prepared in 4x laemmli sample buffer and ddH_2O. Samples were then either immediately taken forward for analysis (OXPHOS), left at room temperature for 24hrs (other mitochondrial proteins) or boiled for 5mins (myofibrillar proteins). Equal amounts of protein (9-30µg) were loaded into 12.5-15% gels and separated by SDS-PAGE for ~1hr at 23mA/gel. Next, protein samples were transferred to a biotrace nitrocellulose membrane (NC; Pall Laboratory, Portsmouth, U.K.) or polyvinylidene difluoride (PVDF; Whatman, Dassel, Germany) membrane at 100V for 1hr. The membranes were then incubated overnight (4°C) with appropriate primary antibodies; total OXPHOS human antibody cocktail (ab110411, diluted 1:1000 in tris-buffered saline with 0.1% Tween 20 (TBST), NC)), muscle ring finger protein 1(MuRF1; sc-398608, 1:1000 in TBST, PVDF), Peroxisome proliferator-activated receptor gamma

coactivator 1-alpha (PGC-1α; ab3242, 1:1000 in TBST, PVDF), total 70 kDa S6 protein kinase (p70S6K1; CST9202, 1:1000 TBST, PVDF), phospho-p70S6K1^{T389} (CST9205, 1:500 5% bovine serum albumin (BSA) in TBST, PVDF), total eukaryotic initiation factor 4E binding protein 1 (4EBP1; CST9452, 1:1000 in TBST, NC), phospho-4E-BP1$^{T37/46}$ (CST9459, 1:500 5%BSA in TBST, NC), total protein kinase B (Akt; CST9272, 1:1000 in TBST, NC), phospho-Akt^{S473} (CST3787, 1:1000 5% BSA in TBST, NC), total AMP activated protein kinase alpha (AMPKα: CST5831, 1:1000 in TBST, PVDF), Phospho-AMPKα^{T172} (CST2535, 1:500 5% BSA in TBST, PVDF), LC3a/b isoform I (LC3ab I; CST12741, 1:1000 in TBST, PVDF), and Caspase-3 (CST9665, 1:500 in 5% BSA in TBST, PVDF). Samples were then incubated for 60-min with an HRP-linked anti-rabbit (CST7074, 1:10000 in TBST) or anti-mouse (CST7076, 1:10000 TBST) IgG. Following IgG binding, immobilon western chemiluminescent HRP substrate (Millipore, Watford, UK) was used to quantify protein content, visualised using a:BOX Chemi XT4 imager with GeneSys capture software (Syngene UK, Cambridge, UK). Band quantification was achieved using a Chemi Genius Bioimaging Gel Doc System (Syngene, Cambridge, UK). Values were corrected to a gel control in the first instance before being corrected to the loading control (ponceau). Following these corrections, where relevant the phosphorylation of proteins, as a proxy of their activation was expressed relative to the total amount of each protein.

3.3.9. Muscle fibre type and CSA

Muscle fibre type and CSA were analysed from biopsy tissue taken prior to feeding, where ~20mg of tissue was placed in Tissue Tek OCT compound and frozen in liquid nitrogen-cooled isopentane utilising a previously published protocol (35, 36). Sections were incubated with Triton X 100 (at 0.02% concentration in 1×PBS) for 5 min and then blocked in 5% normal goat serum (Invitrogen, UK) in 1× PBS for 90 min at room temperature. Sections

were incubated with MHCI (IgG2b, BAF8, DSHB, Iowa, US) and MHCII (IgG1, SC.71) mixed primary antibodies (in 1×PBS) at room temperature overnight. The following day, sections were washed for 3 × 5 min in 1×PBS-T and incubated in secondary antibodies and WGA mixed solutions in 1×PBS for 90min at room temperature. Following secondary antibody incubation, sections were then washed 2 × 5 min with PBS-T and 1 × 5 min with PBS and left to dry. During quantification, all images were obtained with the 20× objective magnification. Quantification of muscle fibre type was performed for type I and type II fibre types, which was obtained manually by using image J Fiji software. On average, approximately 8-10 images were captured per section. Throughout the muscle sections of CTRL and IMMOB groups, an average of 154 ± 13 muscle fibres per section were selected to quantify muscle fibre distribution and muscle CSA.

3.3.10. High Resolution Respirometry

Samples acquired from the pre-feeding biopsy in IMB and CTL were placed in BIOPS (2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 5.77 mM Na_2ATP, 6.56 mM $MgCl_2$-$6H_2O$, 20 mM Taurine, 15 mM Na_2Phosphocreatine, 20 mM Imidazole, 0.5 mM Dithiothreitol, 50 mM MES Hydrate, pH 7.1) and separated into myofiber bundles before permeabilization for 30-mins at 4°C in a BIOPS buffer containing 50µM/ml saponin. Samples (~3mg) were then transferred to an Oxygraph-2k respirometer (Oxygraph 2-K, Oroboros Instruments Corp, Innsbruck, Austria) and analysed in duplicate on the day of collection according to protocols described previously (13). Briefly, complex I linked leak respiration (L_I) was achieved through the titration of 5mM pyruvate,10mM glutamate and 2mM malate. The addition of 5mM ADP coupled the electron transfer to phosphorylation, assessing complex I linked respiration (P_I). Thereafter, maximal phosphorylating respiration through complexes I and II (P_{I+II}) was induced via the addition of 10mM succinate. To control for the outer membrane

integrity cytochrome C (10μM) was added, with samples that demonstrated a significant response (>10% change) excluded from analysis. Next, the titration of 0.5μM carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) uncoupled oxidative phosphorylation in order to assess the maximal electron transfer capacity (E). Finally, non-mitochondrial respiration was inferred through the addition of 5μM antimycin A. All values were measured per mg of weight muscle and corrected for non-mitochondrial respiration.

3.4. Calculations

Myofibrillar and mitochondrial protein synthesis rates were calculated from L-[ring-$^{13}C_6$]-phenylalanine enrichment through the use of the standard precursor-product model:

$$FSR(\%.h^{-1}) = \Delta E_b/E_p \text{ x } 1/t \text{ x } 100$$

ΔE_b describes the difference in bound $^{13}C_6$-phenylalanine enrichment between two biopsies, E_p describes the mean plasma precursor enrichment and t is the time (mins) between muscle biopsies. Postabsorptive MyoPS rates were calculated in the rested/fasted state using the pre-infusion plasma $^{13}C_6$-phenylalanine enrichment as a proxy for postabsorptive muscle protein enrichment, a method that we (33) and others (37) have used previously.

3.4.1. Outcome measures

The primary outcome measure was the change in leg fat-free mass in response to 7d of immobilization. Alterations in isometric and isokinetic strength in response to the 7d intervention as well as Type I and Type II fibre CSA, postabsorptive and postprandial (0-4hrs) MyoPS and MitoPS, mitochondrial respiration, mTOR signalling and markers of oxidative metabolism between the CTL and IMB limbs following 7d of immobilisation were secondary outcome measures. Plasma leucine, plasma phenylalanine, serum insulin and plasma L-[ring-$^{13}C_6$]-phenylalanine enrichment acted as tertiary outcome measures.

3.4.2. Statistical analysis

Data analysis was performed using SPSS version 25 (IBM Corp., USA) and normality checked using GraphPad Prism version 8 (GraphPad Software), all data is presented as mean ± SEM. Baseline participant characteristics were analysed using an independent t-test. Step count was analysed using a two-way mixed model ANOVA, with a single within-group (time; *pre-immobilization vs. during immobilization*) and a single between groups factor (*LEU vs. PLA*). Isometric strength, isokinetic strength and regional body composition were analysed using a three-way mixed model ANOVA, with two within-group factors (time; *pre- vs. post-* and leg; *IMB vs CTL*) and a single between groups factor (*LEU vs. PLA*). Mitochondrial and myofibrillar protein synthesis rates were analysed using a three-way mixed model ANOVA, with two within-group factors (time; *postabsorptive vs. postprandial (0-4h)* and leg; *IMB vs CTL*) and a single between groups factor (*LEU vs. PLA*). The change in protein synthesis rates from postabsorptive to postprandial was calculated and then analysed using a two-way mixed model ANOVA with a single within-group (*CTL vs. IMB*) and between-groups *(LEU vs. PLA*) factor. Markers of mitochondrial function, fibre type and CSA were analysed using a two-way mixed model ANOVA with a single within-group (*CTL vs. IMB*) and between-groups *(LEU vs. PLA*) factor. Intramuscular protein signalling was assessed using a three-way mixed model ANOVA, with two within-group factors (time; *0h vs. 2h vs 4h* and leg; *IMB vs CTL*) and a single between groups factor (*LEU vs. PLA*). Finally, serum insulin, plasma phenylalanine and plasma leucine concentrations were analysed using a two-way mixed model ANOVA with a single within-group (time; *multiple time points during infusion*) and a single between-groups factor (group; *LEU vs. PLA*). A power calculation was performed based on previous data (27, 38) with differences in leg fat-free mass as the primary outcome measure. A power analysis with an error = 0.05, power= 0.8

and Cohen's F effect size= 0.49 was performed using G*power version 3.1 analysis software (Heinrich Hein University, Düsseldorf, Germany) based on the differences in the fat free mass loss between leucine and placebo groups following disuse (27). This produced a minimum sample size of *n*=8 per group to detect a within-group mean loss of 300g in DXA-derived leg fat free mass from pre-to-post immobilization, and a between-group interaction effect (assuming leucine would prevent leg fat-free mass loss). Assumptions for statistical analysis were checked, with the Greenhouse-Geisser correction factor applied if a significant Mauchley's test was noted. For all relevant values, normality was tested using the D'Agostino-Pearson test and if significant at the $P<0.05$ level then data were transformed with the square root of the value. However, non-transformed data is presented in graphical format to enhance clarity. Missing data were not imputed into analysis and *n* numbers for all statistical analysis are reported in figure legends. Where appropriate, Bonferroni's post-hoc test was used to isolate specific differences whenever a is significant interaction or main effect was identified and the results of Bonferroni's post-hoc test are reported herein. The level of significance was considered $P<0.05$.

3.5. Results

3.5.1. Physical Activity and Dietary Intake

Step count significantly reduced during the 7d intervention (8332 ± 818 to 4675 ± 401 steps/d; $P<0.01$), with no differences between groups. During the intervention, independent of supplementation, diet was controlled at: 1.0 ± 0.0, 3.5 ± 0.1, 1.3 ± 0.0 $g \cdot kg^{-1} \cdot d^{-1}$ of protein, carbohydrate and fat, respectively. Inclusive of supplementation, protein intake was 1.2 ± 0.0 $g \cdot kg^{-1} \cdot d^{-1}$ for both groups.

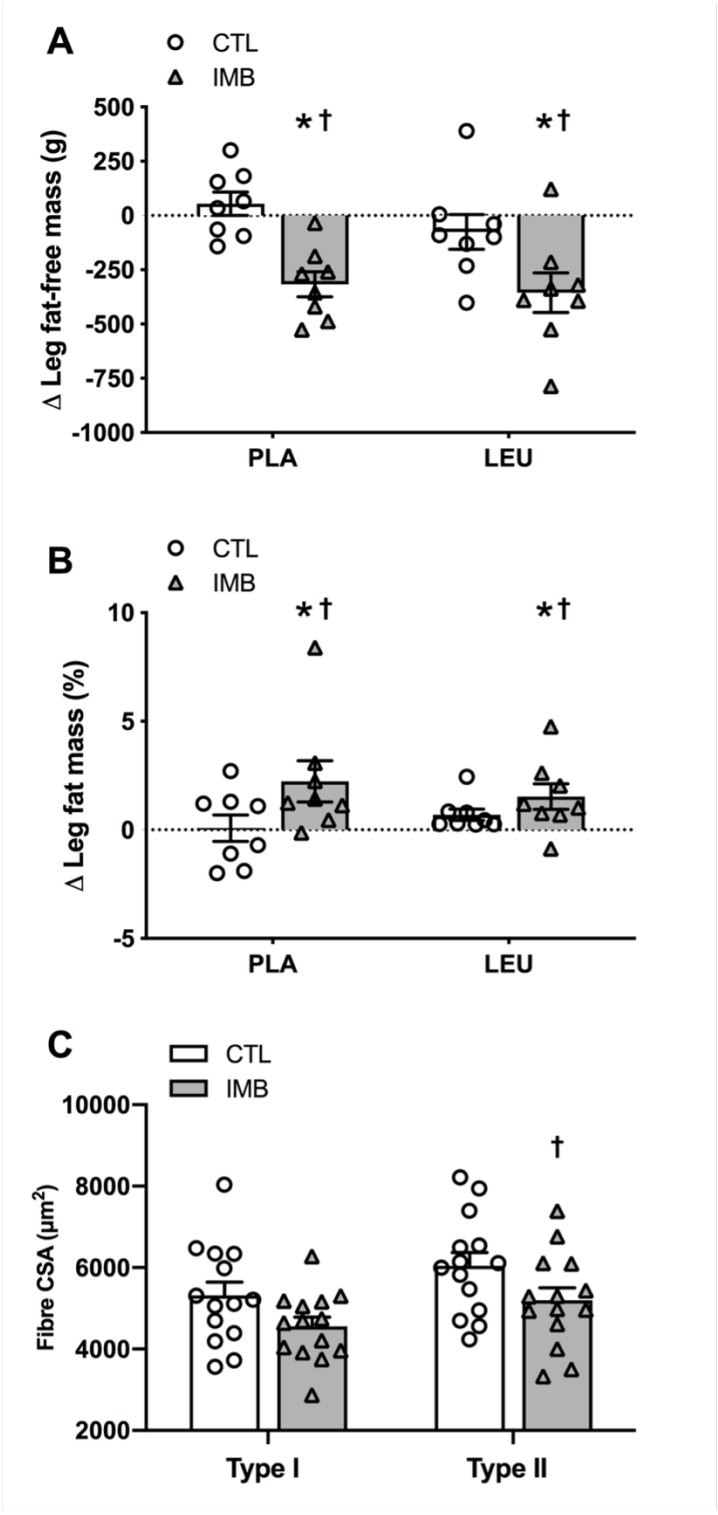

Figure 3.3 Changes in leg fat free mass (A), leg fat mass percentage (B) and leg fibre cross sectional area (C) in response to 7d of immobilization in young males.

Data are presented as mean ± SEM. Data for changes in leg fat free mass and leg fat mass percentage was analysed using a three-way mixed model ANOVA, with *n*=8 in both PLA and LEU

groups. Data for fibre CSA were analysed using a two-way mixed model ANOVA, with *n*=7 in LEU and *n*=8 in PLA. Significance was set at *P*=0.05. * Represents a significant change from pre-intervention to post-intervention; † represents a significant difference between IMB and CTL; CTL limb is detailed by white bars; IMB limb is detailed by grey bars. LEU, $15g \cdot d^{-1}$ leucine supplementation during 7d immobilization; PLA, $15g \cdot d^{-1}$ placebo (non-essential amino acids) during 7d immobilization; CTL, non-immobilized limb; IMB, immobilized limb**.**

3.5.2. Anthropometric and Morphological Assessments

Following 7d of immobilization, leg fat free mass (Figure 3.3A) was significantly reduced in IMB (-3.6 ± 0.5%; *P*=0.030), but not CTL (-0.6 ± 0.5%; *P*=0.970) with no difference between supplement groups (*P*=0.642). Conversely, the percentage of leg fat mass (Figure 3.3B) significantly increased in IMB (1.0 ± 0.4%; *P*=0.017), but not CTL (0.1 ± 0.3%; *P*=0.819) following immobilization, with no difference between supplement groups (*P*=0.540). Type II fibre CSA (Figure 3.3C) was significantly lower in IMB *vs.* CTL (-11± 5%; *P*=0.028), whereas Type I fibre CSA was not (-9 ± 5%; *P*>0.05), with no differences between supplement groups (*P*>0.05).

3.5.3. Leg Strength Assessments

Isometric knee extensor strength (Figure 3.4A) declined following immobilization in IMB (-27.9 ± 4.4%) and CTL (-14.3 ± 4.4%; *P*<0.01 for both), with a significantly greater decline in the IMB *vs.* CTL limb (*P*=0.043) that was the same between supplementation groups (*P*=0.852). Isokinetic knee extensor strength (Figure 3.4B) declined following immobilization in IMB (-28 ± 5%) and CTL (-23 ± 4%; *P*<0.01) with no difference between limbs (*P*=0.982) or supplement groups (*P*=0.888).

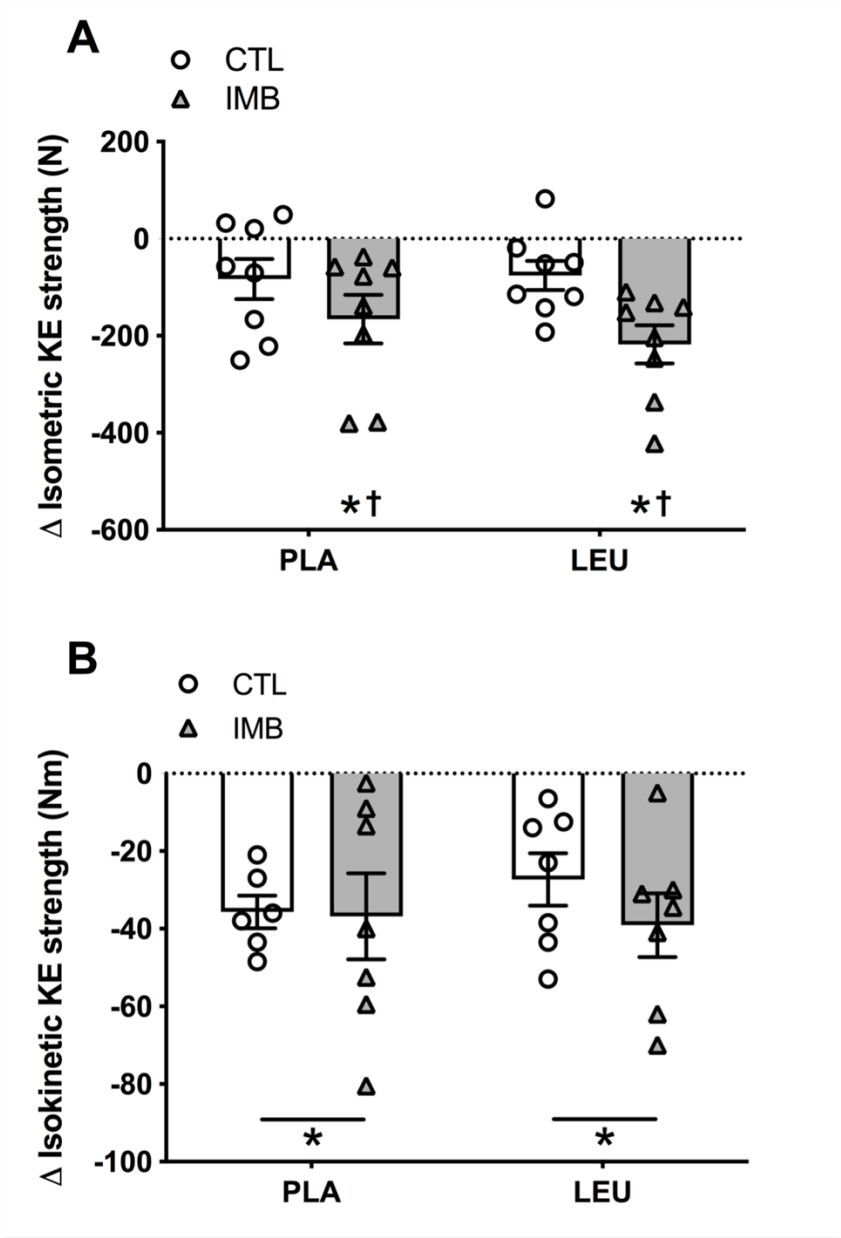

Figure 3.4 Changes in isometric (A) and isokinetic (B) strength in response to a 7d period of immobilization in young males.

Data are presented as mean ± SEM. Data were analysed using a three-way mixed model ANOVA. For measures of isometric strength *n*=8 in the LEU and PLA groups. In measures of isokinetic strength *n*=6 in PLA and *n*=7 in LEU. Significance was set at *P*=0.05. * Represents a significant change from pre-intervention to post-intervention; † represents a significant difference between IMB and CTL; CTL limb is detailed by white bars; IMB limb is detailed by grey bars. LEU, 15g·d-

[1] leucine supplementation during 7d immobilization; PLA, 15g·d^{-1} placebo (non-essential amino acids) during 7d immobilization; CTL, non-immobilized limb; IMB, immobilized limb.

3.5.4. Blood Analyses

Plasma leucine concentrations (Figure 3.5A) were increased compared to postabsorptive (t=-150mins) values from 20-90 mins (all $P<0.05$) following ingestion of 20g of milk protein. Similarly, plasma phenylalanine concentrations (Figure 3.5B) were increased above postabsorptive (t=-150mins) values between 20-60 min post-ingestion of 20g of milk protein (all $P<0.01$). L-[ring-$^{13}C_6$]-phenylalanine enrichments (Figure 3.5D) were elevated above baseline values (t=-150mins) from -90-240 mins of tracer infusion ($P<0.01$). Serum insulin concentrations (Figure 3.5C) were elevated above postabsorptive (t=0 mins) values at 20 min post-ingestion of 20g of milk protein ($P<0.01$). There were no between group differences in measures of plasma leucine, plasma phenylalanine, serum insulin or L-[ring-$^{13}C_6$]-phenylalanine enrichments throughout the period of infusion (all $P>0.05$).

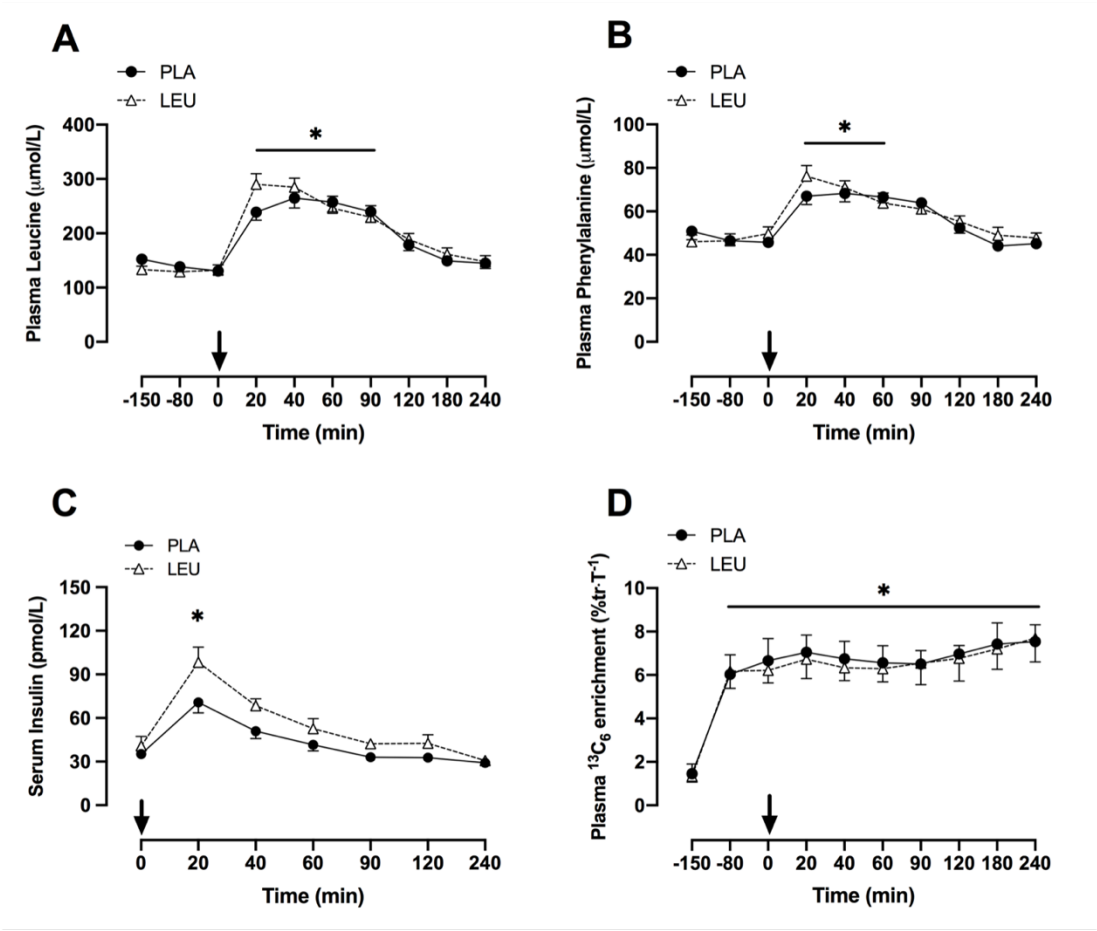

Figure 3.5 Plasma L-[ring-$^{13}C_6$]-Phenylalanine enrichment (A) and plasma insulin (B), plasma leucine (C) and plasma phenylalanine (D) concentrations during postabsorptive (-150-0 mins) and postprandial (0-240 mins) conditions.

t=0 represents the ingestion of a 20g milk protein bolus. Baseline values are considered the first data point represented on the individual figure. *n*=8 for both LEU and PLA for all concentrations. All data were analysed using a two-way mixed model ANOVA. Data are presented as mean ± SEM. * Represents a significant change from baseline values at the $P<0.05$ level. PLA is represented by black circles; LEU is represented by white triangles. LEU, 15g·d^{-1} leucine supplementation during 7d immobilization; PLA, 15g·d^{-1} placebo (non-essential amino acids) during 7d immobilization; CTL, non-immobilized limb; IMB, immobilized limb.

3.5.5. Myofibrillar and Mitochondrial Protein Synthesis

In measures of MyoPS (Figure 3.6A) postabsorptive MyoPS rates (-150-0 mins) were not significantly different between CTL (0.031 ± 0.002 %.h^{-1}) and IMB (0.029 ± 0.002 %.h^{-1}; *P*=0.096). Following ingestion of 20g of milk protein, aggregate postprandial MyoPS (0-240 mins) significantly increased above postabsorptive values in CTL (0.043 ± 0.003%.h^{-1}; *P*<0.01), but not IMB (0.033 ± 0.003 %.h^{-1}; *P*=0.056) and was significantly lower in IMB *vs.* CTL (-22 ± 4%; *P*<0.01). The change in MyoPS from postabsorptive to the postprandial state (Figure 3.6B) was also significantly greater in CTL (0.011 ± 0.002 %.h^{-1}) compared with IMB (0.005 ±0.002 %.h^{-1}; *P*<0.01). In measures of MitoPS (Figure 3.6C), a significant main effect of leg was noted, where IMB (0.030 ± 0.002 %.h-1) had significantly lower MitoPS rates across both postabsorptive (-14 ± 5%) and postprandial (-16 ± 10%) time points compared to CTL (0.036 ± 0.002 %.h-1; *P*<0.01). In response to feeding, a significant main effect of time was noted, where MitoPS significantly decreased in both the CTL (-10 ± 8%) and IMB (-15 ± 10%; *P*=0.039), but there were no significant differences between legs in the postprandial response to feeding (Figure 3.6D; *P*=0.971). There were no significant differences between supplement groups in any measures of MyoPS or MitoPS (*P*>0.05).

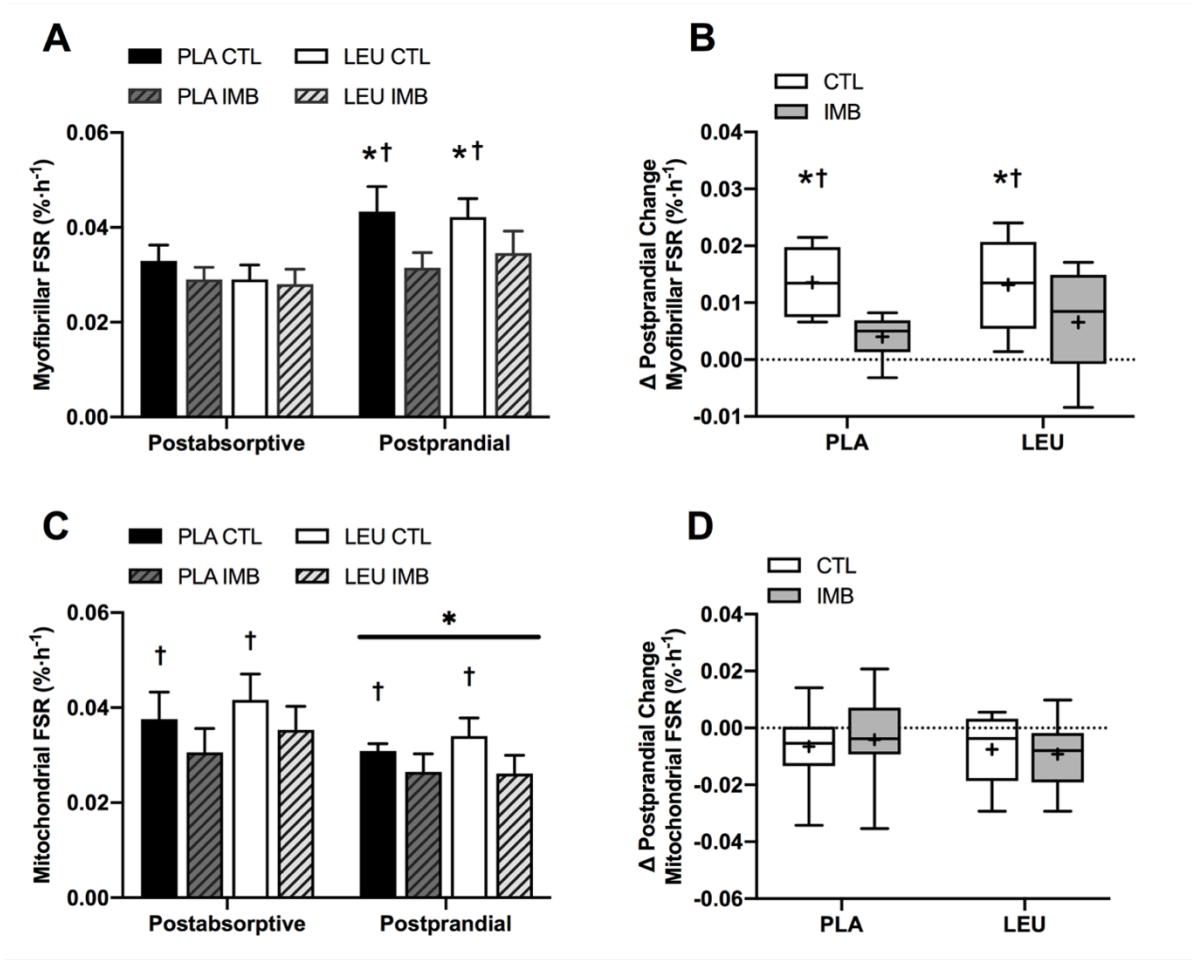

Figure 3.6 Myofibrillar (A) and mitochondrial (C) protein synthesis rates before (-150 to 0 mins) and following (0-240mins) the ingestion of 20g milk protein. Panel B and D demonstrates the change in myofibrillar (B) and mitochondrial (D) from the postabsorptive to postprandial state.

The postprandial change was analysed using a two-way mixed model ANOVA, all other data were analysed using a three-way mixed model ANOVA. Data in figures A and C are presented as mean±SEM. In figures B and D, the boxes represent 25th to 75th percentiles, error bars represent SEM and horizontal lines and crosses within boxes represent medians and means, respectively. In all measures n=8 in LEU, and n=7 in PLA. † represents a significant difference between IMB and CTL; * Represents a significant change from the postabsorptive state (both at the P<0.05 level); PLA-CTL is represented by black boxes, LEU-CTL by white boxes, PLA-IMB by grey-striped boxes and LEU-IMB by light-grey striped boxes. LEU, 15g·d^{-1} leucine supplementation during 7d

immobilization; PLA, 15g·d^{-1} placebo (non-essential amino acids) during 7d immobilization; CTL, non-immobilized limb; IMB, immobilized limb.

3.5.6. Intramuscular Signaling

Phosphorylation of Akt^{S473} (Figure 3.7A) was significantly greater in the CTL *vs.* IMB at all time points (P<0.01), with a significantly greater phosphorylation at 2h *vs.* 4h in both CTL and IMB (P<0.01). The phosphorylation of S6K1^{T389} (Figure 3.7B) increased significantly above postabsorptive values at 2h (P<0.01) and 4h (P=0.049). The phosphorylation of 4EBP1$^{T37/46}$ (Figure 3.7C) increased significantly above postabsorptive values at 2h (P<0.01). There were no significant differences between CTL and IMB or supplement groups in the phosphorylation of Akt^{S473}, S6K1^{T389} or 4EBP1$^{T37/46}$ at any time point (P>0.05). For proteolytic markers, there were no significant differences in LC3ab I (Figure 3.7D), MuRF1 (Figure 3.7F) or caspase (Figure 3.7E) between legs or supplement groups (all P>0.05). AMPKα^{T172} phosphorylation (Figure 3.7G) was not statistically different between time points, legs or supplement groups (all P>0.05). There was a significantly lower PGC1α content (Figure 3.7H) in IMB *vs.* CTL (P=0.047) that was not different between supplement groups (P>0.05). Furthermore, IMB had a significantly lower protein content of OXPHOS complex II (Figure 3.7I) and OXPHOS complex III (Figure 3.7J) compared to CTL at all time points (P<0.01), but there were no significant differences between supplementation conditions (P=0.33 and P=0.11, respectively). Finally, OXPHOS complex V (Figure 3.7K) was not statistically different between time points, legs or supplement groups (all P>0.05). Representative western blot images are presented in Figures 3.8A and 3.8B.

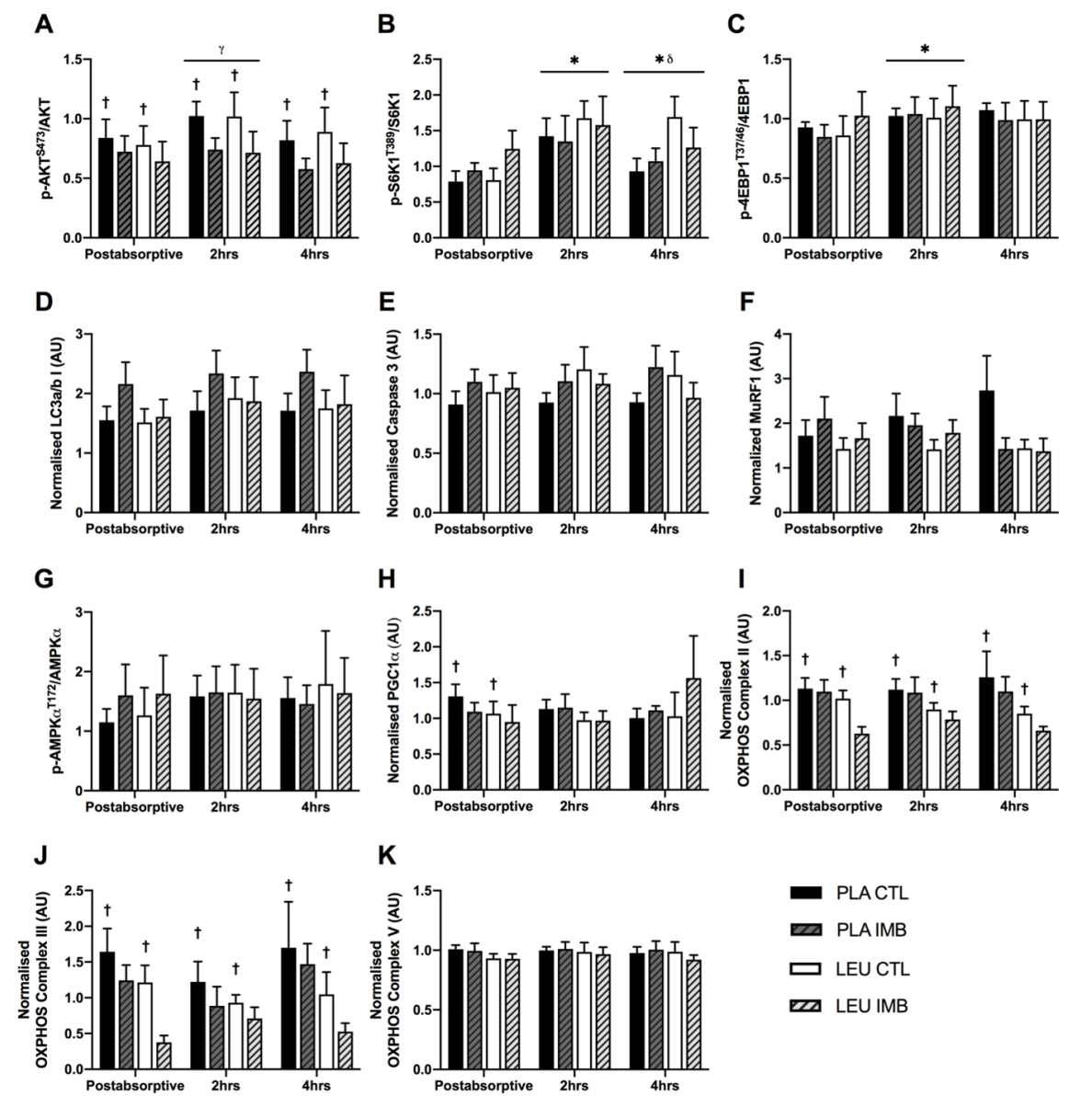

Figure 3.7 Intracellular signalling changes in response to a 20g bolus of milk protein.

(A) phospho protein kinase B at Ser473 (Akt^{S473}; LEU, *n*=8; PLA, *n=7*), (B) phospho 70 kDa S6 protein kinase at Thr389 (S6K1^{T389}; LEU, *n*=7; PLA, *n=7*), (C) phospho eukaryotic initiation factor 4E binding protein 1 at Thr37/46 (4EBP1$^{T37/46}$; LEU, *n*=8; PLA, *n=7*), (D) muscle ring finger protein 1 (MuRF1; LEU, *n*=8; PLA, *n=7*), (E) caspase 3 (LEU, *n*=8; PLA, *n=7*), (F) LC3ab I (LEU, *n*=8; PLA, *n=7*), (G) Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α; LEU, *n*=8; PLA, *n=7*), (H) total AMP activated protein kinase alpha (AMPKα; LEU, *n*=8; PLA, *n=7*), (I) oxidative phosphorylation complex II (OXPHOS CII; LEU, *n*=7; PLA, *n=7*), (J) oxidative phosphorylation complex III (OXPHOS CIII; LEU, *n*=7; PLA, *n*=6) and (K) oxidative

phosphorylation complex V (OXPHOS CV; LEU, *n*=7; PLA, *n*=7). Data are presented as mean ± SEM and were analysed using a three-way mixed model ANOVA. † CTL significantly different from IMB; * Significant change from the postabsorptive state (0hrs); δ Significant change from time 2hrs; γ significant change from time 4hrs; PLA-CTL is represented by black boxes, LEU-CTL by white boxes, PLA-IMB by grey-striped boxes and LEU-IMB by light-grey striped boxes. LEU, 15g·d^{-1} leucine supplementation during 7d of immobilization; PLA, 15g·d^{-1} placebo (non-essential amino acids) during 7d immobilization; CTL, non-immobilized limb; IMB, immobilized limb.

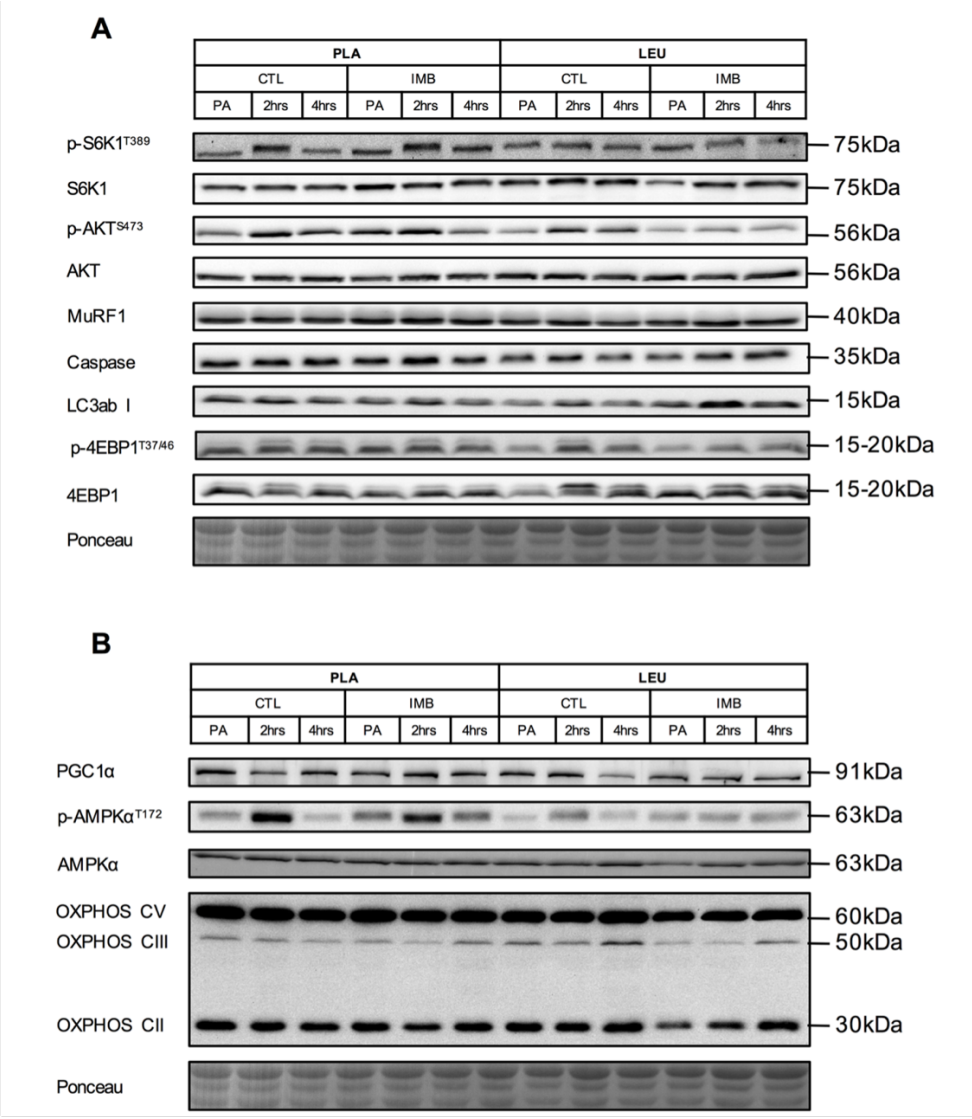

Figure 3.8 . Representative western blot images for signaling proteins.

(A) Proteins implicated in muscle protein synthesis and degradation (referring to data in Fig 6 A-F) and (B) proteins implicated in mitochondrial biogenesis and respiration (referring to data in Figs 6 G-K). kDa, Kilodaltons (molecular weight).

3.5.7. Mitochondrial Respiration

Data for high resolution respirometry is presented in Figure 3.9. In measures of L_I, P_{I+II} and E there were no differences reported between leg or supplementation condition (interaction effect: P=0.809, P=0.083, P=0.111, respectively). A significant group*leg interaction was identified for P_I (P=0.042). However, post-hoc analysis revealed no differences in P_I in IMB *vs.* CTL in either the PLA (FC=1.20 ± 0.19, P=0.06) or LEU (FC=0.87 ± 0.27, P=0.87) group.

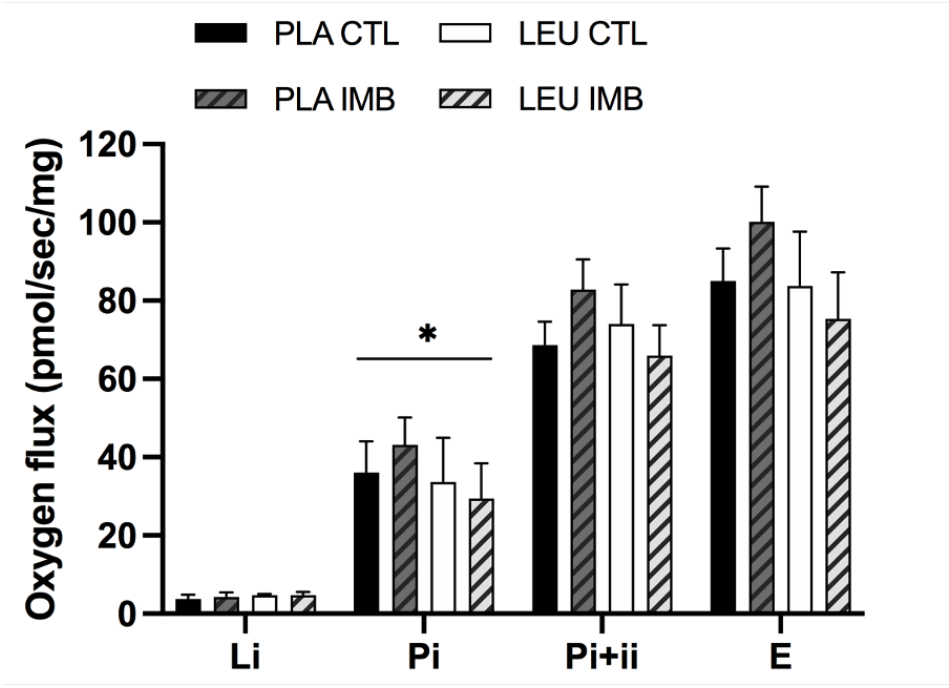

Figure 3.9 Changes in mitochondrial respiration in the IMB vs. CTL following 7d of immobilization in young males.

Data are presented mean ± SEM and were analysed using a two-way mixed model ANOVA, with n=5 in LEU *and* n=5 in PLA, with the exception of L_i where there was an n=5 in LEU and n=4 in

PLA. PLA-CTL is represented by black boxes, LEU-CTL by white boxes, PLA-IMB by grey-striped boxes and LEU-IMB by light-grey striped boxes. LEU, 15g·d^{-1} leucine supplementation during 7d immobilization; PLA, 15g·d^{-1} placebo (non-essential amino acids) during 7d immobilization; CTL, non-immobilized limb; IMB, immobilized limb. * Represents a significant group*leg interaction at the $P<0.05$ level.

3.6. Discussion

Skeletal muscle atrophy during the first week of disuse can prolong recovery of normal physiological function. Since the majority of unavoidable periods of disuse are less than one week in duration (39), there is a need for effective strategies to offset muscle deterioration during this time frame. In line with previous findings (2, 26), we report that 7d of single-leg immobilization in healthy young males significantly reduced leg fat-free mass (FFM; -3.6 ± 0.5%) and isometric strength (-27.9 ± 4.4%) in IMB, but not CTL, with no effect of high-dose leucine supplementation compared with placebo. Mechanistically, declines in FFM occurred in parallel with a significantly smaller type II fibre CSA (-11 ± 5%) and impaired MyoPS and MitoPS rates in IMB compared with CTL.

Disuse-induced muscle atrophy is attributed, primarily, to a blunted MyoPS response to protein intake (3-5). Leucine is a potent stimulator of MyoPS (14, 29, 40) and, therefore, could potentially offset disuse-induced decrements in postprandial MyoPS (41). We hypothesised that applying the same supplemental strategy that improved integrated MyoPS rates in an older population (29), where an underlying muscle anabolic resistance was likely present (42), would prevent declines in FFM and strength during 7d of immobilization in young males, but this was not the case. Although the current study did not directly investigate whether high-dose leucine supplementation maintained postprandial MyoPS rates, it is clear that anabolic resistance to protein intake was present in the IMB limb and a driving factor in the loss of FFM. Therefore, irrespective of whether high-dose leucine supplementation

augmented postprandial MyoPS during immobilization, it is apparent that this strategy was not able to fully overcome the negative effect of disuse on muscle protein turnover. With the exception of lower Akt phosphorylation in IMB vs. CTL, we found no difference in the phosphorylation of mTOR-mediated signaling to explain the observed anabolic resistance in IMB. The absence of disuse-induced impairment in mTOR signaling has previously been reported (4) and suggests that disuse-induced anabolic resistance of MyoPS may be influenced by other signaling pathways (see (43) for review).

Given recent evidence that thrice daily consumption of a mixed amino acid cocktail (containing 4g of leucine) attenuated muscle atrophy during 7d of limb immobilization in young adults (44), a full complement of amino acids may be required to protect MyoPS, muscle mass and strength during disuse in younger adults. Nonetheless, leucine supplementation may still be an effective strategy in middle-aged and older adults, where disuse may exacerbate underlying age-related muscle anabolic resistance (27, 28). Another possible explanation for the absence of any sparing effect of leucine supplementation on disuse-induced FFM loss, could be the timing of leucine ingestion. In the current and previous (26) investigations, participants were provided leucine supplementation alongside each meal, to act as an anabolic trigger to maintain postprandial MyoPS rates during immobilisation. However, providing leucine alongside meals was recently reported to blunt postprandial plasma leucinemia (45) that may be critical for MyoPS stimulation (46). Therefore, future studies should consider the optimal timing of leucine supplementation across the day to maximize integrated MyoPS rates.

In contrast to previous observations (6), we did not detect any difference in postabsorptive MyoPS rates or mTOR signaling between IMB and CTL. Although we used pre-infusion plasma [$^{13}C_6$] phenylalanine enrichment as a proxy for muscle protein enrichment, this approach has been shown to yield comparable results to the two biopsy

approach and is unlikely to explain the lack of difference in postabsorptive MyoPS between IMB and CTL (47). Alternatively, it is possible that any potential difference in postabsorptive MyoPS between IMB and CTL could have been diminished by a reduction in physical activity in both limbs. In line with our observation that step-count was reduced by ~44% during immobilization, others have demonstrated that 7d of single-leg immobilization resulted in a ~35% reduction in physical activity (48) and integrated MyoPS rates (encompassing postabsorptive and postprandial MyoPS) in young males (48)). Congruent with our findings, others have observed impaired postprandial, but not postabsorptive, MyoPS rates following disuse (5, 49). Collectively, these data suggest that impaired postprandial MyoPS is a central driver of disuse-induced muscle atrophy and a focal point for targeted therapeutic interventions. It should also be considered that correcting potential declines in postprandial MyoPS, that may occur in different models of disuse, could also help to mitigate disuse-induced muscle atrophy and strength loss.

Mitochondria are mechanically (12) and nutritionally sensitive (50) and are critical to contractile function (13). In response to short-term disuse, significant reductions in mitochondrial respiratory capacity (7), the protein content of mitochondrial complexes I-V (7) and integrated MitoPS rates (11) have been noted. Herein, we report significant decrements in both postabsorptive (-14 ± 5%) and postprandial (-16 ± 10%) MitoPS and the protein content of PGC1α, OXPHOS complex II and III in IMB *vs.* CTL following 7d of immobilization. Mitochondrial respiratory capacity remained unchanged, possibly due to the time-frame of immobilization. Specifically, intrinsic mitochondrial capacity has been reported to increase in the first 5d of disuse (2, 51), whereas declines in respiratory function are noted after 14d of disuse (10), which suggests that our observations at 7d may represent the transition phase from an increase to a decrease in respiratory capacity. This suggestion is further supported by the reduction in protein content of key markers of oxidative metabolism

and MitoPS rates; alterations which likely precede the decline in mitochondrial respiratory capacity reported elsewhere (10). Furthermore, others have demonstrated a reduction in submaximal respiration following 3 and 14d of immobilization, measured through extensive ADP titrations (52). Therefore, it is plausible that in the current study immobilization reduced ADP sensitivity. Since we were unable to measure this directly, further research is warranted to determine the exact timeline of altered mitochondrial function with immobilization.

We hypothesized that leucine supplementation would prevent adverse mitochondrial adaptations to disuse, through preserving mitochondrial turnover (as in (25)), but this was not the case. Surprisingly, we noted a significant decline in postprandial MitoPS in response to 20g of milk protein in both CTL and IMB, which was confirmed by the absence of any significant time or group effects in markers of oxidative metabolism. These findings may explain why high-dose leucine supplementation had no impact on markers of oxidative metabolism. Combined with the observation that a mixed meal containing 18g of egg protein did not stimulate MitoPS rates at rest (53), our findings suggest that moderate-to-low dose protein ingestion (i.e. ≤20g) may not be sufficient for MitoPS stimulation, whereas a continuous amino acid IV infusion (50) or protein-carbohydrate co-ingestion (54) appears to be more potent. The absence of any difference in postprandial MitoPS between CTL and IMB could indicate that disuse-induced alterations in mitochondrial kinetics originated primarily from postabsorptive declines in MitoPS. However, we are unable to speculate whether these alterations in mitochondrial turnover are secondary to a reduced energetic demand of inactivity or whether there is cross-talk between mitochondrial and myofibrillar compartments. Nevertheless, our findings suggest that nutritional interventions to mitigate disuse-induced muscle atrophy during disuse events, where contraction is not possible, may need to target both MyoPS and MitoPS.

A methodological limitation of the current study was the use of DXA to measure FFM. Although strongly correlated with magnetic resonance imaging/computerized tomography (55-58) there is a 1.2% precision error (expressed as %CV) variance associated with measurement (31). When combined with the small sample size this may have reduced the statistical power and impaired our ability to detect small, but potentially important, attenuations in FFM following the supplementation intervention. Nevertheless, the current study applied a strict scanning protocol (outlined in methods) allowing the determination of the hypothesized question (whether high-dose leucine could prevent immobilization-induced declines in FFM). Similarly, the lack of measurement of integrated MyoPS rates (via deuterium water), in combination with the implementation of 20g milk protein ingestion alone in our experimental trial, precludes our ability to determine whether the 5g of supplemental leucine enhanced/maintained postprandial or integrated MyoPS in IMB. Furthermore, although the inclusion of a number of measurements increases the mechanistic insight into the negative physiological adaptations to disuse, this approach increases the type I error rate that results from testing numerous study outcomes. Nonetheless, our study provides important clinical insights into the mechanisms underpinning disuse-atrophy and the inability of high-dose leucine to prevent these alterations.

In summary, 7d of immobilization caused significant declines in FFM and strength in healthy young males, which occurred alongside blunted postprandial MyoPS and postabsorptive MitoPS rates. Supplementation with $15g \cdot d^{-1}$ of leucine did not prevent disuse-induced decrements in FFM or strength.

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4. EPICATECHIN AND IT'S COLONIC METABOLITE HIPPURIC ACID PROTECT AGAINST DEXAMETHASONE-INDUCED ATRHOPY IN SKELETAL MUSCLE CELLS

Disclaimers

This research has been submitted for publication. If published this chapter will be in verbatim.

Author declarations

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4.1. Abstract

Cocoa flavanols have been shown to improve muscle function and may offer a novel approach to protect against muscle atrophy. Hippuric acid (HA) is a colonic metabolite of (-)-epicatechin (EPI), the primary bioactive compound of cocoa, and may be responsible for the associations between cocoa supplementation and muscle metabolic alterations. Accordingly, we investigated the effects of EPI and HA upon skeletal muscle morphology and metabolism within an *in vitro* model of muscle atrophy. Under atrophy-like conditions (24h 100μM dexamethasone (DEX)), C2C12 myotube diameter was significantly greater following co-incubation with either 25μM HA (11.19±0.39μm) or 25μM EPI (11.01±0.21μm) compared to the vehicle control (VC; 7.61±0.16μm, both $P<0.001$). In basal and leucine-stimulated states, there was a significant reduction in myotube protein synthesis (MPS) rates following DEX treatment in VC (P=0.024). Interestingly, co-incubation with EPI or HA abrogated the DEX-induced reductions in MPS rates, whereas no significant differences *vs.* control treated myotubes (CTL) were noted. Furthermore, co-incubation with EPI or HA partially attenuated proteolysis, preserving LC3 α/β II:I and caspase-3 protein expression in atrophy-like conditions. In response to DEX treatment, the protein content of PGC1α, ACC and TFAM (regulators of mitochondrial function) was significantly reduced vs. CTL (all P<0.050). However, co-incubation with EPI or HA was unable to prevent these DEX-induced alterations. For the first time we demonstrate that EPI and HA exert anti-atrophic effects on C2C12 myotubes. Accordingly, our data highlights the importance of HA, the colonic metabolite of EPI, in the association between flavanol supplementation and muscle health.

4.2. Introduction

Skeletal muscle atrophy accompanies many physiological processes such as ageing, immobility and disease (1). It is also a prominent consequence of corticosteroid treatments for chronic inflammatory and autoimmune conditions (2). Muscle atrophy is underpinned by alterations to muscle protein turnover, resulting from a decline in basal and postprandial muscle protein synthesis rates (MPS) and/or a concomitant increase in muscle protein breakdown (MPB) (3-5). Impaired mitochondrial metabolism has also been associated with muscle atrophy (6) and has been implicated in mediating the atrophic pathology (7).

Polyphenolic compounds are regularly consumed in the human diet (8). Flavanols are a type of flavonoids, a class of polyphenolic secondary metabolites found in plants. Cocoa is rich in flavanols (9) and has been shown to improve muscle function (10), exercise capacity (11) and recovery from exercise-induced damage (12). (-)-Epicatechin (EPI) is the most abundant flavanol in cocoa (13), representing ~35% of total polyphenols in cocoa powder (14). As a result, many of the health-promoting benefits of cocoa supplementation have been attributed to EPI (15). *In vitro* experimental models suggest that EPI exerts positive effects on skeletal muscle metabolism through enhancing mitochondrial function (16). There is also evidence to suggest that cocoa promotes myogenesis and increases C2C12 myotube width (17). Furthermore, in C2C12 myotubes subjected to clinorotation (an *in vitro* model of atrophy), EPI treatment was associated with the downregulation of the key atrogene, muscle ring finger protein 1 (MURF1) (18). Since oxidative stress, mitochondrial dysfunction and an imbalance between protein anabolism and catabolism occurs in muscle atrophy (4, 7, 19, 20), it is plausible that cocoa flavanols could abrogate muscle atrophy through maintaining mitochondrial function and supporting net muscle protein balance through the activities of its main bioactive compound, EPI. However, the specific role and mechanisms of cocoa flavanols in protecting against muscle atrophy remains to be determined.

EPI has a poor oral bioavailability and, as a consequence, is only present in systemic circulation at extremely low concentrations and typically in a sulphated, methylated or glucuronidated forms (20). Unabsorbed EPI is metabolised by colonic microbiota to form smaller structurally related metabolites (20). Although research determining the role of EPI metabolites on peripheral metabolism is limited, it is possible that these smaller bioactive compounds may ultimately be responsible for the *in vivo* short-to-long term metabolic effects associated with native flavanol supplementation. The 3/1-carbon ring fission metabolite (3/1C RFM), hippuric acid (HA) is a colonic metabolite derived from EPI and proanthocyanidins (flavanol oligomers of EPI and catechin) following cocoa supplementation. In the 12-48h following cocoa ingestion, HA reaches a high circulating systemic concentration (in the mM range) (21) and may possess potent metabolic properties relevant to muscle atrophy. *In vitro*, HA has been suggested to stimulate glucose metabolism and preserve mitochondrial function following insult in C2C12 myotubes (22), which is in agreement with evidence demonstrating similar effects following supplementation with a native flavanol (e.g., (16, 23)). Since mitochondrial dysfunction and insulin resistance are implicated in the aetiology of muscle atrophy, further research is warranted to assess the impact of EPI metabolites, in particular HA, as a strategy to offset muscle atrophy.

Accordingly, we investigated the effects of EPI and HA on skeletal muscle morphology, muscle protein turnover and mitochondrial metabolism within an *in vitro* model of skeletal muscle atrophy. Our hypotheses were twofold; (1) under atrophy-inducing conditions, concomitant treatment with EPI would prevent declines in myotube diameter through the preservation of MPS rates, a reduction in catabolic signalling and the preservation of mitochondrial metabolism and (2) under atrophy-inducing conditions, the colonic metabolite HA would also preserve myotube diameter, through the preservation of MPS and catabolic signalling processes.

4.3. Methods

4.3.1. C2C12 cell culture

Mouse skeletal muscle C2C12 myoblasts were maintained in Dulbecco's Modified Eagle's Medium (DMEM; #11966025 Gibco) supplemented with 10% *(v/v)* fetal bovine serum (FBS; #F9665 Invitrogen), 5 mM glucose (#G7021 Sigma), 1 mM sodium pyruvate (#S8636 Sigma), 1mM GlutaMax (#35050-038 Gibco), 100 U/mL penicillin and 100 µg/mL streptomycin (#15070-063 Invitrogen). Once ~70-80% confluence was obtained, cells were seeded to 6-well or 24-well plates at a density of $2x10^5$ or $5x10^4$ cells/well, respectively. Upon reaching ~90-95% confluence, cells were washed into fresh DMEM containing 2% *(v/v)* horse serum (#16050-122 Gibco), 5 mM glucose, 1 mM sodium pyruvate, 1mM GlutaMax, 100 U/mL penicillin and 100 µg/mL streptomycin and differentiated for 6d. On day 6 of differentiation cells were washed into fresh serum-free DMEM containing 5 mM glucose, 1 mM sodium pyruvate, 1 mM GlutaMax, 100 U/mL penicillin and 100 µg/mL streptomycin for 1h prior to commencing the 24h treatment protocol as detailed below. Cell passage numbers 10-13 were used for experimentation. Visual inspection utilising inverted light microscopy confirmed that myotubes were fully differentiated at the time of treatment.

4.3.2. Treatment protocol

The full treatment protocol is detailed in Figure 4.1 and the rationale underpinning the treatment protocol is outlined extensively in the Chapter 1.8 of this thesis. Briefly, to identify anti-atrophic effects of EPI and HA, C2C12 myotubes were subdivided into six groups for the 24h treatment protocol; (i) VC-CTL, with cells incubated in serum free medium (as above), (ii) VC-DEX, with cells incubated in 100 µM dexamethasone (#D4902 Sigma), (iii) EPI-CTL, with cells incubated with 25 µM EPI (#E1753 Sigma), (iv) EPI + DEX, with cells co-incubated in 25 µM EPI and 100 µM DEX. (v) HA-CTL, with cells incubated in 25 µM HA (#112003

Sigma) and, (vi) HA + DEX, with cells co-incubated with 25 µM HA and 100 µM DEX. All treatment conditions were dissolved in ethanol and matched for ethanol concentrations (0.2% of cell media). To enhance the translatability to *in vivo* physiology, a 25 µM treatment dose was selected due to it's upper-limit representation of both circulating natural flavanol (i.e. EPI) and subsequent phenolic acid (i.e. HA) concentrations *in vivo* (21) and successful implementation in previous *in vitro* research (22). An [100 µM] DEX was selected based on previous research that demonstrated significantly increased levels of protein degradation following 24h or 48h incubation at 100 µM DEX (24).

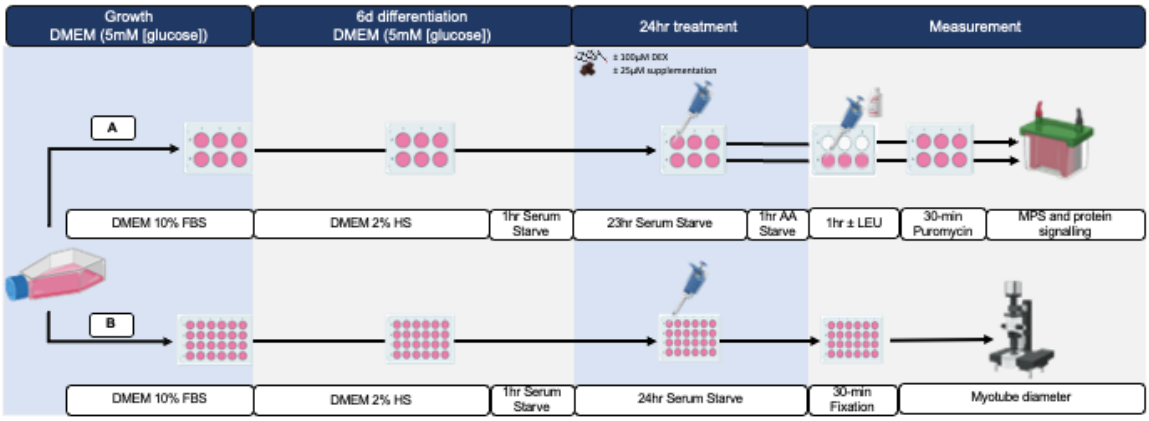

Figure 4.1 Experimental protocol for the primary outcome measures of myotube protein synthesis (MPS; A) and myotube diameter (B).

DEX, Dexamethasone, DMEM; Dulbecco's Modified Eagle Medium, FBS; Fetal Bovine Serum, HS; Horse serum, LEU; Leucine.

4.3.3. Biochemical analysis

4.3.3.1. Immunofluorescence analysis

The immunofluorescence protocol has been outlined previously (25). Briefly, immediately following the 24h treatment period (Figure 1), culture medium was removed and myotubes

were fixed for 30mins in 2% (v/v) formaldehyde in Dulbecco's phosphate-buffered saline (dPBS). Myotubes then underwent 10mins of permeabilization in 100% methanol, before being washed 3X in dPBS and blocked for 30min in 5% (v/v) goat serum in dPBS. Subsequently, myotubes were incubated for 1h with a primary anti-desmin antibody (#D8281 Sigma: 1:100 1% (v/v) bovine serum albumin (BSA): dPBS) before undergoing a wash step (as above) and incubation in the dark for 1h with a secondary goat anti-rabbit IgG H+L Alexa Fluor 488 antibody (#P-2771MP Fisher: 1:200 in dPBS). Wells were then washed once in dPBS before a 5min incubation with DAPI (#4083 CST: 1:5000 in dPBS) in the dark. Finally, cells were washed once with dPBS, before 10 μL of mountant was added to each well and a coverslip added.

4.3.3.2. MPS analysis

In the final hour of the 24h treatment protocol (described above), C2C12 myotubes were nutrient deprived in amino acid and serum free low-glucose DMEM medium (#19052222 US Biological) made according to the manufactures instructions and supplemented with 1 mM sodium pyruvate, 1 mM GlutaMax, 100 U/mL penicillin and 100 μg/mL streptomycin. Following the period of nutrient deprivation, each treatment condition underwent a 90min period of stimulation with 5 mM L-leucine (LEU; #L8912 Sigma) or a ddH_2O volume matched control to provide both a basal and LEU-stimulated measure of MPS. To obtain a proxy measurement of MPS, the Surface Sensing of Translation (SUnSET) technique developed by Goodman et al. (26) was utilised and puromycin (1 μM; #P8833 Sigma) added to cell media in the final 30mins of the acute stimulation period. Subsequently, myotubes were washed and lysed with RIPA lysis buffer (#20118 Merk-Millipore) and supplemented with one cOmplete™ mini protease inhibitor (#4693159001 Roche) tablet and one phosSTOP ™ (#4906845001 Roche) tablet. MPS rates were determined from cell lysates during western blot analysis utilising an anti-puromycin antibody (#MABE343 Merk-Millipore) as detailed below.

Protein extraction and expression analysis. Protein expression was measured via western blot analysis performed on total cell lysates following centrifugation (4,500rpm, 10mins, 4°C) in accordance to previously published protocols (27, 28). Gels were loaded according to the protein concentration assessed by the DC protein assay (Bio-Rad), before western blot aliquots of 2µg/1µL were prepared in 4x laemmli sample buffer and ddH_2O. To ensure membrane integrity, samples were then left to denature overnight at room temperature before subsequent analysis. Equal amounts of total protein (18-30µg) were loaded onto either in-house 10-15% or Criterion™ TGX™ Precast Midi protein gels (Bio-rad, Hertfordshire, UK) and separated by SDS-PAGE using a constant voltage of 100V for 10min and 150V for a subsequent 1h. Separated proteins were then transferred to a Protran nitrocellulose or polyvinylidene difluoride (PVDF) membrane at 100V for 1h. The membranes were then incubated overnight (4°C) with appropriate and validated primary antibodies; anti-puromycin (#MABE343, 1:1000 in 5% BSA:TBST), muscle ring finger protein 1 (MuRF-1; sc-398608, 1:1000 in TBST), muscle atrophy f-box (MAFbx; AM-3141, 1:1000 in 5% BSA: TBST), caspase-3 (ECM14220, 1:1000 in 5% BSA:TBST), microtubule-associated proteins 1A/1B light chain 3B (LC3 α/β; CST127415 in TBST), total protein kinase B (Akt; CST9272, 1:1000 in TBST), p-Akt^{S473} (CST4060,1:1000 5% BSA in TBST), p-Akt^{T308} (CST9275, 1:5000 in TBST), total mechanistic target of rapamycin complex 1 (mTORC1; CST2983, 1:1000 in 5% BSA TBST), p-mTORC1^{S2448} (CST2971, 1:1000 in 5% BSA TBST), total ribosomal protein S6 kinase beta-1 (S6K1; CST9202, 1:1000 in 5%BSA:TBST), p-S6K1^{T389} (CST9205, 1:500 in 5%BSA:TBST), total ribosomal protein S6 (RPS6; CST2217, 1:1000 in 5% BSA:TBST), phosphor RPS6$^{S240/244}$ (CST5364, 1:500 in 5% BSA:TBST), total eukaryotic initiation factor 4E binding protein 1 (4EBP1; CST9452, 1:1000 in TBST), p-4EBP1$^{T37/46}$(CST9459, 1:500 5% BSA in TBST), total OXPHOS rodent antibody cocktail (ab110413, 1:1000 in 5% BSA:TBST), total 5' AMP-activated protein kinase (AMPKα; CST2757, 1:1000 in TBST), p-

AMPKα^{T172} (CST2535, 1:1000 in TBST), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α; MM3248419, 1:1000 in 5% BSA:TBST), citrate synthase (CST143095 1:1000 in TBST), total acetyl-CoA carboxylase (ACC; CST3676 in TBST), p-ACC^{S79} (CST36615 in TBST) and mitochondrial transcription factor A (TFAM; SAB1401383 1:1000 in 5%BSA: TBST). Samples were then washed 3X 5min in TBST before undergoing a 60min incubation with a previously validated horseradish peroxidase (HRP)-linked anti-rabbit (CST7074, 1:10 000 in TBST), anti-mouse (CST7076, 1:10 000 in 5% BSA:TBST), or anti-rat (CST7077,1:1000 in 5% BSA:TBST) IgG. Subsequently, Immobilon western chemiluminescent HRP substrate (Millipore) was used quantify protein content, visualised using a:BOX Chemi XT4 imager with GeneSys capture software (Syngene UK, Cambridge, UK). Band quantification was achieved using a Chemi Genius Bioimaging Gel Doc System (Syngene, Cambridge, UK). Values were corrected to both a gel control and a loading control (ponceau). Following these corrections, where appropriate, the phosphorylation of proteins, as a proxy of their activation was expressed relative to the total amount of each protein. Data are presented as fold changes from the VC-CTL treatment condition.

4.3.4. Quantification and statistical analysis

4.3.4.1. Quantification of myotube diameter

Immunofluorescence stained C2C12 myotubes were imaged using a epifluorescence/brightfield microscope (Leica DMI6000). To improve reproducibility, for each passage triplicate wells were utilised for each treatment condition. For the quantification of myotube diameter, a total of 10 images were acquired per well at a magnification of 20x and the analysis of myotube diameter was conducted using Image J (v1.51 NIH). A myotube was classified as a desmin positive object, containing 3 or more DAPI positive nuclei. The myotube diameter of each individual myotube was determined as the average of 5

measurements acquired along its full length. For each image, the centremost 5 myotubes were utilised, with each passage ($n=5$) having a total of 750 measurements across 150 myotubes analysed per treatment condition.

4.3.4.2. Outcome measures

The primary outcome measures included the change in both myotube diameter and MPS rates in response to EPI or HA treatment in atrophy-like conditions. Alterations in intracellular signalling in response to EPI or HA treatment in atrophy-like conditions acted as a secondary outcome measure.

4.3.4.3. Statistical analysis

Data are presented as means ± SEMs. Data analysis was performed using SPSS version 25 (IBM Corp.) and statistical assumptions were checked prior to analysis. Myotube diameter were analysed using a 2-factor mixed-model ANOVA, with a single between group factor (supplementation; VC *vs.* EPI *vs.* HA) and single within-group factor (atrophy treatment; CTL *vs.* DEX). Measures of MPS and protein expression were analysed using a 3-factor mixed-model ANOVA, with a single between-groups factor (supplementation; VC *vs.* EPI *vs.* HA) and two within-group factors (atrophy treatment; CTL *vs.* DEX and stimulation; basal *vs.* LEU-stimulated). Missing data were not imputed into analyses and *n* numbers are reported in each figure legend. Where appropriate, Bonferroni's post-hoc test was used to isolate specific differences whenever a is significant interaction or main effect was identified and the results of Bonferroni's post-hoc test are reported herein. The level of significance was considered $P \leq 0.050$.

4.4. Results

4.4.1. Myotube Diameter

As shown in Figure 4.2B, the DEX-induced decline in myotube diameter was attenuated following treatment with HA and EPI (Figure 4.2C), with significantly greater myotube diameter in EPI-DEX (11.01 ± 0.21 µm; P<0.001) and HA-DEX (11.19 ± 0.39 µm; P<0.001) *vs.* VC-DEX (7.61 ± 0.16 µm). However, in the CTL treated myotubes, myotube diameter was significantly reduced in both EPI-CTL (11.85 ± 0.20 µm) and HA-CTL (12.12 ± 0.22 µm) *vs.* VC-CTL (13.03 ± 0.19 µm, P=0.005 and P=0.027, respectively).

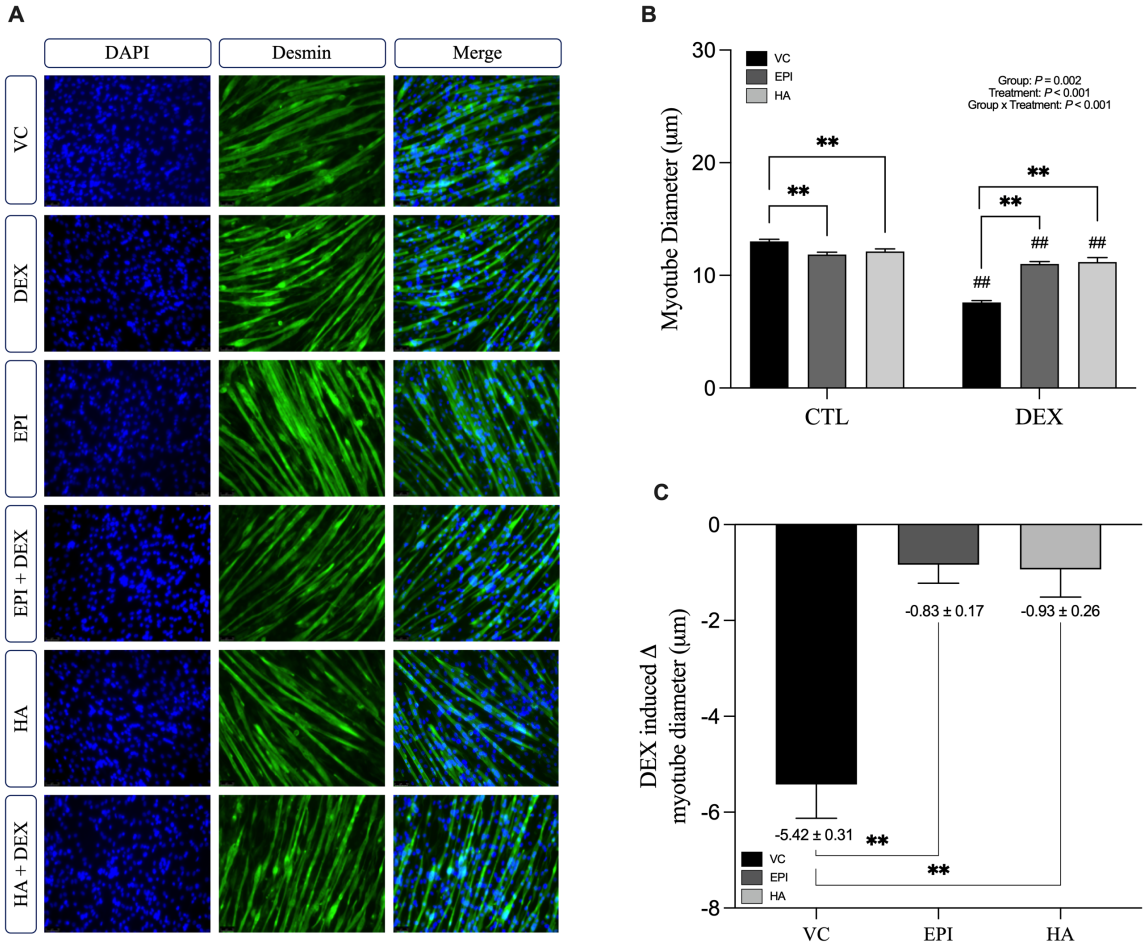

Figure 4.2 Myotube diameter of C2C12 myotubes.

Measures of myotube diameter following the 24h treatment protocol (A, B). Figure C represents the change in myotube diameter between DEX treated and CTL treated C2C12 myotubes. Data is

presented as mean ± SEM. The DEX induced change in myotube diameter was analysed using a single factor independent ANOVA; all other data were analysed using a two -actor mixed-model ANOVA. In all measures *n*=5. ** Represents a significant difference between supplementation groups at the P<0.01 level, ## DEX treated significantly different from CTL treated at the P<0.01 level. CTL; 24h control (no atrophic stimulus), DEX; 24h, 100μM dexamethasone treatment, EPI; 24h, 25μM epicatechin supplementation (dark grey bars), HA; 24h, 25μM hippuric acid supplementation (light grey bars), VC; Ethanol matched vehicle control (black bars).

4.4.2. Muscle protein turnover

4.4.2.1. MPS

The current study utilised the SUnSET methodology (26) to determine the ability of EPI and HA to prevent DEX-induced changes in MPS rates. The expression of puromycin bound proteins was significantly reduced in VC-DEX (fold change: 0.62 ± 0.07) *vs.* VC-CTL (fold change: 1.00 ± 0.18, P=0.024). However, the DEX-induced decline in MPS was mitigated following EPI and HA treatment with no significant differences in the expression of puromycin bound proteins between HA-CTL (fold change: 1.19 ± 0.31) and HA-DEX (fold change: 1.34 ± 0.40, P=0.205) or EPI-CTL (fold change: 0.70 ± 0.10) and EPI-DEX (fold change: 0.89 ± 0.16, P=0.148, Figure 4.3A). Furthermore, there was a significant increase in puromycin bound proteins from the basal to the LEU-stimulated state (main effect: P=0.007, Figure 4.3B) with no significant difference across all 6 treatment conditions.

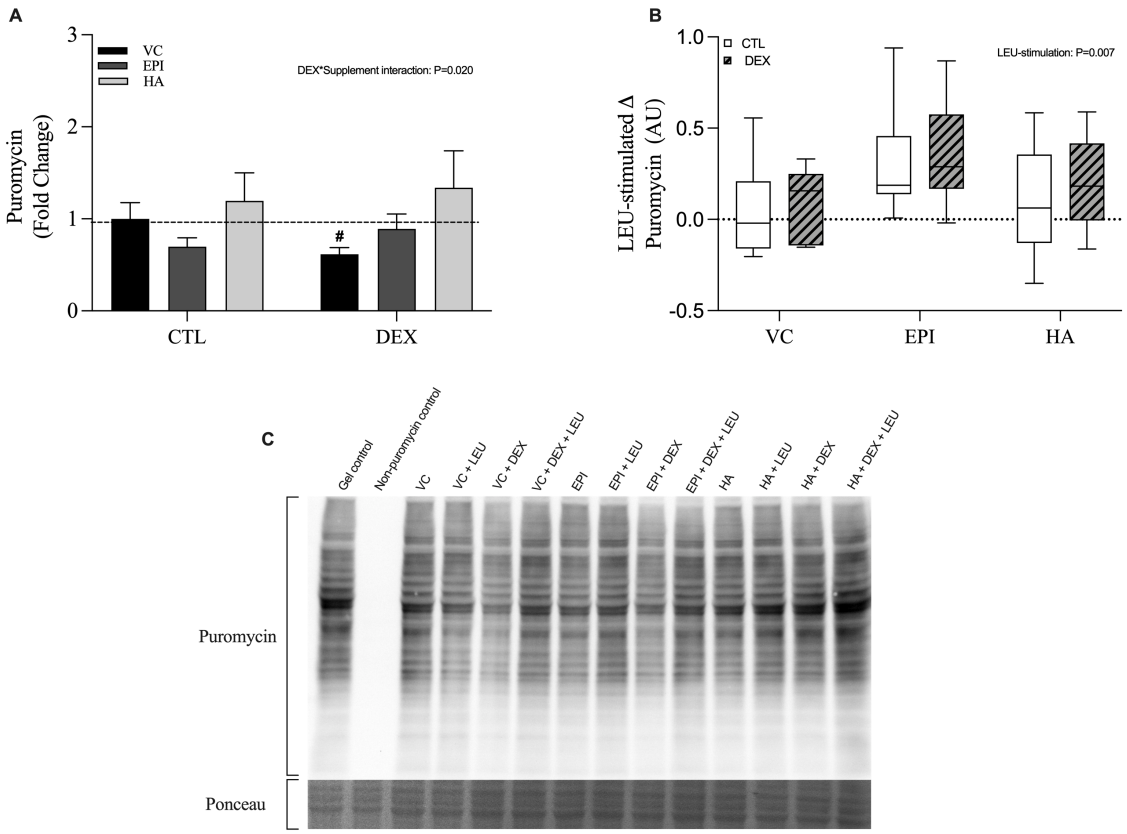

Figure 4.3 Myotube protein synthesis (MPS) rates of C2C12 myotubes.

The incorporation of puromycin labelled proteins following the 24h treatment protocol as a proxy of MPS rates (A,C). Figure B represents the change in puromycin bound proteins between the basal and LEU-stimulated states. In figure A, data is presented as mean ± SEM. In figure B, the boxes represent the 25th-75th percentile, the error bars represent min-max values and the lines represent medians. Data was analysed using a three-factor mixed-model ANOVA. In all measures experimental repeats *n*=6. Black bars represent VC, dark grey bars represent EPI, light grey bars represent HA, white bars represent CTL and grey striped bars represent DEX. $^{\#}$ DEX treated significantly different from CTL treated at the P<0.05 level. CTL; 24h control (no atrophic stimulus), DEX; 24h, 100µM dexamethasone treatment, EPI; 24h, 25µM epicatechin supplementation, HA; 24h, 25µM hippuric acid supplementation, LEU; 90mins of 5mM leucine stimulation, VC; Ethanol matched vehicle control.

4.4.2.2. Anabolic protein signalling pathways.

Protein expression alterations in response to the 24h treatment protocol were assessed by western blotting. Compared to CTL, DEX treatment significantly reduced the phosphorylation of mTORC1^{S2448} (main effect: P=0.013, Figure 4.4B), AKT^{S473} (main effect: P<0.001, Figure 4.4F), S6K1^{T389} (main effect: P<0.001, Figure 4.4H) RPS6$^{s240/244}$ (main effect P<0.001, Figure 4.4J) and AKT^{T308} (main effect: P<0.001, Figure 4.4K). The protein content of 4EBP1 (Figure 4.4D) was also significantly reduced following DEX treatment in EPI (P=0.004), but not in VC (P=0.230) or HA (P=0.653). There was no further significant differences noted between treatment conditions (i.e., HA, EPI and VC) in the protein expression of key anabolic signalling intermediates (all P>0.050). Compared to CTL, DEX treatment significantly reduced the phosphorylation of mTORC1^{S2448} (main effect: P=0.013, Figure 4.4B), AKT^{S473} (main effect: P<0.001, Figure 4.4F), S6K1^{T389} (main effect: P<0.001, Figure 4.4H), RPS6$^{s240/244}$ (main effect P<0.001, Figure 4.4J), and AKT^{T308} (main effect: P<0.001, Figure 4.4K). In response to LEU-stimulation, DEX treatment did not impair leucine stimulated anabolism across any measure of anabolic protein expression, with no significant reductions in the protein expression or phosphorylation of targets in DEX *vs.* CTL treated myotubes between the basal and LEU-stimulated conditions (all P>0.050, Figure 4.4A-K). In fact, in measures of S6K1 protein content (Figure 4.4C), mTORC1^{S2448} phosphorylation (Figure 4.4G), and RPS6$^{s240/244}$ phosphorylation (Figure 4.4J) there was a significant increase following LEU-stimulation compared to basal in DEX treated myotubes (all P<0.050) that was not observed in CTL treated myotubes (all P>0.050).

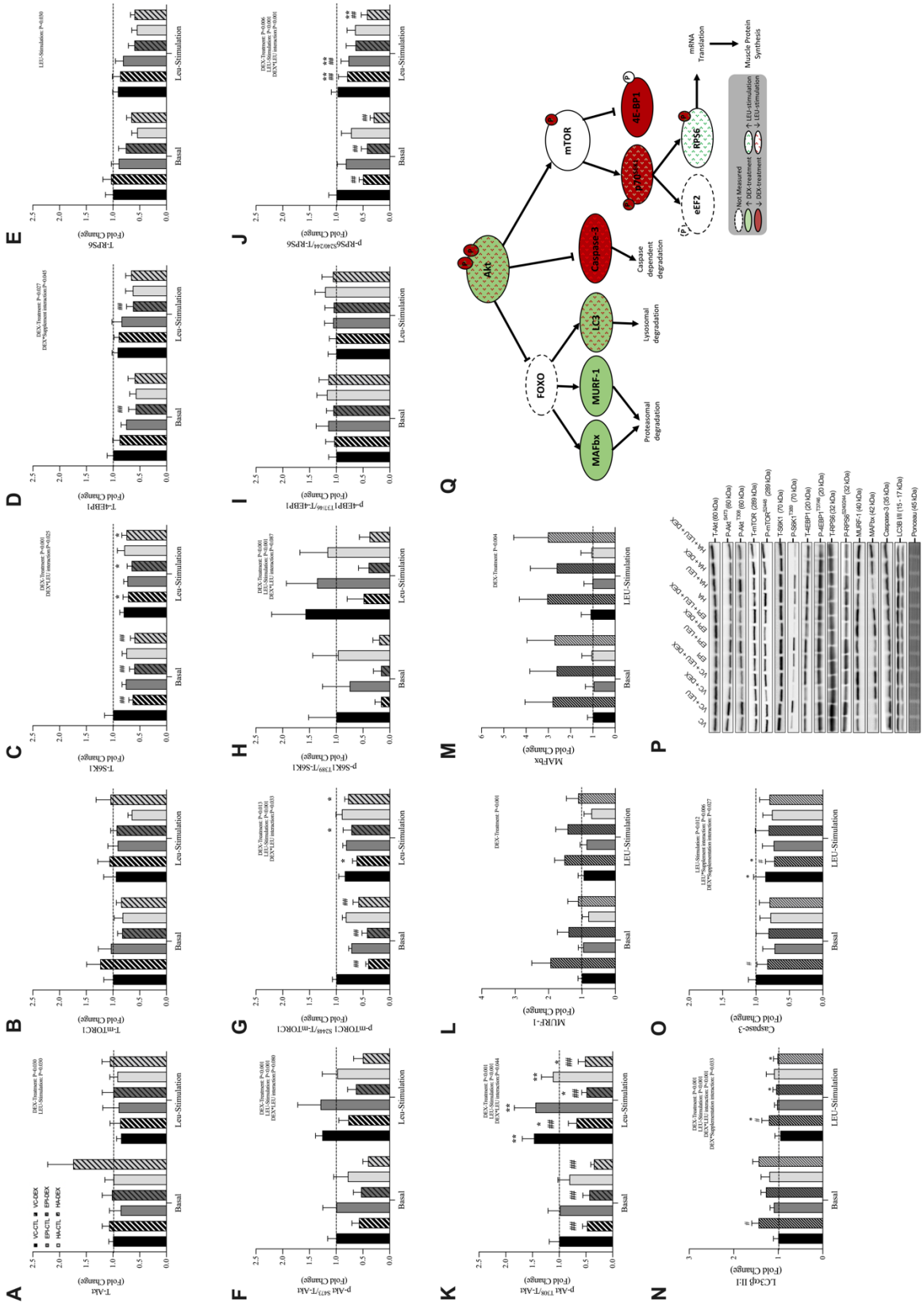

Figure 4.4 Anabolic and catabolic protein expression in response to the 24h treatment protocol.

(A) total Akt (*n*=5), (B) total mTORC1(*n*=5), (C) total S6K1(*n*=6), (D) total 4EBP1(*n*=6), (E) total RPS6 (*n*=6), (F) p-AKT^{S473}/T-Akt (*n*=5), (G) p-mTORC1^{S2448}/t-mTORC1 (*n*=5), (H) p-S6K1^{T389}/T-S6K1 (*n*=6), (I) p-4EBP1$^{T37/46}$/T-4EBP1 (*n*=6), (J) p-RPS6$^{S240/244}$/T-RPS6 (*n*=6), (K) p-Akt^{T308}/T-Akt (*n*=5), (L) MURF-1 (*n*=6), (M) MAFbx (*n*=6), (N) LC3 α/β II:I (*n*=6), (O) Caspase-3 (*n*=6), (P) western blot representative image of anabolic and catabolic signalling proteins, (Q) schematic overview of measured targets in the anabolic and catabolic signalling pathways following 24h treatment. Data are presented as means ± SEMs and were analysed using a 3-factor mixed-model ANOVA. Black bars represent VC, dark grey bars represent EPI, light grey bars represent HA, striped bars represent DEX and plain bars represent CTL. * and ** LEU-stimulated significantly different from basal at the P<0.05 level and P<0.01 level, respectively. $^{\#}$ and $^{\#\#}$ DEX treated significantly different from CTL treated at the P<0.05 level and P<0.01 level, respectively. 4EBP1; eukaryotic initiation factor 4E binding protein 1, Akt; total protein kinase B, CTL; 24h control (no atrophic stimulus), DEX; 24h, 100μM dexamethasone treatment, EPI; 24h, 25μM epicatechin supplementation,
HA; 24h, 25μM hippuric acid supplementation, LC3 α/β; Microtubule-associated proteins 1A/1B light chain 3B, LEU; 90mins of 5mM leucine stimulation, MAFbx; muscle atrophy f-box, mTORC1; total mechanistic target of rapamycin, MURF-1; muscle ring finger protein 1, RPS6; ribosomal protein S6, S6K1; ribosomal protein S6 kinase beta-1, VC; Ethanol matched vehicle control.

4.4.2.3. Catabolic signalling pathways

No significant differences were noted between supplemented conditions (i.e., EPI, HA or VC) in measures of MURF-1 (Figure 4.4L) or MAFbx (Figure 4.4M) protein expression. However, the protein expression of MURF-1 and MAFbx were significantly greater in response to DEX treatment *vs.* CTL treated myotubes (main effect: P=0.001 and P=0.004, respectively). The ratio of LC3 α/β II:I (Figure 4.4N) was significantly increased in VC-DEX *vs.* VC-CTL (P<0.001). However, the DEX-induced alteration in LC3 α/β II:I was mitigated following

treatment with HA and EPI, with no significant differences noted between EPI-DEX *vs.* EPI-CTL (P=0.181) or HA-DEX *vs.* HA-CTL (P=0.278). Likewise, the DEX-induced decline in the protein expression of caspase-3 (Figure 4.4O) in VC-DEX *vs.* VC-CTL (P=0.018) was not observed following treatment with EPI or HA, with no significant differences noted in measures of caspase-3 protein expression between EPI-DEX *vs.* EPI-CTL (P=0.163) or HA-DEX *vs.* HA-CTL (P=0.654).

4.4.3. Mitochondrial protein expression

In CTL treated myotubes, treatment with HA significantly reduced total OXPHOS content (Figure 5A) compared to VC (HA-CTL *vs.* VC-CTL: P=0.041). Similarly, total OXPHOS content was significantly lower in HA-DEX compared to both VC-DEX and EPI-DEX (P=0.003 for both). Compared to the basal state, LEU-stimulation significantly decreased total OXPHOS protein content (Figure 4.5A) in both EPI (P=0.041) and HA (P=0.017) supplemented conditions, but not in VC (P=0.364). Indeed, total OXPHOS content was significantly lower following LEU-stimulation in HA *vs.*VC (P=0.002). In CTL treated myotubes, treatment with EPI (EPI-CTL *vs.* VC-CTL; P=0.038) or HA (HA-CTL *vs.* VC-CTL; P=0.002) significantly reduced TFAM protein content compared to VC (Figure 4.5D). Following DEX treatment there was a significant reduction in TFAM protein content (main effect: P<0.001), with no significant group (i.e. HA, EPI or VC) differences noted in TFAM protein content in DEX treated myotubes (all P>0.050). The final between group difference was noted in T-AMPKα, where treatment with HA significantly reduced T-AMPKα *vs.* VC (P=0.043; Figure 4.5E). There were no between group (i.e., HA, EPI or VC) differences noted in measures of PGC1α (Figure 4.5B), AMPKα^{T172} phosphorylation (Figure 4.5F) T-ACC (Figure 4.5G), or ACC^{S79} phosphorylation (Figure 4.5 H; all P>0.050). However, compared to CTL, DEX treated myotubes had a significantly reduced protein expression of PGC1α (main

effect: P=0.012, Figure 4.5B) and T-ACC (main effect: P<0.001, Figure 4.5G). Conversely, the protein expression of citrate synthase was significantly increased in DEX *vs.* CTL treated myotubes (main effect: P=0.009, Figure 4.5C).

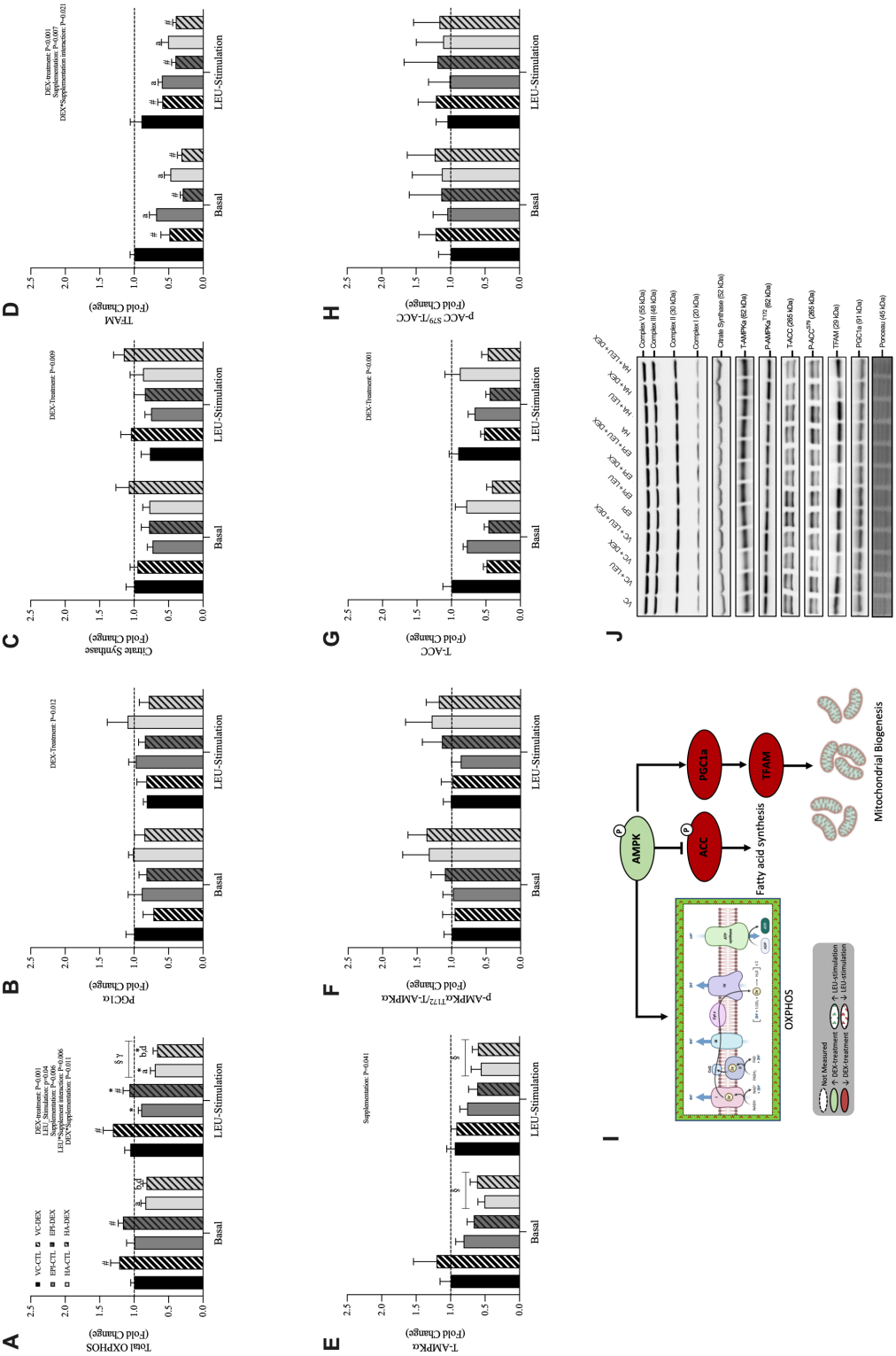

Figure 4.5 Oxidative metabolism protein expression in response to 24hr treatment.

(A) total OXPHOS (*n*=6), (B) PGC1α (*n*=5), (C) citrate synthase (*n*=6), (D) TFAM (*n*=6), (E) total AMPKα (*n*=6), (F) p-AMPKα^{T172}/T-AMPKα (*n*=6), (G) total ACC (*n*=6), (H) p-ACC^{S79}/T-ACC (*n*=6), (I) schematic overview of alterations in measured oxidative metabolism markers following 24hr treatment, (G) western blot representative image of oxidative metabolism markers. Data are presented as means ± SEMs and were analysed using a 3-factor mixed-model ANOVA. Black bars represent VC, dark grey bars represent EPI, light grey bars represent HA, striped bars represent DEX and plain bars represent CTL. * LEU-stimulated significantly different from basal at the P<0.05 level. $^{\#}$ DEX-treated significantly different from CTL-treated at the P<0.05 level. § supplementation group significantly different from VC group at the P<0.05 level. $^{\gamma}$ supplementation group significantly different from EPI group at the P<0.05 level. ^{a} significantly different from VC-CTL at the P<0.05 level. ^{b} significantly different from VC-DEX. ^{d} significantly different from EPI-DEX at the P<0.05 level. ACC; acetyl-CoA carboxylase, AMPKα; 5' AMP-activated protein kinase, CTL; 24hr control (no atrophic stimulus), DEX; 24hr, 100μM dexamethasone treatment, EPI; 24hr, 25μM epicatechin supplementation, HA; 24hr, 25μM hippuric acid supplementation, LEU; 90mins of 5mM leucine stimulation, PGC1α; peroxisome proliferator-activated receptor gamma coactivator 1-alpha, TFAM; Mitochondrial transcription factor A, VC; Ethanol matched vehicle control.

4.5. Discussion

Skeletal muscle atrophy results in declines of physiological functioning and metabolic health, highlighting a need for effective strategies to combat its progression. Here, we report that 24h DEX treatment significantly reduced myotube diameter in fully differentiated C2C12 myotubes and concomitant treatment with EPI or HA abrogated these DEX-induced alterations in myotube diameter. Mechanistically, DEX-induced reductions in myotube diameter were accompanied by declines in basal MPS *vs.* CTL treated cells. However, these DEX-induced declines in basal MPS were mitigated following a co-incubation with EPI or

HA treatment, whereas there were no significant differences from CTL treated myotubes. Finally, mitochondrial metabolism was altered in the atrophy-like conditions, with the protein expression of PGC1α, ACC and TFAM significantly reduced following 24h DEX treatment. However, concomitant treatment with EPI or HA did not prevent these DEX-induced alterations in mitochondrial metabolism.

Polyphenolic compounds have been identified as effective anti-atrophic agents following DEX treatment (29-32). Here we investigated the ability of the native flavanol EPI to preserve myotube diameter in atrophy-like conditions. Due to the reduced bioavailability of EPI *in vivo* (33) and the importance of colonic metabolites in the manifestation of phenotypic changes following flavanol supplementation (22, 33), we also investigated the ability of the 3/1C RFM metabolite HA to protect against DEX-induced atrophy. In line with previous literature (34), we report that 24h DEX treatment was successful at inducing a ~41% decline in myotube diameter *vs.* CTL. Interestingly, in CTL treated myotubes there was a marginal, albeit significant decline in myotube diameter following concomitant treatment with EPI or HA, compared to VC. However, in atrophy-like conditions (i.e. co-incubation with DEX), EPI or HA treatment abrogated DEX-induced declines in myotube diameter compared to VC. The marginal discrepancy between the CTL and DEX states suggest that the ability of flavanols to positivity influence protein metabolism may be context dependent and is suggestive that in these C2C12 myotubes flavanol metabolites act to offset atrophy through pathways divergent to those promoting hypertrophy in non-catabolic conditions (i.e. through alterations in catabolic signalling as discussed below). Since HA is present in circulation from 12-48h following cocoa ingestion at a relatively high concentration (21), these novel results indicate that HA may account for a fraction of the observed bioactivities following flavanol supplementation, especially in chronic supplementation protocols where cocoa is provided every 12-24h (i.e.(10)). Therefore, our findings extend on previous

investigations (5, 6) that highlight the importance of the splanchnic metabolism of cocoa following oral ingestion and offer novel insights as to how cocoa supplementation could support skeletal muscle health in conditions of atrophy, despite the poor bioavailability of its main bioactive compound, EPI.

A decline in postabsorptive, postprandial and exercise-induced MPS rates, combined with normal and/or dysregulated MPB, underscores the skeletal muscle atrophy that accompanies aging (35), inactivity (36, 37) and diseased states (38). We hypothesised that EPI and its metabolite HA would similarly maintain myotube diameter in response to DEX treatment, through the preservation of basal and LEU-stimulated MPS rates. We report that the 24h DEX treatment induced a ~34% decline in basal MPS, which was accompanied with significant declines in the phosphorylation of AKT^{S473}, AKT^{T308}, $mTORC1^{S2448}$, $S6K1^{T389}$ and $RPS6^{s240/244}$ in response to DEX treatment *vs.* CTL treated myotubes. Concomitant treatment with EPI or HA was able to abrogate the DEX-induced reductions in MPS rates to a similar extent. However, the protective effects of EPI and HA against the DEX-induced decline in MPS was not reflected in the phosphorylation of key anabolic signalling intermediates. This finding was perhaps surprising since previous literature reported significant increases in markers of myogenesis following EPI treatment (17). Nevertheless, our novel finding that HA was able to preserve MPS rates to a similar extent as the natural flavanol EPI furthers our understanding to the importance of EPI metabolites present in circulation for relatively long durations (~24h) following supplementation in the regulation of myotube metabolism. Furthermore, these results may explain discrepancies between acute and chronic *in vivo* supplementation interventions. Acute interventions have measured MPS rates over a 4-6h period post cocoa ingestion (i.e. (39)). Due to the differences in absorption rates of gut metabolites, these acute interventions likely only measure the effectiveness of the fast-absorbing structurally related epicatechin metabolites (SHREMs) on skeletal muscle

metabolism (33). However, it is likely, due to the differences in the chemical structure between metabolites, that each metabolite will have divergent effects on skeletal muscle metabolism and may explain why chronic studies (which account for all metabolites) have shown improvements in muscle function (10, 11), yet acute interventions do not show any alterations in muscle protein metabolism despite preferential changes to muscle vasculature (39). As such, the present findings highlight the need for further research to determine the different roles of flavanol metabolites on *in vivo* skeletal muscle protein metabolism in order to optimise supplementation protocols to support adaptation.

Given evidence of the central role of impaired muscle anabolic responsiveness in conditions of atrophy (3-5, 28), we also examined the ability of EPI and HA treatment to offset DEX-induced alterations in LEU-stimulated MPS rates to provide some translation to evidence from *in vivo* human models. We have recently demonstrated the utility of this approach, detecting a reduced anabolic responsiveness to an acute LEU-treatment (5mM) in C2C12 myotubes treated in old *vs.* young serum (40). Herein, we were unable to detect any DEX-induced alterations in LEU-stimulated MPS rates or the protein expression of key anabolic signalling markers *vs.* CTL treated myotubes. Similarly, previous research has demonstrated that 1h of LEU treatment (5mM) was sufficient to overcome DEX-induced decrements in the protein expression of key markers of anabolic signalling (41). Therefore, it is plausible that DEX treatment does not induce declines in anabolic responsiveness, instead exerting an atrophic effect primarily by dampening basal MPS. The fact that myotube atrophy occurred independently of any 'anabolic resistance' in the present study, could be viewed as a limitation when utilising this model to examine the effectiveness of treatments to offset muscle atrophy *in vivo*. That said, declines in basal MPS play an important role in the manifestation of muscle atrophy in human disease (5, 42, 43), highlighting the utility of this model as a means to investigate potential countermeasures to muscle atrophy.

Muscle atrophy under catabolic conditions is also regulated by an increase in muscle protein breakdown, where rates exceed those of MPS promoting a negative net protein balance (44, 45). Within skeletal muscle, two main degradation systems are suggested to be activated under atrophy-inducing conditions; (1) the ubiquitin proteasome system and, (2) the autophagy lysosome system (44). In line with previous literature, which noted significant increases in MPB following DEX treatment (46), we hypothesised that 24h DEX treatment would significantly increase the protein expression of key markers of catabolic signalling and concomitant treatment with EPI or HA would prevent such alterations. Here, we report that 24h DEX treatment significantly increased the protein expression of key markers in the ubiquitin proteasome system (i.e. MAFbx and MURF1) and promoted significant alterations in the autophagy lysosome system (i.e. LC3 α/β II:I and caspase-3) compared to CTL treated myotubes. Similar to previous research in DEX and lipopolysaccharide treated C2C12 myotubes, concomitant treatment with EPI or HA was unable to prevent alterations in MAFbx or MURF1 protein expression (18, 47). However, the concomitant treatment did abrogate the DEX-induced alterations in LC3 α/β II:I and caspase-3 protein expression. This is important as the autophagy lysosome system is active under catabolic conditions to degrade and recycle proteins (48). Furthermore, under conditions of oxidative stress, autophagy can trigger the selective degradation and removal of mitochondria, through mitophagy (for a review see (49)). Previously, EPI has been demonstrated to exhibit anti-oxidative properties stimulating the protein expression of multiple anti-oxidant enzymes (i.e. SOD and catalase (50)). In combination with the aforementioned data, it is plausible that flavanols may act to preserve myotube diameter partially through this oxidative stress-autophagy mechanism, although research is warranted to examine this further.

Mitochondria are critical to contractile function (51, 52) and are proposed to play an important role in muscle protein turnover, which has high ATP requirements (53). In atrophy-

like conditions, previous research reports significant declines in mitochondrial protein synthesis (28, 54) as well as alterations in mitochondrial metabolism (55-58). Polyphenolic compounds have been demonstrated to enhance mitochondrial function (16) and protect against mitochondrial insult (22). Therefore, we hypothesised that EPI or HA treatment would preserve myotube diameter, partially through the maintenance of mitochondrial metabolism in atrophy-like conditions (i.e. DEX treatment). In line with previous research, we report alterations to mitochondrial metabolism in atrophy-like conditions. Specifically, we report a significant reduction in the protein expression of PGC1α, ACC and TFAM in response to 24h DEX treatment *vs.* CTL treated myotubes. In contrast to previous literature (16, 22), we did not detect any preservation of these markers in myotubes co-incubated with EPI or HA. These results were somewhat surprising and may be due to differences in culture conditions between studies. For example, previous literature has utilised immortalised cell lines, cultured in media containing higher glucose concentrations (e.g. (16, 17, 32)), which promotes a heavy reliance upon anaerobic glycolysis (59) and mitochondrial dysfunction (60-63), compared with the more oxidative phenotype of immortalized cell lines cultured in media containing lower glucose concentrations (64). Therefore, we speculate that the contrasting findings between the current and previous studies in response to DEX treatment may be secondary to differences in basal metabolism during growth and differentiation. To limit type I errors, the current study focused on the measurement of key markers of mitochondrial function. Therefore, we were unable to measure the protein expression of markers involved in Ca^{2+} handling or oxidative stress, both which have been purported as secondary mechanisms of mitochondrial function improvements in basal conditions following treatment with polyphenolic compounds (50, 65). Therefore, in combination with the aforementioned alterations in autophagic signaling it is possible that flavanol metabolites

could improve myotube metabolism via these mechanisms, although this remains to be elucidated.

Despite the importance and novelty of the current findings, the authors note that a limitation of the current study was the absence of measurement of additional EPI metabolites (i.e., SHREMs or additional RFMs) due to the lack of commercial availability of these compounds. Further research utilizing an *ex vivo* approach with multiple serum samples obtained over the 12h post cocoa supplementation may now be required to extend on the current findings. That said, this study provides important and novel insights into the role of the 3/1C RFM metabolite HA in preserving myotube diameter and MPS rates during atrophy-like conditions in C2C12 myotubes.

In summary, our data demonstrate that EPI was able to exert anti-atrophic effects on C2C12 myotubes and the colonic metabolite HA was able to preserve myotube diameter and MPS rates to a similar extent to its natural flavanol (i.e., EPI). Alterations in basal MPS and myotube diameter following co-incubation of EPI/HA, occurred independently to alterations in markers of mitochondrial function, with a possible attenuation of MPB markers also contributing to preserve myotube diameter. Overall, our data provides important insight on the role of flavanol gut metabolites on skeletal muscle. Furthermore, the current study highlights the significance of accounting for slow-absorbing colonic metabolites (e.g., HA) in experimental protocols, providing evidence against the use of acute *in vivo* interventions to determine the effectiveness of cocoa supplementation to alter muscle metabolism. Future research needs to be conducted in an *in vivo* model of atrophy to confirm the ecological validity of chronic cocoa supplementation.

4.6. References

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5. GENERAL DISCUSSION

5.1. Introduction

Musculoskeletal disuse is associated with a plethora of adverse physiological consequences, including a loss of muscle mass (1-6) and impaired aerobic capacity (7, 8). Disuse induced atrophy is a consequence of an impairment in MyoPS, characterised by a decline in the anabolic responsiveness of skeletal muscle to nutritional and/or exercise stimuli (5, 9, 10). Primarily, anabolic resistance is suggested to be driven by alterations in mTORC1 signalling (10, 11), with secondary alterations in oxidative metabolism recently purported to also be involved (12-15). Although there has been significant advancements in our understanding of the mechanisms underpinning these alterations in MyoPS rates, research continues to investigate suitable countermeasures to prevent disuse induced muscle atrophy. Therefore, the purpose of this thesis was assess the mechanisms of and nutritional countermeasures to overcome disuse muscle atrophy. Specifically, we aimed to:

- Determine the effect of 7d SR on mitochondrial function and skeletal muscle insulin signalling in young adults (**Chapter 2**).
- Determine the effectiveness of a novel leucine supplementation strategy to offset disuse induced atrophy in young adults (**Chapter 3**).
- Determine the ability of EPI and its metabolite HA to offset alterations in myotube metabolism in an *in vitro* model of atrophy (**Chapter 4**).

The aim of this chapter is to provide a brief summary of the key findings noted in each of the experimental chapters described in this thesis. Based on the data outlined in **Chapter 2** we will discuss the effect of musculoskeletal disuse on mitochondrial functioning and formulate directions for future research. For the data outlined in **Chapter 3**, we will discuss the effectiveness of leucine to offset disuse induced alterations in myofibrillar and mitochondrial protein synthesis rates and formulate recommendations for future research in this area.

Finally, we will discuss the findings noted in **Chapter 4** and formulate directions for the translation of results into a human experimental model.

5.2. Musculoskeletal disuse and mitochondrial function

Mitochondria are critical to contractile function (16) and fuel utilisation (17, 18), with dysfunctional mitochondria implicated in the pathology of insulin resistance (19) and reductions in functional capacity (20-22). More recently, dysregulated mitochondria have also been implicated in the pathology of disuse atrophy (12-15). In severe models of disuse (i.e. bed rest and limb immobilisation), significant alterations in respiratory capacity (10) and the protein content of key proteins involved in oxidative metabolism (7, 12, 23, 24) have been noted. However, the metabolic consequences of less severe physical inactivity (i.e. short term SR) remain to be established. Understanding whether short-term SR dampens mitochondrial functioning is vital since daily step count is ~750 steps.d^{-1} during acute periods of illness at home or in hospitalisation (25). Therefore, in **Chapter 2** we conducted a study that aimed to determine whether the protein expression of key skeletal muscle markers of mitochondrial/oxidative metabolism and insulin-mediated signalling would be altered over 7d of SR in young healthy males. Our data indicate that 7d of SR in young and healthy males reduced the activity of citrate synthase without altering the protein expression of key markers of skeletal muscle oxidative metabolism or insulin mediated signalling.

There has been much debate regarding the role of mitochondria in the onset of disuse-induced atrophy. Mitochondria are critical to contractile functioning (16), and are suggested to influence rates of myofibrillar protein synthesis (MyoPS) through the production of reactive oxygen species (ROS; (26)). In **chapter 2**, we did not note any significant alterations in mitochondrial protein content despite a reduction in citrate synthase activity and a reduction in daily MyoPS rates (see (27) for further data). Collectively, this suggests that in a

younger population undergoing a period of 7d SR alterations in MyoPS occurred independently to alterations in mitochondrial functioning, highlighting that alterations in abundance of mitochondrial proteins are not a necessity for reductions in MyoPS rates, at least in this specific population. Despite the lack of alterations in mitochondrial functioning noted in **Chapter 2**, we cannot rule out a role of mitochondria in the pathology of atrophy in more severe models of disuse (i.e. limb immobilisation/bed rest). In **Chapter 2**, we did not note any significant alterations in the protein content of CAMKII, PERM1, PGC1α (Figure 2.3), markers of oxidative stress (i.e. NOS, mnSOD; Figure 2.3) or in markers of skeletal muscle insulin signalling (e.g. IR, Akt, GLUT4; Figure 2.5). This suggests that the reduction in physical activity was not significant enough to alter calcium handling, fuel utilisation or oxidative stress in skeletal muscle, which are key signalling mechanisms linking dysfunctional mitochondria and disuse atrophy (28, 29). However, in models where physical activity is ceased, it may be speculated that these pathways are disrupted.

A secondary aim of **Chapter 3** was to determine whether 7d lower limb immobilisation in young adults altered mitochondria functioning. Here, we noted significant declines in MitoPS rates (Figure 3.6) and a decline in the protein content of PGC1α and OXPHOS complex II and III (Figure 3.7)**.** Interestingly in **Chapter 3**, we also noted declines in postprandial MyoPS but not postabsorptive MyoPS (Figure 3.6). Others have confirmed these findings, noting a similar decline in MitoPS that occurred simultaneously to declines in MyoPS following 10d immobilisation (30). Overall, this suggests that in more severe models of disuse where physical activity is completely ceased, mitochondrial metabolism may have some role in the development of atrophy. However, further research is still warranted to determine whether the disruption in mitochondrial signalling that occurs in models of severe disuse promotes reductions in MyoPS and muscle mass, or whether it is just an accompanying consequence of a reduction in ATP requirements. Nevertheless, due to the

importance of mitochondria for optimal whole-body metabolism (17, 18) and functional capacity (20-22), the decrements in mitochondrial metabolism noted in this thesis cannot be ignored and targeted interventions that aim to attenuate these alterations must be of priority.

5.3. Leucine supplementation as a countermeasure for musculoskeletal disuse atrophy

Leucine is an essential amino acid that is suggested to be an important mediator of the MyoPS response to feeding (31-35). However, the role of leucine to prevent alterations in muscle metabolism during disuse events is debated. In previous research, 2.5 $g.meal^{-1}$ of supplemented leucine was unable to attenuate the loss of muscle mass and function following short-term disuse (36), but high doses of leucine (i.e. 0.06 $g.kg.^{-1}.meal^{-1}$) are suggested to attenuate muscle atrophy and strength losses during short-term bed rest in middle aged and older adults (37, 38). Since $15g.d^{-1}$ of supplemental leucine enhanced integrated MyoPS rates in free-living conditions, independent to contractile activity (39), **Chapter 3** of the current thesis aimed to determine whether applying a $15g \cdot d^{-1}$ leucine supplementation protocol would attenuate disuse atrophy in a young adult population. Here, we report that 7d of limb immobilization in young adults significantly reduced leg fat free mass by ~4% (Figure 3.3) and isometric strength by ~28% (Figure 3.4) in the immobilized limb, with no significant reductions noted in the control limb. Mechanistically, these declines in fat free mass occurred in parrel to a significant decline in type II fiber CSA and impaired postprandial MyoPS and postabsorptive MitoPS rates (Figure 3.6) in the immobilized limb compared to the control. Furthermore, our data indicated that even a high-dose leucine supplementation protocol ($15g.d^{-1}$) did not appear to attenuate the declines in fat free mass or strength associated with the 7d immobilization intervention in young healthy male adults.

Despite the potency of leucine to stimulate MyoPS in a free-living population (39), the data presented in **Chapter 3** in combination with previous research (i.e. (36)) provides

little evidence to support the use of leucine alone as a dietary intervention to prevent or even attenuate muscle atrophy in a young healthy population. In free living conditions, it has been suggested that leucine acts to potentiate the MyoPS response through the activation of mTORC1 signalling components (40) and this may explain why leucine has failed to improve MyoPS in young adults during disuse. In models of disuse, it is evident that mTORC1 signalling does not appear impaired in a younger population, either during fasted conditions (5, 9, 13, 41, 42) or in response to feeding (3, 9, 13). Our data in **Chapter 3** agree with this hypothesis as we did not note any alterations in mTORC1 signalling, despite significant reductions in postprandial MyoPS. Collectively, these data question whether the mechanisms underpinning atrophy are simply the reverse to those promoting hypertrophy, or whether they are indeed divergent. If this hypothesis holds true, it may explain why nutritional interventions (e.g., leucine supplementation) that are successful in promoting a positive NBAL in free-living conditions do not translate to promote a positive NBAL in periods of disuse.

The differences in signalling patterns in free-living conditions *vs.* musculoskeletal disuse may be overcome using a leucine rich essential amino acid supplement. There is evidence to suggest that the thrice daily consumption of an essential amino acid mixture (containing 4g leucine) can attenuate disuse induced muscle atrophy in young adults (43), suggesting that a full complement of amino acids may be required to protect MyoPS and muscle mass during disuse in a young adult population. Indeed, it is suggested that a positive NBAL cannot be achieved without a complete essential amino acid profile (44) and therefore, it is likely that leucine alone may not be sufficient to potentiate the convergent signalling pathways to attenuate muscle atrophy during disuse in young adults.

5.4. Polyphenol supplementation as a countermeasure to prevent musculoskeletal atrophy

Cocoa is a food that is rich in polyphenols and has been suggested to promote clinically relevant improvements in cardiovascular related outcomes (45) and offer protection against cognitive aging (46). EPI is the most abundant flavanol in cocoa (47) and is suggested to promote increases in myotube width (48) and reduce atrogene expression (49). However, EPI has a poor bioavailability *in vivo* and is metabolised into smaller bioactive compounds (50). HA is a long-acting EPI metabolite that present in circulation for up to 48h post EPI ingestion (50). *In vitro*, HA shown favourable observations *in vitro* through the stimulation of glucose metabolism and preservation of mitochondrial function following insult in C2C12 myotubes (51). Consequently, in **Chapter 4** we investigated the effects of EPI and HA on skeletal muscle morphology, muscle protein turnover and mitochondrial metabolism within an *in vitro* model of skeletal muscle atrophy. In **Chapter 4**, we reported that 24h DEX treatment significantly reduced C2C12 myotube diameter and concomitant treatment with EPI or HA abrogated these DEX induced alterations (Figure 4.2). DEX induced reductions in myotube diameter were accompanied by declines in basal MPS *vs.* CTL treated cells (Figure 4.3). However, these DEX induced declines in basal MPS were mitigated following a co-incubation with EPI or HA treatment, whereas there were no significant differences from CTL treated myotubes (Figure 4.3). Finally, mitochondrial metabolism was altered in the atrophy-like conditions, with the protein expression of PGC1α, ACC and TFAM significantly reduced following 24h DEX treatment (Figure 4.5). However, concomitant treatment with EPI or HA did not prevent these DEX induced alterations in mitochondrial metabolism.

The primary outcome measure in **Chapter 4** was to assess whether EPI/HA could offset DEX induced atrophy in C2C12 myotubes. Our data suggests that EPI/HA treatment protected against DEX induced atrophy through the preservation of basal MPS rates (Figure

4.3) and autophagic signalling (Figure 4.4). Interestingly, in the absence of DEX, both EPI and HA treatment promoted a small, but significant decline in myotube diameter (Figure 4.2). Due to the oxidative potential of these supplements, this result is perhaps unsurprising, but it does suggest that EPI/HA are likely only beneficial in attenuating atrophy under catabolic conditions (i.e., treatment with DEX) and not in promoting hypertrophy under 'normal' conditions, albeit in an *in vitro* model. Another interesting observation noted in **Chapter 4**, is that we did not witness a recovery in mTORC1 signalling following co-incubation with EPI or HA and DEX (Figure 4.4)., despite the attenuation of DEX induced declines in MPS rates. This finding was perhaps surprising and warrants further mechanistic research to reconcile the mechanisms to explain how flavanol supplementation can attenuate declines in MPS rates independently to mTORC1 signalling. Nevertheless, the success of both EPI and HA to offset DEX induced declines in myotube diameter and MPS rates confirms the importance of translating this research into a human model.

To induce a model of atrophy, we utilised the corticosteroid DEX, which was selected over *ex vivo* approaches (e.g., the use of human serum) as we wanted to isolate atrophy from systemic factors. However, in **Chapter 4,** the DEX treatment protocol did not induce declines in leucine stimulated MPS rates, despite inducing a reduction in basal MPS rates. Anabolic resistance is an important mechanism underpinning disuse atrophy in humans (5, 9, 10), so it was surprising that DEX did not induce similar declines. However, this does highlight that atrophy in response to differing physiological conditions (i.e. ageing, disuse, disease and corticosteroids) likely doesn't manifest from a standalone common mechanistic pathway, with differences in intracellular mechanisms potentially diverging between physiological conditions. Therefore, this must be considered in *in vitro* mechanistic studies to ensure the 'end' population in question has the best representation in culture, with the

exception that no *in vitro* model will be able to recapitulate the integrative human model it seeks to emulate.

The use of a glucose concentrations of 5mM was utilised to enhance the physiological relevance of the data produced in **Chapter 4**. However, we speculate that the lack of improvements in mitochondrial metabolism in response to EPI or HA treatment may be the result of our culture conditions. As previously outlined, prior research has utilised 25mM [glucose], which enhances the reliance on anaerobic glycolysis (52) and promotes a basal level of mitochondrial dysfunction (53-56), compared with the more oxidative phenotype of immortalized cell lines cultured in media containing lower glucose concentrations (57). Therefore, research that has previously described alterations in mitochondrial function in response to EPI or HA treatment likely suggest that EPI/HA can protect against mitochondrial dysfunction in a metabolically compromised tissue. Consequently, the differences noted between the current study and previous literature (e.g. (58)) are likely secondary to differences in the basal metabolic state of the myotubes (i.e. healthy *vs.* metabolically compromised).

5.5. Clinical and practical implications

In **Chapter 2,** we reported that 7d of SR in young and otherwise healthy individuals did not alter the abundance of skeletal muscle proteins involved in mitochondrial metabolism or insulin sensitivity. The lack of alterations in these key signaling intermediates is clinically promising, as it suggests even a small amount of activity (i.e. a brief, slow walk each day) during a period of illness may help to prevent alterations in mitochondrial functioning in this population, which is crucial for the maintenance of functional capacity (20-22).

In **Chapter 3** we reported that high-dose leucine supplementation did not prevent disuse-induced losses in fat free mass and strength. The clinical implications of this finding

are that leucine supplementation (even at a high dose) should not be used in practice to offset the negative physiological adaptations to disuse in a young and otherwise healthy population.

In **Chapter 4**, we reported that co-incubation with EPI or HA attenuated DEX induced declines in myotube diameter, through the preservation of MPS rates and autophagic signaling. These findings provide rationale to conduct research examining whether cocoa supplementation can offset muscle atrophy in a human model in order to generate clinically relevant outcomes. Furthermore, the lack of significant differences between EPI and HA groups highlights the importance of HA to mediate the positive effects of cocoa supplementation on skeletal muscle metabolism. Due to the peak of HA in circulation occurring at 12-24h following cocoa ingestion, the present data suggests that research should utilize a chronic experimental intervention, where cocoa supplementation is provided 1-2x/d at regular 12-24h intervals to maximize the effectiveness of this supplementation intervention to offset muscle atrophy.

5.6. Experimental limitations

In **Chapter 2**, we were unable to assess mitochondrial respiratory capacity and the activity of OXPHOS complexes due to the requirement of fresh/permeabilised tissue (e.g. oroboros) and technical failures (e.g. additional enzymatic assays). Therefore, although this study provides significant mechanistic insight into mitochondrial functioning following short-term SR, whether alterations in ATP production accompany the reduction in citrate synthase activity (noted in Figure 2.2) remains to be determined. In **Chapter 3,** we were unable to assess integrated MyoPS rates (via D_2O) due to financial constraints and we also did not measure the acute MyoPS response to the supplementation (i.e. 5g of leucine). Together, this precluded our ability to determine whether the 5g of leucine provided as a supplement maintained/enhanced MyoPS rates during the immobilisation intervention. Nonetheless, we

did provide important clinical insights into the inability of high-dose leucine to prevent alterations in muscle mass and strength. Both **Chapter 2** and **Chapter 3** were conducted utilizing males only, this is an experimental limitation as it reduces the applicability of this research to a real world population and further research should be conducted including both males and females. A limitation of **Chapter 4** was that we were unable to assess the impact of additional metabolites (i.e. SHREMs and additional RFMs) on skeletal muscle metabolism due to the lack of commercial availability of these compounds. Therefore, although we were able to outline the importance of the metabolite HA to offset muscle atrophy, we were unable to determine whether additional metabolites had a similar effectiveness. Despite the experimental limitations of the experimental chapters outlined above, this thesis provides a significant advancement in understanding of the mechanisms underpinning disuse atrophy as well as the effectiveness of leucine supplementation and potential effectiveness of cocoa flavanols as countermeasures against disuse atrophy.

5.7. Future research

To further our understanding regarding the ATP requirements of SR, future research should aim to utilise high-resolution respirometry to determine maximal and submaximal respiration rates in response to short-term reduced ambulation in a young population. Furthermore, research determining the effectiveness of an essential amino acid mixture that contains 5g leucine to offset disuse atrophy in young adults following short-term immobilisation is warranted. This knowledge will enable us to determine whether the full essential amino acid cocktail would be more successful to offset atrophy in this population, compared to the 5g of leucine alone. Finally, an original aim of this thesis was to translate **Chapter 4** into a human immobilisation model and to acquire primary cultures from human participants to further enhance our mechanistic understanding. However, due to the COVID-19 pandemic this final

experimental approach was not feasible. Notwithstanding, our promising *in vitro* results lay the foundation for future research to conduct a whether cocoa supplementation can attenuate disuse induced alterations in NBAL and attenuate atrophy under catabolic conditions (i.e., disuse).

5.8. Conclusions

In this thesis we have explored the mechanisms of and potential countermeasures to musculoskeletal disuse atrophy. The original findings in this thesis suggest that in response to 7d SR in young adults, there are no significant alterations in the protein content of skeletal markers of oxidative metabolism or insulin sensitivity, despite a significant reduction in citrate synthase activity. Furthermore, in response to 7d of immobilisation in young adults, we found a significant reduction in leg fat free mass and strength, which was underpinned by significant reductions in both postprandial MyoPS and basal MitoPS measures. Our results indicate that 15g.day^{-1} leucine supplementation was unable to counteract the disuse induced decline in fat free mass and strength. In an *in vitro* model, our data demonstrate that a 24h treatment with DEX induces a significant decline in myotube diameter and MPS rates, underpinned by alterations in anabolic and catabolic signalling pathways. Furthermore, we demonstrated that a 24h co-incubation with EPI or HA attenuated these declines in myotube diameter and MPS rates but did so independently to alterations in anabolic signalling processes.

Collectively, this thesis offers a significant insight into the mechanisms and counter mechanisms to disuse atrophy. We were the first to establish that mitochondrial protein content does not appear to be impaired by 7d SR in young adults. Furthermore, we provide novel data to suggest a high-dose leucine supplementation does not attenuate disuse atrophy in young adults. Finally, our novel data on EPI and HA treatment in an *in vitro* model of

atrophy provides significant rationale for a human intervention to determine whether cocoa flavanols can offset muscle atrophy. These original findings improve our understanding of the mechanisms underpinning disuse atrophy and the use of nutritional countermeasures to attenuate atrophy in a young adult population.

5.9. References

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