THE IMPACT OF n-3 PUFA SUPPLEMENTATION ON HUMAN SKELETAL MUSCLE METABOLISM.

By

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DEDICATION

I dedicate it all to the loving memory of my *best* friend…

ABSTRACT

The time course of this increase in muscle n-3 PUFA composition and anabolic protein expression is currently unknown. In **Chapter 2** of this thesis ten healthy male participants consumed $5g \cdot d^{-1}$ of n-3 PUFA-enriched fish oil for 4 weeks. Muscle biopsies samples were collected in the fasted, rested state 2 weeks prior, immediately before (Week 0), at Week 1, Week 2 and Week 4 after initiation of fish oil supplementation for assessment of changes in lipid composition and expression of anabolic signalling proteins over time**.** Muscle lipid profile, (% total n-3 PUFA/total fatty acids) increased from W0 to W2 (3.8 ± 0.2 to 5.1 ± 0.3 %) and continued to rise at W4 (6.7 ± 0.4 %). Total protein content of FAK increased from W0 to W4 (3.9 ± 1.5 fold) whereas total mTOR was increased from W0 at W1 (2.4 ± 0.6 fold) with no further significant increases at W2 and W4. For the first time this study demonstrates that oral fish oil consumption results in an increase of n-3 PUFA levels in human skeletal muscle that is associated with increases in the expression of anabolic signalling proteins.

Our understanding of the anabolic signalling process that underpins muscle protein synthesis has been advanced by the application of the WB technique. However, the semi-quantitative nature and poor dynamic range associated with the WB technique may lead to incongruence regarding the molecular response of skeletal muscle to anabolic stimulation. **Chapter 3** of this thesis developed and applied a quantitative *in vitro* [γ-^{32}P] ATP kinase assay (KA) alongside a traditional WB methodology to assess p70S6K1 signalling responses in human skeletal muscle to RE and protein feeding. Following validation in tissue culture with rapamycin and optimization of the assay in human skeletal muscle, this methodology was tested in a physiologically relevant context. In this regard, six males performed unilateral resistance exercise (RE) followed by the consumption of 20 g of protein. Skeletal muscle biopsies were

obtained at pre-RE, at 1 h and 3 h post-RE. In response to RE and protein consumption, p70S6K1 activity was significantly increased from pre-RE at 1 h and 3 h post-RE (8.84 ± 0.78 to 17.18 ± 2.62 and 15.62 ± 3.12 μU/mg). However, phosphorylated p70S6K1^{thr389} was not significantly elevated. To assess if a combined stimulus of RE and feeding can influence AMPK activity we directly measured AMPK activity. AMPK activity was suppressed from pre-RE at 3 h post-RE (24.15 ± 1.6 to 15.64 ± 1.07 mU/mg), whereas phosphorylated ACC^{ser79} was unchanged. These data therefore highlight the utility of the KA to study skeletal muscle plasticity.

Previous studies have shown that ingestion of n-3 PUFA potentiates the phosphorylation of mTORC1 and associated kinases in response to nutrition. However, no study has identified whether n-3 PUFA supplementation potentiates anabolic kinase activity when RE is performed prior to nutrient provision. In **Chapter 4** of this thesis, twenty healthy males consumed 5g·d^{-1} of either fish oil (FO) or coconut oil (CO) capsules for 8 weeks. Muscle biopsy samples were collected in the fasted, rested state before and after 8 weeks of supplementation for assessment of changes in lipid composition. Following 8 weeks of supplementation muscle samples also were obtained at rest (Rest), post RE in both the exercise leg (Post-RE) and the rested leg (Pre-FED) and also at 3 h post RE and protein feeding from both the exercise leg (3 h post-REF) and rested leg (3 h post-FED). There was a 2-fold increase in muscle (5.53 ± 0.3 to 11.16 ± 0.45 % of total fatty acids) n-3 PUFA composition after supplementation in the FO group but no change in the CO group. Following supplementation there was an increase in p70S6K1 activity at 3 h post-REF from Rest in the CO group (5.6 ± 1.4 to 12.2 ± 2.1 μU/mg) but no change in the FO group. In the CO group, AMPKα2 was significantly increased at Post-RE from Rest (3.7 ± 0.7 to 9.9 ± 2.0 mU/mg). These data show that 8 weeks of n-3 PUFA enriched fish oil supplementation suppresses the activity of p70S6K1 in response to RE and protein feeding.

Thank you…

I would like to thank my supervisors Kevin, Stuart and Lee for their guidance and compassion during my Ph.D. studies. I also would like to thank James for getting me started and sparking my fascination in the study of skeletal muscle metabolism. I am a product of my education to which you all have contributed and I will not forget the many lessons that I have learnt both inside and outside the lab.

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Finally, and most importantly, I would like to thank my family, Mum, Dad, Kate and Andrew for all your help. To my Dad, a rock of integrity, stability and an exemplary role model for character, above all I hope that I have made you most proud. I will now move forward with a smile on my face, equipped with all the lessons that I have learnt, essentially that *balance* and *family* are key.

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ABBREVIATIONS

4EBP1	4E-binding protein-1
AA	Arachidonic acid
ACC	Acetyl-CoA carboxylase
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
Deptor	DEP domain-containing mTOR-interacting protein
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
eEF	Eukaryotic elongation factor
eIF	Eukaryotic initiation factor
EPA	Eicosapentaenoic acid
FAK	Focal adhesion kinase
GTP	Guanosine-5'-triphosphate
HUFA	Highly unsaturated fatty acid
mLST8	Mammalian lethal with SEC13 protein 8
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
mRNA	Messenger RNA
mTOR	Mechanistic target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
NPB	Net protein balance
p70S6K1	Ribosomal protein of 70 kDa S6 kinase 1
PDK1	Phosphoinositide-dependent kinase-1
PGF2α	Prostaglandin F2α
PKB	Protein kinase B
PRAS40	Proline-rich Akt substrate of 40 kDa
PUFA	Polyunsaturated fatty acid
Raptor	Regulatory-associated protein of mTOR
RE	Resistance exercise
Rheb	Ras homolog enriched in brain
RNA	Ribonucleic acid
TSC	Tuberous sclerosis complex
WB	Western blot

LIST OF PUBLICATIONS

Published

McGlory C, Galloway SDR, Hamilton DL, McClintock C, Breen L, Dick JR, Bell JG, Tipton KD (2014) Temporal changes in lipid composition and anabolic proteins in human skeletal muscle with fish oil supplementation. Prostaglandins Leukot Essent. Fatty Acids. http://dx.doi.org/10.1016/j.plefa.2014.03.001i

McGlory C, White A, Treins C, Drust B, Close GL MacLaren DPM, Campbell IT, Philp A, Schenk S, Morton JP, Hamilton DL (2014) Application of the (γ-^{32}P) ATP kinase assay to study anabolic signalling in human skeletal muscle. J Appl Physiol 116:504-513.

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Abstracts

McGlory C, White A, Treins C, Drust B, Close GL MacLaren DPM, Campbell IT, Philp A, Schenk S, Morton JP, Hamilton DL. Application of the (γ-^{32}P) ATP kinase assay to study anabolic signalling in human skeletal muscle. *Proceedings of the European College of Sports Science, Barcelona, July 2013*.

McGlory C, Galloway SDR, Hamilton DL, McClintock C, Breen L, Dick JR, Bell JG, Tipton KD. Temporal changes in lipid composition of anabolic signalling proteins in human skeletal muscle with fish oil supplementation. *Proceedings of the Physiological Society, Edinburgh, July 2012*.

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CHAPTER 1 GENERAL INTRODUCTION

1.0 Introduction

Skeletal muscle plays a critical role in locomotion [1], metabolic health [2], and longevity [3]. Strategies to enhance skeletal muscle mass and strength therefore have obvious implications in both the athletic and clinical settings. One strategy proven to enhance skeletal muscle mass is resistance exercise (RE [for abbreviated nomenclature see page viii]). The mechanistic basis of RE-induced skeletal muscle hypertrophy is underpinned by the coordinated interaction between MPS and MPB. In healthy humans, RE results in a mild stimulation of MPB but a greater increase in MPS, such that muscle NPB is increased [4]. However, when RE is conducted in close temporal proximity to amino acid feeding, MPS is synergistically stimulated potentiating NPB [4-6]. Hence, repeated, episodic bouts of RE and protein feeding result in skeletal muscle hypertrophy over time that can occur at a typical rate of ~0.2% per day [7-9].

Although temporal changes in both MPS and MPB underpin skeletal muscle mass homeostasis, in many studies only assessments of changes in MPS in response to stimulation are made. The primary reason for choosing to assess rates of MPS in response to perturbation is that it is changes in MPS, and not MPB, that ultimately drive increases in skeletal muscle mass. Support for this contention emanates from data that demonstrate in response to stimulation, there is a significant (~80%) increase in MPS with minimal associated changes in MPB [5,10,11]. Moreover, the acute response of MPS to RE and feeding is known to be predictive of long-term increases in skeletal muscle mass [12]. However, it has been shown that some forms of dietary intake are known to affect MPB but these data are scarce [13].

Another reason that data pertaining to changes in MPB with nutrition and exercise are few is that assessing MPB, particularly during exercise, is technically challenging [14]. The requirement for a steady state rate of blood flow is oftentimes a prerequisite for the assessment of MPB [15] and therefore difficult during exercise. As such, it is important not to completely discount the role of MPB when evaluating the efficacy of an exercise intervention in studies that have assessed MPS alone.

In addition to amino acids, other nutritional interventions also may enhance the adaptive response to RE. Indeed, data from cell [16], rodent [17,18] and human models [19-22] all reveal a potential anabolic influence of n-3 PUFA on skeletal muscle. Studies conducted in rodents have shown that diets rich in n-3 PUFA can attenuate muscle atrophy during immobilisation [17] as well as enhancing anabolic signalling events [23]. Furthermore, two recent studies in humans from the same laboratory show that supplementing humans with n-3 PUFA-enriched fish oil for 8 weeks potentiates MPS in response to a hyperaminoacidemic-hyperinsulinemic clamp [21,22]. Those authors speculated that the anabolic influence of n-3 PUFA supplementation was related to changes in the n-3 PUFA composition of skeletal muscle that were induced by n-3 PUFA supplementation. However, the exact time course of the change in skeletal muscle lipid composition associated with n-3 PUFA supplementation remains unknown. In addition, no study has adequately assessed the impact of altered n-3 PUFA muscle lipid profiles on human skeletal muscle anabolism in response to a combined stimulus of RE and protein feeding.

Although a wealth of research now exists characterising the impact of RE and various nutritional interventions on muscle anabolism [4,7,22,24-27], the molecular mechanisms that underpin these adaptive responses are yet to be fully elucidated. What is known is that, in the context of MPS, a series of phosphorylation cascades involving the mTORC1/p70S6K1

signalling pathway play a crucial role [28-30]. These, along with other associated signalling events, facilitate the binding of ribosomes to mRNA [31,32], elongation of polypeptides and incorporation of the complete protein into skeletal muscle [4,33]. Under basal conditions ribosomes are positioned approximately 100 nucleotides apart on the mRNA [32] but in response to stimulation they can stack 27-30 nucleotides apart [34]. It is for this reason that translation initiation is widely considered the primary rate-limiting step in translational control [33]. As a result, many studies examining the impact of interventions on MPS have focused on translation initiation. Many measures of skeletal muscle anabolism revolve around these processes and it is therefore crucial to the rationale of this thesis that an overview of translational control is provided.

In this chapter the reader is initially presented with a brief overview of translation initiation as well as an outline of the molecular signals that mediate the creation of new muscle proteins in response to RE and amino acids. Subsequently, a critical review of existing studies that characterise the impact of n-3 PUFA on muscle anabolic responses will be made. In this regard, the aim of this chapter is to provide the reader with an introduction and rationale as to the nature of the studies contained within this thesis. This chapter will review human studies but where appropriate, data from other experimental models will be cited to substantiate the discussion points.

1.1 Translational control

Translational control can be defined as the molecular events that act in unison to regulate the rate of translation of an mRNA [35]. Eukaryotic translation is a complex process encompassing a series of intricate molecular events that are broadly divided into three stages:

initiation, elongation and termination. As translation initiation is believed to be the major rate-limiting step of translation [32], translation initiation will be a primary focus of this Chapter.

Translation initiation is regulated by proteins known as eIFs (Figure 1.0), [32]. Initiation begins with the binding of eIF3 and eIF1A to the 40S ribosomal subunit (Step 1), [36]. Subsequently, eIF2, and GTP-Met-tRNA bind together with the 40S ribosomal subunit (Step 2). In order to assemble on the 5' end of the mRNA the 43S preinitiation complex requires the hydrolysis of ATP as well as the binding of a series of proteins contained within the eIF4 family, (Step 3). Following the formation of the 43S preinitiation complex, eIF4A unwinds the secondary structure in the 5'UTR enabling the 43S ribosomal subunit to scan the UTR of the mRNA until it reaches a start codon [37]. Upon reaching this start codon the 43S subunit forms a 48S initiation complex and associated initiation factors are released, a process that is mediated by GAP and eIF5, which facilitate hydrolysis of GTP by eIF2 (Steps 4 and 5). Full disassociation of initiation factors promotes the joining of the 60S ribosomal subunit and the commencement of translation elongation (Step 6). Both eIF2 and eIF4F are then recycled to facilitate the next round of translation initiation (Steps 7 and 8) [38].

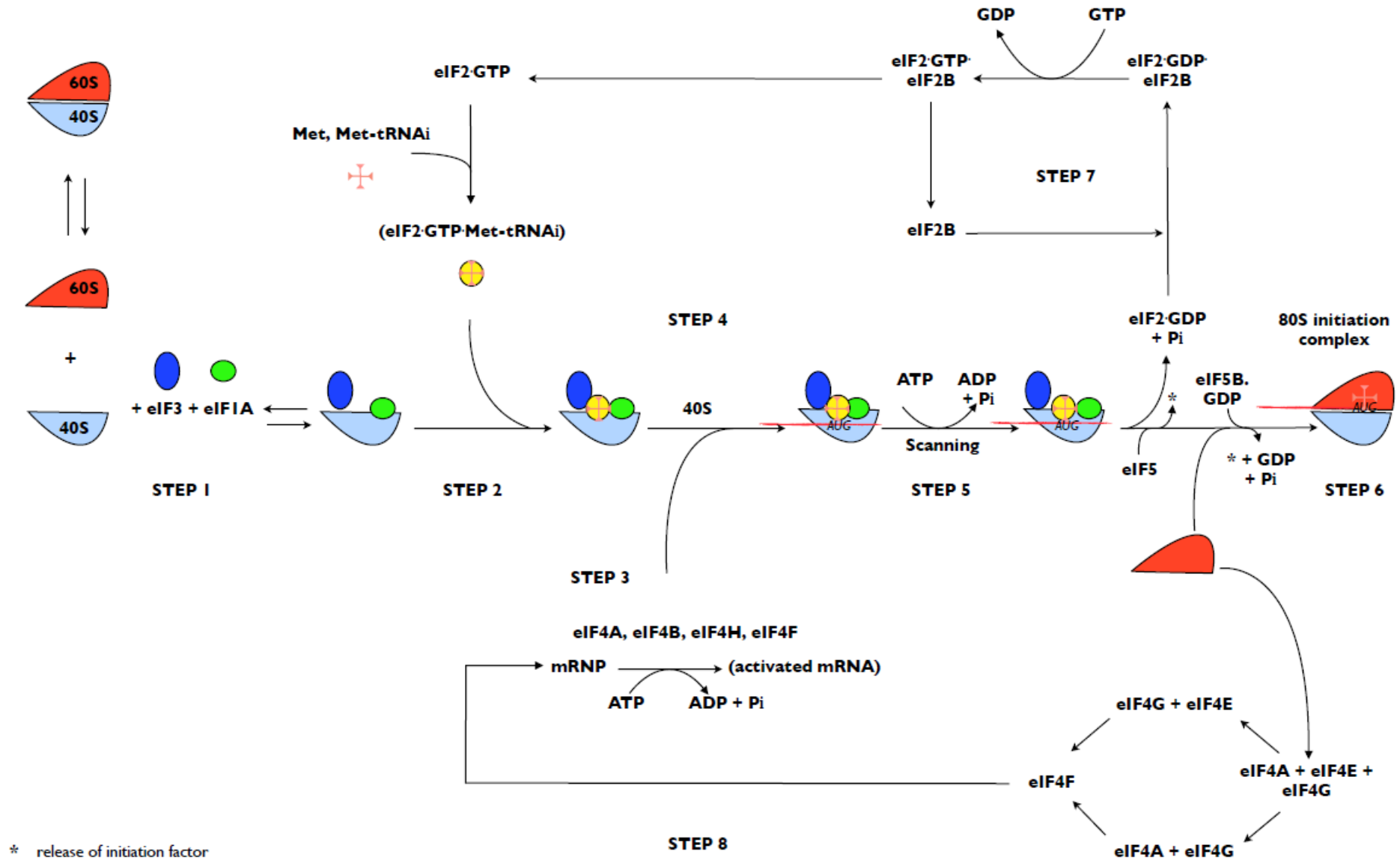

Figure 1.0 Schematic illustration of the eukaryotic translation initiation pathway redrawn from Merrick [39].

1.2 The roles of 4EBP1, p70S6K1 and mTORC1 in translational control

Of critical importance to cap dependant translation is the regulation of eIF4E by 4EBPs [32]. Both 4EBPs and eIF4Gs compete for a mutual binding site on eIF4E [40]. It is known that unphosphorylated 4EBP1, a member of the 4EBP family, has a high affinity for eIF4E and blocks its association with eIF4G thereby inhibiting the formation of the 43S preinitiation complex [41]. Liberation of eIF4E occurs when 4EBP1 is phosphorylated on Thr37 and Thr46 by an upstream kinase. Free eIF4E then interacts with eIF4G to form the active complex eIF4F [42]. Disassociation of 4EBP1 and eIF4E also may stimulate eIF4E translocation to the nucleus [43]. Entry of eIF4E into the nucleus is stimulatory to cell growth and is strongly associated with cellular transformation [44]. As such, phosphorylation of 4EBP1 is widely recognised as an important regulatory event in the control of eukaryotic protein synthesis.

Another key regulator of protein synthesis is p70S6K1 [30,45]. Indeed, knockout of p70S6K1 in mice reduces cell size by ~15% [46]. Furthermore, myotubes harvested from p70S6K1 knockout mice display a reduction in size of ~20% and demonstrate an inability to increase myotube diameter in response to stimulation compared to controls [47]. In a classic study by Baar and Esser [29], p70S6K1 phosphorylation was shown to be significantly elevated following high-resistance contractions in rodents. Moreover, this increase in p70S6K1 phosphorylation correlated strongly (r=0.998) with the percent change in muscle mass after 6 weeks of RE training. Data from human studies also corroborate these findings. One study demonstrates that RE-induced hypertrophy can be predicted by the acute phosphorylation changes in p70S6K1 30 min following the cessation of exercise [48] whereas another shows that post-RE p70S6K1 phosphorylation increases with training volume [49], a key determinant in the MPS response to RE [50]. The regulation of translation initiation by p70S6K1 is through direct phosphorylation of eIF4B [51]. eIF4B facilitates the unwinding of

the 5' UTR of mRNA enabling binding of the ribosome to mRNA [32]. Moreover, p70S6K1 is known to enhance peptide elongation by phosphorylating eEF2K relieving the inhibition of eEF2K on eEF2 [52]. This indirect activation of eEF2 by p70S6K1 is a key process facilitating the translocation step in translation elongation [32].

Another way in which p70S6K1 may influence translational control is through the up regulation of ribosomal transcripts. Indeed, distinct to many ribosomal RNAs is a unique terminal oligopyrmidine tract sequence (5'TOP). It is known that rapamycin supresses the up regulation of these transcripts an effect that is blocked by the expression of a rapamycin-resistant form of p70S6K1 [53]. Interestingly, overexpression of this rapamycin-resistant form of p70S6K1 also blocks the phosphorylation of 4EBP1 but not the p70S6K1 substrate S6 [54]. As such, it appears that 5'TOP mRNA translation is primarily driven by p70S6K1 and not 4EBP1. However, there are data to show that in transgenic mice, disrupting p70S6K1 has little impact on 5'TOP transcription [55] whilst knock-in of a non-phosphorylatable form of S6 in mice has little impact on the translation of 5'TOP mRNAs [56]. Although, expression of dominant negative p70S6K1 has been shown to repress the translation of 5'TOP transcripts, an effect rescued by rapamycin resistant p70S6K1 [57]. Reconciling the contrasting findings between studies is difficult due to differences in experimental models and methodologies. As such, the role of p70S6K1 in regulating translation of 5'TOP mRNAs remains a subject of conjecture and it appears that more work is required before the mechanisms that underpin p70S6K1-mediated translation of mRNAs that contain the 5'TOP sequence become fully elucidated.

Both 4EBP1 and P70S6K1 are regulated by a protein complex called mTORC1 (Figure 1.1). mTORC1 is an evolutionarily conserved threonine/serine protein kinase that regulates cell growth [58], cell proliferation [59], protein synthesis [60] and ribosomal biogenesis [61,62].

mTORC1 is composed of the catalytic subunit mTOR and it's regulatory adapter proteins Raptor, mLST8, PRAS40 and an inhibitory protein Deptor [62,63]. mTORC1 can be activated by a multitude of cellular cues indicative of nutrient availability and mechanical strain [64-66]. Activation of mTORC1 can occur via phosphorylation of PKB by PDK1 [67]. In response to insulin, PDK1 and PKB translocate to the membrane where PDK1 phosphorylates PKB [68]. Phosphorylation of PKB has been shown to directly activate mTORC1 on Ser2448 [67] or indirectly activate mTORC1 via TSC1/2 [69]. Although PKB has been shown to regulate mTORC1 activation, the role of PKB in mediating skeletal MPS in humans is currently a topic of intense debate [70].

Another kinase that has been implicated in the regulation of mTORC1 is AMPK [71]. AMPK is a key sensor of cellular energy status [72]. AMPK is a heterotrimeric complex that consists of a catalytic α subunit and two distinct regulatory β and γ subunits [73]. In humans, two to three isoforms of each subunit exist (α1, α2, β1, β2, γ1, γ2 and γ3) each with differential regulatory functions [74,75]. The kinase activity of AMPK is dependent upon the phosphorylation of the Thr172 site located in the α subunits [74]. AMPK activation is activated in response to contraction due to perturbations in the ATP:AMP [76] that may exert a repressive effect on mTORC1 activity [77]. Indeed, AMPK phosphorylates TSC2 on Ser1345 thus inhibiting the association of Rheb with mTORC1 that subsequently results in a reduction in mTORC1 activity [77]. Furthermore, phosphorylation of the mTORC1 adaptor protein Raptor at Ser792 by AMPK also inhibits its association with mTORC1 [78]. Data in humans have demonstrated that endurance exercise increases AMPK phosphorylation that is associated with a suppression of RE-induced increases in mTOR phosphorylation [79]. It could be contended that exercise bouts that induce the greatest degree of AMPK phosphorylation may therefore exert a diminishing impact on muscle anabolism in response to anabolic stimulation. However, it is important to reiterate that changes in AMPK

phosphorylation are dependent upon the cellular energy status of the muscle [80]. Thus, strategies such as feeding may alter the magnitude and amplitude of AMPK activation and therefore the adaptive response to exercise.

A significant amount of our knowledge regarding the multiple biological roles of mTORC1 has come from pharmacological/knockout studies in mice and cell-based systems [60,81]. From this body of work it has been established that the mTORC1 associated protein Raptor, binds to p70S6K1 on its TOS signalling motif to bring it in close proximity to mTOR to then become phosphorylated on Thr389 [82]. The phosphorylation of p70S6K1 on Thr389 results in the creation of a docking site for PDK1 allowing PDK1 to phosphorylate p70S6K1 on Thr229 [56,83], thus fully activating p70S6K1. Similar to p70S6K1, mTORC1 also targets 4EBP1 at Thr37/46 [32,42]. As previously discussed, phosphorylation at these sites causes 4EBP1 to disassociate from eIF4E thereby facilitating the creation of the active eIF4F complex [32]. These studies taken together with other contemporary articles [62,63] highlight the critical role of mTORC1 signalling in the regulation of cell size and skeletal muscle growth.

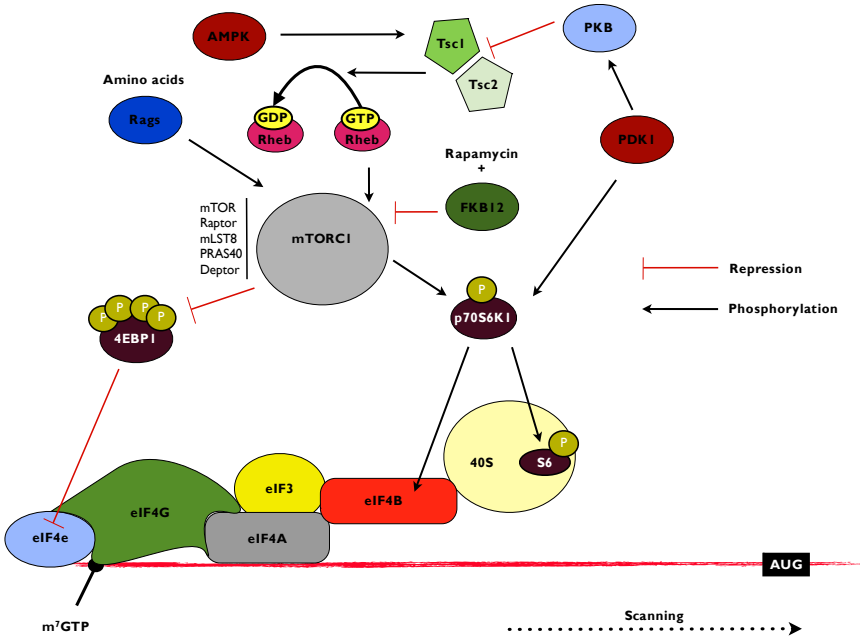

Figure 1.1 Schematic illustration of mTORC1-mediated regulation of translation initiation redrawn from Gkogkas et al. [35].

1.3 The impact of resistance exercise on anabolic signalling

The impact of RE on the signalling pathways described above is now a common theme of the exercise sciences [84-86]. The majority of studies show the phosphorylation, and presumably activation [87], of the mTORC1 signalling pathway is increased following an acute bout of RE [88,89]. The activation of mTORC1, p70S6K1 and 4EBP1 occurs within minutes [49,90] and is sustained for hours [88] following the cessation of exercise that is associated with elevations in MPS [88]. The assessment of the activation of mTORC1 and its associated proteins in response to RE is therefore used to gain a mechanistic insight into the molecular regulation of RE-induced skeletal muscle hypertrophy [25,91,92].

Despite the wealth of data indicating a role for mTORC1 signalling in MPS in humans, to date, the literature has been largely descriptive. However, in a unique experiment, one group provides strong evidence to show that mTORC1 signalling is essential to the functional regulation of RE-induced increases in MPS [30]. In this study, the authors treated human participants with rapamycin (a highly specific mTORC1 inhibitor) prior to a bout of high-intensity-RE. As hypothesised, treatment with rapamycin resulted in no increase in MPS during the 2 h post-RE recovery period. Rapamycin also inhibited mTOR phosphorylation and completely blocked the phosphorylation of p70S6K1 at Thr421/Ser424 as well as eEF2 at Thr56 1 h post-RE. However, in the control group, there was a significant increase in MPS of ~40% that was accompanied by a ~5-fold increase in p70S6K1 phosphorylation at Thr389 and a ~25% reduction in eEF2 phosphorylation at Thr56 1 h post-RE. To this author's knowledge, this single study is the only one to employ rapamycin in a human exercise trial.

The use of rapamycin in humans provides strong evidence that mTORC1 signalling is an active regulator of RE-induced increases in MPS. Although, how anabolic stimuli such as RE, regulate mTORC1 activity is not fully understood. Some data have emerged to suggest that

localisation of mTOR and its associated signalling proteins to the lysosome membrane may play a role [93,94]. In a recent study, Jacobs et al. [65] show that eccentric RE in mice results in the phosphorylation of TCS2 on RxRxxS/T consensus motifs. It is known that TSC2 possesses a GTPase activating protein that converts the mTORC1 activator Ras-homologue enriched in brain (Rheb) from a GTP to a GDP bound state that inhibits mTORC1 activation [95]. However, growth factor-mediated phosphorylation of TCS2 releases its inhibition on Rheb enabling it to activate mTOR [96]. The key finding of Jacobs et al. [65] was that the phosphorylation of TSC2 also resulted in the movement of TSC2 away from the lysosome towards the cytoplasm, therefore disassociating itself from Rheb. Furthermore, these authors show that at 1 h post-RE mTOR moves towards the lysosome where it interacts with Rheb to form the active mTORC1 complex. As such, this study provides novel insights as to the spatial and temporal arrangement of cellular signalling processes following a bout of RE.

The studies of Drummond et al. [30] and Jacobs et al. [65] represent significant advancements in our understanding of the molecular events that mediate skeletal muscle hypertrophy. Yet, despite these advancements, the key mechanical signal that transmits RE-induced tension to the translational machinery remains elusive. One potential candidate is FAK. FAK is a tyrosine kinase protein [97] and is situated in the sarcolemma linking the extracellular matrix to the cytoplasmic cytoskeleton [97]. FAK phosphorylation is known to be sensitive to loading of the muscle. Indeed, the total protein content and activity of FAK has been shown to increase in response to overload in chicken *anterior latissimus dorsi* [98]. In humans, following 10 days of disuse atrophy, FAK phosphorylation in the postabsorptive state is decreased by ~30% that is associated with a concomitant reduction in myofbrillar MPS [99]. Furthermore, 14 days of immobilisation also results in a ~23% decrease in FAK phosphorylation along with a decline in muscle cross sectional area and peak isometric torque of ~5% and 25% respectively [100]. The exact role of FAK in mediating these changes in

skeletal muscle is currently unknown. Mechanistically, there are data to suggest that FAK phosphorylation may act to target p70S6K1 [101], perhaps via TSC2-mTORC1 signalling [102]. Thus, FAK could potentially act as the mechanical signal that results in TSC2 localisation away from the lysosome [103] but it is highly plausible that there are a multitude of other signals which lie upstream of mTORC1, in addition to FAK, that could modulate RE-induced perturbations in anabolic signalling processes [85].

1.4 The impact of amino acid feeding on anabolic signalling

Amino acid provision, particularly of the essential amino acids, results in a robust increase in MPS [45]. An increase in MPS in response to amino acid provision is accompanied by elevations in the activation of proteins contained within the mTORC1-p70S6K1 signalling axis [27,104]. However, such studies rely on data that are largely associative and fail to provide an adequate cause and effect relationship. In an adapted version of their original rapamycin experiment, Dickinson and colleagues [45] employed rapamycin to identify if putative increases in MPS to amino acid provision also are mTORC1 dependent. The primary finding of this investigation was that prior treatment with rapamycin completely blocked increases in MPS during a 2 hour postabsorptive state. The authors also identified that rapamycin blocked increases in mTOR phosphorylation at Ser2448 at 2 hour post-ingestion as well as attenuating the increase in p70S6K1 and 4EBP1 phosphorylation at 1 hour post-ingestion. However, it is important to recognise that rapamycin treatment does not completely inhibit basal rates of MPS [105]. This finding would therefore suggest there are other, unrelated, processes that influence MPS, independent of mTORC1-signalling in response to amino acid feeding. [106]

The consumption of amino acids, especially in large doses [27], is known to induce an increase in blood insulin concentration [107]. This increase in insulin concentration with

amino acids indirectly activates PKB that is associated with increases in MPS [27]. Data from Biolo and colleagues [108] provide evidence to assert that in the basal state, insulin has a stimulatory impact on MPS. In addition, there are data to show that insulin attenuates MPB thus enhancing NPB in the basal state [109]. However, the role of insulin in the regulation of post-RE MPS has been questioned [109,110]. Indeed, one study has shown that although insulin exerts a stimulatory impact on MPS at rest, this effect is absent during a post-RE recovery period [108]. Furthermore, the addition of carbohydrate, which stimulates increases in plasma insulin concentration, to a protein-containing drink has no added effect on exercise-induced increases in MPS [111]. There are studies in cell culture models demonstrating that a total absence of insulin inhibits mTORC1 signalling to amino acid provision [112,113] and it is known that amino acid-induced changes in mTORC1 signalling are enhanced in the presence of insulin [106]. However, in humans the consumption of amino acids results in a ~3-fold increase in plasma insulin concentration above basal conditions [27,107] and increasing plasma insulin concentration 30 times above basal has no enhancing effect on MPS even in the presence of hyperaminoacidemia [109]. Furthermore, the stimulatory impact of carbohydrate on rates of MPS at rest is far below that as induced by amino acid consumption [13] and when considered together, these data would suggest that the mechanisms by which amino acids stimulate MPS are independent to insulin action.

There are data to show that amino acids may directly target mTORC1 signalling. Indeed, amino acids can influence mTORC1 activity via the class 3 phosphoinositol kinase Vps34 [112], MAPK43 [114] or direct to eIF2B [60,115]. Alternatively, others have shown in C2C12 cells that the amino acid leucine promotes mTOR translocation to the lysosome via the Rag-Ragulator complex pathway [93,94,116]. Once at the lysosome mTOR may then interact with Rheb to form the active mTORC1. This finding would suggest that amino acids influence cellular trafficking of signalling molecules, in addition to direct phosphorylation

events. However, the localisation of mTOR to the lysosome is yet to be observed in humans and future work is clearly required in order to experimentally address this particular observation.

It is well established that performing RE potentiates MPS to protein feeding [5,6,26,91]. One study has shown that consuming 25 g of whey protein following a bout of unilateral RE results in a ~160% increase in MPS in the rested fed limb but in the fed exercising limb, MPS is much greater, ~ 220% above rest [6]. It appears that the potentiation of MPS following RE to amino acid provision also is underpinned by concomitant increases in both p70S6K1 and mTOR phosphorylation [26,86,88]. Interestingly, it is known that the enhanced anabolic response of muscle to amino acids following RE is in fact dose-dependant. In the first study of its kind Moore and colleagues [25] show that following a bout of high-intensity RE, MPS over a 4 h period increases in a stepwise fashion with graded protein ingestion up until 20 g of protein. In extending these findings Areta et al. [26] recently demonstrated that consuming 20 g of protein every 3 h post-RE is a more superior strategy for optimising MPS over a 12 h period compared with either 10 g every 1.5 h or 40 g every 6 h. Collectively, these data demonstrate that consuming 20 g of protein immediately post-RE is sufficient to maximally stimulate MPS for $\leq$ 4 h with excessive protein consumption resulting in a significant stimulation of whole-body leucine oxidation [25].

1.5 The impact of n-3 PUFA on skeletal muscle anabolism

The impact of amino acid ingestion on MPS is well established. However, there is comparably less information regarding the impact of other nutritional interventions on human muscle. With respect to cardiovascular health the consumption of foods rich in n-3 PUFA ingestion is a topic of intense investigation [117-119]. Data from many studies now suggest that consumption of foods rich in n-3 PUFA, such as some types of fish, lowers the risk of

cardiovascular disease [117], improves cognitive function [120] and enhances insulin sensitivity [121]. It appears that the beneficial impacts of n-3 PUFA on human health may be related to the anti-inflammatory properties of the n-3 PUFA class of fatty acids, EPA and DHA [122]. As a result, diets rich in n-3 PUFA are now recommended by the U.K government as means to enhance the general health and well being of the population [123].

Despite the efficacy for n-3 PUFA ingestion to improve cardiovascular health [117], only recently has significant research been conducted to examine the potential of n-3 PUFA ingestion to influence skeletal muscle metabolism (Table 1.0). An early report from a study in growing steers [124] demonstrated that n-3 PUFA supplementation potentiates insulin action and induces phosphorylation of the mTOR-p70S6K1 signalling pathway in response to a hyperinsulinaemic clamp. More recently, data in rodents have shown that dietary fish oil rescues the deleterious loss of skeletal muscle mass during a period of enforced immobilisation [17]. In that study the attenuation of muscle mass loss with n-3 PUFA supplementation was associated with the phosphorylation of p70S6K1 and similar findings have now been replicated in other rodent [18] and cell-based systems [16]. Although these data are of interest, the mechanism by which the consumption of n-3 PUFA regulates the molecular events that orchestrate skeletal MPS remains largely unknown.

Ingestion of n-3 PUFA raises the n-3 PUFA composition of whole blood [125], adipose tissue [123] and that of the skeletal muscle membrane [21,22]. The incorporation of n-3 PUFA into the cellular membrane is thought to be at the expense of n-6 PUFA [122,126]. Both n-3 PUFA and n-6 PUFA serve as substrates for the production of eicosanoid and eicosanoid-like mediators [126] that regulate inflammation [127], and other biological processes [126]. One such eicosanoid is PGF2α. PGF2α is synthesised from an n-6 PUFA called arachidonic acid (AA) and the incorporation of n-3 PUFA into cellular membranes has been proposed to

reduce the expression of PGF2α [126]. In this regard, extant data show that dietary fish oil reduces the expression of PGF2α in rodents that is associated with the inhibition of the recovery of atrophied skeletal muscle [128]. Additionally, this reduction in PGF2α content was concomitant to reduced p70S6K1 phosphorylation. Thus, it could be suggested that dietary fish oil reduces PGF2α content that subsequently serves to inhibit anabolic signalling processes.

However, it is important to recognise that measurement of changes in the lipid profile of skeletal muscle were not made in the aforementioned study [128]. This point is particularly relevant as increases in human skeletal muscle [21,22] and blood [125] n-3 PUFA composition with fish oil supplementation occur in the absence of changes in n-6 PUFA composition. These data therefore suggest that there are other, as yet unknown, mechanisms by which n-3 PUFA supplementation alters anabolic signalling in human skeletal muscle. In addition, it also is interesting to note that one study has shown dietary fish oil inhibits muscle hypertrophy during remobilisation [128] whereas others suggest dietary fish oil enhances muscle anabolism following nutrition [21,22,32]. It is therefore reasonable to conclude that the impact of fish oil supplementation on skeletal muscle may therefore be dependant upon the nature of stimulation, i.e. nutrition vs. loading. Clearly, more work is now needed in humans in order to experimentally address these confounding reports.

Table 1.0 Summary of studies characterising the impact of various n-3 PUFA protocols on skeletal muscle metabolism.

Study	Model	Supplementation protocol	Key findings
Smith et al. (2011a)[21]	Human	8 wk, 1.86 g EPA and 1.50 g DHA daily.	n-3 PUFA supplementation potentiated MPS and mTOR-p70S6K1 to a hyperaminoacidemic-hyperinsulinemic clamp in young volunteers.
Smith et al. (2011b)[22]	Human	8 wk, 1.86 g EPA and 1.50 g DHA daily.	n-3 PUFA supplementation potentiated MPS and mTOR-p70S6K1 to a hyperaminoacidemic-hyperinsulinemic clamp in elderly volunteers.
Rodacki et al. (2012)[19]	Human	60 d before and during 90 d of strength training, 0.4 g of EPA and 0.3 g DHA daily.	Peak torque and rate of torque development for knee flexor and extensor, plantar and dorsiflexor) was enhanced by n-3 PUFA supplementation from pre to post training.
You et al. (2010)[17]	Rodent	14 d of a diet that contained 5% cod liver oil followed by 14 d immobilisation.	Dietary n-3 PUFA alleviated myosin heavy chain losses during 14 d of immobilisation compared to control (-22.7% vs. 34.5%).
Kamolrat et al. (2013)[18]	Rodent	8 wk, chocolate- derived sweets containing 49.6% EPA and 50.4% DHA.	In response to an aminoacidemic-insulinemic infusion, n-3 PUFA supplementation enhanced the phosphorylation of PI3K and p70S6K1.
Gingras et al. (2007)[124]	Steers	5 wk of abomasal infusion with 4 % menhaden oil (high n-3 PUFA).	n-3 PUFA potentiated insulin action, whole body amino acid disposal and mTOR-p70S6K1 signalling were enhanced in response to a hyperinsulinaemic–euglycaemic–euaminoacidaemic clamp.

Some studies show that provision of n-3 PUFA has a direct influence on muscle mass homeostasis in the clinical setting. Indeed, supplementing the diet with > 2 g of EPA has been known to increase survival [129], attenuate muscle cachexia [130] and stabilise weight loss in advanced cancer patients [131]. One study in particular has demonstrated that supplementing the diet of lung cancer patients with fish oil (2.2 g EPA per day) maintained or increased muscle mass (0.5 ± 1.0 kg) as assessed by computed tomography [132]. This finding is more remarkable considering that in the control group mean average muscle mass loss was ~2.3 kg. It is important to acknowledge that cancer cachexia is characterised by excessive inflammation that may accelerate rates of MPB [133]. The salvaging effect of n-3 PUFA supplementation in this setting could therefore be related to the attenuation of MPB associated with the anti-inflammatory actions of n-3 PUFAs rather than, or in addition to, the potentiation of MPS. Although, the authors did not assess MPS and any potential impact of n-3 PUFA on MPS cannot be excluded. Furthermore, no measurement of changes in the lipid composition of the muscle was made. Nevertheless, taken together with previous work [19] these data provide strong evidence that in humans, n-3 PUFA has the capacity to exert a positive influence on muscle mass. More work is now required outwith the clinical environment to establish the efficacy of n-3 PUFA to enhance muscle anabolism in the general population.

In healthy humans it is the response of MPS, not MPB, to stimulation by nutrition and exercise that is the primary determinant of NPB [134]. However, to date, only one study has directly assessed the impact of n-3 PUFA supplementation on rates of MPS in humans [21]. In this study the authors supplemented nine 25-40 year old adults with 1.9 g and 1.5 g of EPA and DHA, respectively, for 8 wk. Before and after supplementation basal rates of MPS and rates of MPS in response to a hyperaminoacidemic-hyperinsulinemic infusion were assessed as well as the phosphorylation status of proteins contained within the mTOR-p70S6K1

signalling axis. The main findings from this investigation were that neither basal MPS nor the basal phosphorylation status of anabolic signalling agents was altered by n-3 PUFA supplementation. However, in response to the hyperaminoacidemic-hyperinsulinemic infusion, rates of MPS were potentiated, as was the phosphorylation status of both mTOR and p70S6K1. Furthermore, protein to DNA ratio and muscle protein concentration also was increased. Interestingly, the authors have replicated similar findings in elderly humans [22] and speculate that the potentiation of MPS and mTOR-P70S6K1 signalling in response to the simulated feeding could be related to increases in the n-3 PUFA composition of skeletal muscle. Indeed in both studies supplementation with n-3 PUFA resulted in ~ 2 fold increase in the n-3 PUFA composition of the cell membrane. It therefore appears that altering the n-3 PUFA composition of skeletal muscle could be exploited to enhance the adaptive response of skeletal muscle to exercise.

1.6 Limitations of existing research

Collectively, the studies outlined in this Chapter point to a beneficial influence of supplementing humans with n-3 PUFA on muscle anabolism. However, there are a number of caveats in the field that need to be experimentally addressed in order to advance our current understanding of how n-3 PUFA supplementation influences skeletal muscle metabolism. Firstly, although time course changes in n-3 PUFA composition have been established in blood [125,135] adipose [123] and numerous other biological tissues [136,137], to date, no study has provided a time course change in n-3 PUFA muscle composition with n-3 PUFA supplementation [121,138]. Given recent reports of enhanced muscle anabolism [21,22], as well as many other relevant health claims with n-3 PUFA supplementation, such a time course may provide critical information for future researchers who wish to alter the n-3 PUFA lipid profile of skeletal muscle for experimental purposes. Secondly, the influence of n-3

PUFA supplementation on muscle anabolism appears to be underpinned by alterations in the mTOR-p70S6K1 signalling cascade [16-18,22,124]. However, to the author's knowledge, no study has characterised a time course change in the expression of these proteins in human skeletal muscle during a period of n-3 PUFA supplementation. The results of such a study may provide a greater mechanistic insight as to how n-3 PUFAs confer anabolic influence at the molecular level in humans.

It has now been reported that n-3 PUFA supplementation enhances muscle strength in response to RE [19] as well as MPS and mTOR-p70S6K1 signalling following amino acid infusion [21,22]. However, in order to maximise both MPS and mTOR-p70S6K1 signalling, amino acid feeding and RE must be combined [6]. To date, no study has examined the impact of a combined RE and amino acid feeding stimulus on mTOR-p70S6K1 signalling following a period of n-3 PUFA supplementation in humans. In addition, although the impact of amino acid provision on mTORC1-p70S6K1 signalling following n-3 PUFA supplementation has already been examined, in that study, amino acids were provided via a hyperaminoacidemic-hyperinsulinemic clamp for 3 h. The administration of amino acids in this manner is not reflective of habitual dietary practices and results in more sustained plasma amino acid concentrations compared with the those induced by consumption of an amino acid bolus [107]. A differential plasma amino acid profile in response to nutrients may alter the anabolic response of muscle to feeding [139]. Thus, characterising the impact of n-3 PUFA supplementation on mTOR-P70S6K1 signalling in response to RE and a bolus of amino acids would provide information pertinent to a physiologically relevant context.

1.7 Aims and objectives

The overall aim of this thesis is to address current knowledge gaps in the literature regarding how n-3 PUFA supplementation impacts skeletal muscle metabolism. Specifically, the aims of this programme of work are to identify how n-3 PUFA supplementation influences muscle lipid profiles, anabolic protein expression, as well as the response of those proteins to RE and amino acid consumption. These aims will be achieved by the successful completion of the following objectives in chronological order:

1. **To determine a time course change in muscle and blood lipid profiles in response to n-3 PUFA supplementation.**

2. **To determine a time course change in anabolic signalling protein phosphorylation and expression in response to n-3 PUFA supplementation.**

3. **a) To characterise the influence of n-3 PUFA supplementation on the molecular response of human skeletal muscle to the ingestion of a protein bolus and b) to the ingestion of a protein bolus following a bout of RE.**

CHAPTER 2 Temporal changes in lipid composition and anabolic proteins in human skeletal muscle with fish oil supplementation

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2.0 Abstract

Ingestion of n-3 PUFA has been shown to increase n-3 PUFA muscle composition that subsequently appears to render skeletal muscle more anabolically sensitive. The time course of this increase in muscle n-3 PUFA composition is currently unknown. Changes in the lipid profile of red blood cells and muscle tissue along with muscle expression of anabolic signalling proteins in human skeletal muscle were examined in response to 4 wk of fish oil supplementation. Ten healthy male participants consumed $5g \cdot d^{-1}$ of n-3 PUFA-enriched fish oil for 4 wk. Muscle biopsies and venous blood samples were collected in the fasted, rested state 2 wk prior (W-2), immediately before (W0), at week 1: (W1), 2 (W2) and 4 (W4) after initiation of fish oil supplementation for assessment of changes in lipid composition and expression of anabolic signalling proteins over time**.** Muscle lipid profile, (% total n-3 PUFA/total fatty acids) increased from W0 to W2 (3.8 ± 0.2 to 5.1 ± 0.3 %) and continued to rise at W4 (6.7 ± 0.4 %). Blood lipid profile (% total n-3 PUFA/total fatty acids) was increased from W0 to W1 (5.8 ± 0.3 to 9.5 ± 0.4 %) and remained elevated for the remaining time points with no further increase. Total protein content of FAK increased from W0 to W4 (3.9 ± 1.5 fold) whereas total mTOR was increased from W0 at W1 (2.4 ± 0.6 fold) with no further significant increases at W2 and W4. However, there was no increase in total p70S6K1 or 4EBP1. There were no significant changes in the phosphorylation of $FAK^{tyr576/577}$, $mTOR^{ser2448}$, $p70S6K1^{thr389}$ or $4EBP1^{thr37/46}$. This study shows that oral fish oil consumption results in an increase of n-3 PUFA levels in human skeletal muscle and blood that is associated with increases in the expression of anabolic signalling proteins.

2.1 Introduction

The consumption of food rich in n-3 PUFA is thought to be beneficial for cognitive function [120], the attenuation of skeletal muscle atrophy during cancer cachexia [20] and cardiovascular health [140]. The impact of n-3 PUFA intake for cardiovascular health is now well established [119,141] and changes in the n-3 PUFA composition of blood correlate inversely with cardiovascular disease risk [142]. Consequently, the n-3 PUFA composition of blood is now considered as a surrogate marker for cardiovascular health [117,119,142].

Whereas much is known about other cardiovascular health, far less is known about the impact of n-3 PUFA supplementation on skeletal muscle. Specifically, work in rodents has shown that dietary fish oil alleviates soleus muscle atrophy during a period of enforced immobilisation [17]. In humans, supplementation with n-3 PUFA-rich fish oil for 8 weeks is known to enhance MPS in response to a hyperaminoacidemic-hyperinsulinemic infusion in both the young and elderly [21,22]. Mechanistically, these anabolic influences are proposed to be mediated by mTOR-p70S6K1 signalling following n-3 PUFA supplementation [21,22]. These changes in mTOR-p70S6K1 signalling with fish oil supplementation also were accompanied by increases in the n-3 PUFA composition of skeletal muscle. Thus, it appears that n-3 PUFA supplementation increases the n-3 PUFA composition of skeletal muscle, conferring its anabolic influence, in part, via mTOR-p70S6K1 signalling.

Although data exist characterizing changes in the n-3 PUFA composition of muscle following 8 wk of n-3 PUFA supplementation [21,22], these studies are limited to pre- and post-supplementation measurements with little temporal resolution. Data are available on the time course of n-3 PUFA changes in blood [125] with n-3 PUFA supplementation. However, to our knowledge, no study has established the time course of n-3 PUFA changes in human

skeletal muscle. Given the potential beneficial impact of altering the n-3 PUFA composition of skeletal muscle, the aim of this chapter was to identify the time course of n-3 PUFA change in skeletal muscle over 4 weeks of n-3 PUFA-enriched fish oil supplementation. Additionally, as previous reports demonstrate changes in mTOR-p70S6K1 signalling with n-3 PUFA supplementation, a secondary aim was to determine whether 4 weeks of fish oil supplementation modified the expression and or phosphorylation of key intramuscular anabolic signalling proteins.

2.2 Experimental methods

2.2.1 Participants

Ten healthy, moderately active males (aged 21 ± 3 years; body mass 76 ± 4 kg, mean ± SEM) from the University of Stirling and the surrounding area volunteered to participate in the present investigation. Following health screening, participants were excluded if they were engaged in any form of dietary supplementation or were taking any prescribed medication. This study was conducted according to the guidelines laid down in the Declaration of Helsinki (2008) and the Local Ethics Committee, University of Stirling, approved all procedures. Written, informed consent was obtained prior to the commencement of the experiment.

2.2.2 Experimental Design

In a one-way, repeated measures design, participants reported to the laboratory on five separate occasions. Initial baseline assessment of muscle and blood lipid profiles was conducted at -2 (W-2) and 0 (W0) weeks, to determine changes in muscle and blood lipid profiles over a period of habitual diet and physical activity thus, the participants served as their own internal control (Table 1). Following this baseline control period, participants consumed 5 $g \cdot d^{-1}$ of n-3 PUFA-rich fish oil capsules (providing 3500 mg EPA [20:5n-3], 900

mg DHA [22:6n-3], 100 mg DPA [22:5n-3] vitamin E 0.1 mg, Ideal Omega-3, Glasgow Health Solutions Ltd, UK) for 4 weeks. The supplemental fish-oil dose and participant number were chosen based on previous work showing that a similar dose in ten males can induce significant changes in the lipid profile of human blood over a 4 weeks period [125]. Participants were required to complete a 7 day food and physical activity diary prior to baseline testing (W-2). This diary was presented back to the participants who were then asked to replicate a similar pattern of food consumption and physical activity for the remainder of the experiment. During each visit to the laboratory, participants were verbally requested to confirm the pattern of oily fish consumption in an attempt to ensure that changes in free-living oily fish consumption did not influence muscle and blood lipid profiles during the study. Each participant's height, nude body mass, resting skeletal muscle samples and duplicate 5 mL venous blood samples were obtained at 0700 following a 10 hours overnight fast at W-2, W0, 1 week (W1), 2 week (W2) and 4 week (W4) of supplementation. Supplementation compliance was assessed via a blind capsule count. Resting, fasting venous blood samples were analysed for glucose concentration to assess compliance with the overnight fast.

2.2.3 Venous blood and muscle biopsy procedures

Blood samples were obtained from an antecubital forearm vein. All samples were drawn into evacuated 5 mL vacutainers containing ethylenediaminetetraacetic acid (EDTA), (Vacutainer Systems, Becton, Dickinson and Company. U.K). An aliquot of blood was removed and centrifuged at 3000 $rpm \cdot min^{-1}$ for 15 min and the plasma stored at -80°C until further analysis. Plasma glucose was determined using an ILAB automated analyser (Instrumentation laboratory, Cheshire, UK).

Muscle biopsies were obtained from the lateral portion of the *vastus lateralis*. Initially the site was cleaned before an incision into the skin and fascia was made under local anaesthetic (2 % lidocaine). A 5 mm Bergstrom biopsy needle was inserted to extract ~60-100 mg of skeletal muscle tissue. Muscle samples were rinsed with ice-cold saline, blotted dry and any visible fat or connective tissue was removed. Muscle samples were separated into two Eppendorf tubes, before being snap-frozen in liquid nitrogen and stored at -80°C pending further analysis. All subsequent muscle biopsies i.e. 0, 1, 2 and 4 weeks, were obtained from the contralateral limb to the previous sample.

2.2.4 Lipid extraction of muscle biopsy samples

Total lipid was extracted from the muscle by a modification of a method previously described [143]. The frozen muscle biopsy samples (20-60 mg) were placed in a pre-weighed reacti-vial and an accurate tissue mass recorded. The reacti-vials were capped and placed on ice and then 1 mL of chloroform/methanol (C:M, 2:1 v/v) was added to each vial. Each muscle sample was homogenised using a hand-held IKA-Werke Ultra-turrax T8 homogeniser (Fisher, Loughborough, UK). The probe was rinsed with 3 mL of C:M, 2:1 and added to the reacti-vial before being placed on ice for 1 h. Then 1 mL 0.88 % KCl was added, shaken and allowed to stand for 10 min to remove non-lipid impurities. The vials were then centrifuged at 400 *g* for 5 min before removal of the aqueous layer. The lower solvent layer was removed using a Pasteur pipette and filtered through a 5.5 mm Whatman No. 1 filter, pre-washed with C:M, 2:1 into a 7 mL bottle, and dried under N_2. The lipid was then re-dissolved in 1 mL of C:M, 2:1 and transferred to a pre-weighed 1.7 mL bottle. The 1.7 mL bottle then was rinsed with 0.5 mL C:M, 2:1 and this was added to the 1.7 mL bottle. The lipid was dried under N_2 and desiccated overnight in a vacuum desiccator after which the lipid was reweighed and

dissolved in C:M, 2:1 + 0.01 % butylated hydroxyl toluene (BHT), (v/v) at a concentration of 2 $mg \cdot mL^{-1}$.

2.2.5 Lipid extraction of whole blood samples

Samples of whole blood were placed onto two circular collection spots on Whatman 903 blood collection cards (GE Healthcare Ltd, Forest Farm Industrial Estate, Cardiff, CF 14 7YT, UK). The cards were left open and allowed to dry for 3 h after which the dried whole blood sample was detached from the collection device using forceps and placed into a screw-cap vial containing 1 mL of methylating solution (1.25M methanol/HCl). The vials were placed in a hot block at 70°C for 1 h. The vials were allowed to cool to room temperature before 2 mL of distilled water and 2 mL of saturated KCl solution were added. Fatty acid methyl esters (FAME) were then extracted using 1 × 2 mL of isohexane + BHT followed by a second extraction using 2 mL of isohexane alone. This extraction method has been previously validated as a reliable measure of whole blood fatty acid composition in our own laboratories [144].

2.2.6 Analysis of fatty acid methyl esters (FAME)

FAME were separated and quantified by gas-liquid chromatography (ThermoFisher Trace, Hemel Hempstead, England) using a 60 m x 0.32 mm x 0.25 µm film thickness capillary column (ZB Wax, Phenomenex, Macclesfield, UK). Hydrogen was used as carrier gas at a flow rate of 4.0 $mL.min^{-1}$ and the temperature program was from 50 to 150°C at 40°C·min^{-1} then to 195°C at 2°C·min^{-1} and finally to 215°C at 0.5°C·min^{-1}. Individual FAME were identified compared to well-characterised in house standards as well as commercial FAME mixtures (Supelco™ 37 FAME mix, Sigma-Aldrich Ltd., Gillingham, England).

2.2.7 Western blot

Muscle tissue (20-40 mg) was homogenised in ice-cold homogenization buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 10 mM ß-glycerophosphate, 50 mM NaF, 0.5mM activated sodium orthovanadate (all Sigma Aldrich, St Louis, MO, USA) and a complete protease inhibitor cocktail tablet (Roche, Indianapolis, IN, USA). Homogenates were centrifuged at 2200 *g* for 10 min at 4°C before recovery of supernatants representing the sarcoplasmic protein pool for analysis by WB. Protein concentration was determined using the BCA protein assay (Thermo Fisher Scientific, Ontario, Canada). Equal aliquots of protein (20 μg) were boiled in Laemmli sample buffer (250 mM Tris-HCl, pH 6.8; 2 % SDS; 10 % glycerol; 0.01 % bromophenol blue; 5 % β-mercaptoethanol) and separated on SDS polyacrylamide gels (range 10-15 %) for 1.5 h at 140 V. Following electrophoresis, proteins were transferred to a PVDF membrane at 100 V for 1 h. Following 1 h of blocking in 5 % milk powder in TBST (Tris Buffered Saline and 0.1 % Tween-20; both Sigma-Aldrich, Poole, UK), membranes were incubated overnight at 4°C with the appropriate primary antibody diluted in TBST. Primary antibodies were; $mTOR^{ser2448}$ (Cell Signalling, #2971, 1:1000), total mTOR (Cell Signalling, #2972, 1:1000), $p70S6K1^{thr389}$ (Cell Signalling, #9234, 1:1000), total p70S6K1 (Cell Signalling, #9202, 1:1000), $4EBP1^{thr37/46}$ (Cell Signalling, #2855, 1:1000), total 4EBP1 (Cell Signalling, #9452, 1:2000), $FAK^{tyr576/577}$ (Santa Cruz Biotechnology, #21831, 1:5000), total FAK (Santa Cruz, #558, 1:5000) and α-tubulin (Sigma-Aldrich, #T6074, 1:2000). The following morning the membrane was rinsed three times for 5 min in TBST. The membrane was then incubated for 1 h at room temperature in horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody diluted in TBST (New England Biolabs, UK, 1:10000). The membrane was then cleared three times for 5 min in TBST. Antibody binding was detected using enhanced chemiluminescence (Millipore, Billerica, MA). Band visualization was carried out using a Chemidoc XRS system (Bio-Rad, Hemel

Hempstead, UK) and quantification using densitometry (ImageJ v1.34s 281 software, rsbweb.nih.gov/ij/). Molecular signalling proteins were determined with n = 10. Phospho-proteins were expressed relative to total protein by stripping the membrane using Restore Western Blot Stripping Buffer (Thermo Scientific, FL, USA, #21059) and re-probing for total protein. Phosphorylated antibodies were removed from all membranes using Restore Stripping Buffer (Thermo Fisher Scientific, Ontario, Canada) prior to probing for total protein. Total protein was expressed relative to α-tubulin as a protein loading control.

2.2.8 Data presentation and statistical analysis

All statistical analyses were performed using Minitab 17 statistical software (Minitab Ltd., Coventry, UK). Paired t-tests were used to detect differences in lipid profiles of blood and muscle between W-2 and W0. As no differences were detected between baseline measurements (W-2, W0) for lipid profiles of the blood or muscle, all further statistical analyses were performed using only W0 as the baseline measurement. A single-factor (week of supplementation), repeated measures ANOVA was employed to evaluate changes in lipid composition of both the blood and muscle as well as changes in protein signalling from W0 to W4. In order to comply with the assumptions of the ANOVA, when required, protein signalling data were initially log transformed. If a main effect of time was observed a Tukey's post-hoc analysis was applied to detect differences between weeks. Regression analysis was performed to identify correlation coefficients between blood and muscle n-3 PUFA composition over time. Statistical significance was set at $P<0.05$. Protein signalling data are presented as mean (cross), median with interquartile-range and range. All lipid profiling data are expressed as mean relative percentage of total fatty acids ± SEM unless otherwise stated.

2.3 Results

2.3.1 Dietary analysis

Daily energy intake was 2244 ± 132 kcal (55 ± 5 % carbohydrate, 15 ± 2 % protein, 30 ± 4 % fat). Participants' fasted, plasma glucose samples across all trials ranged from 4.1 to 5.5 mM and mean fasted plasma glucose across all trials was 4.81 ± 0.12 mM.

2.3.2 Lipid profile changes in muscle

There was no significant change in the total lipid content of muscle (1.38 ± 0.04 [W-2] to 1.25 ± 0.05 [W0] to 1.22 ± 0.02 [W1] to 1.36 ± 0.08 [W2] to 1.20 ± 0.05 [W4], mg/100 mg of muscle) at any time point. Thus, in order to enable meaningful comparisons between changes in blood and muscle n-3 PUFA composition as well with previously published studies [21,22,123,125] all lipid data are presented as % of total lipid composition unless otherwise stated. A full breakdown of muscle fatty acid profiles is presented in Table 2.0. Lipid composition of blood and muscle did not differ between W-2 and W0, (Table 2.1). The difference in mean % EPA + DHA/total fatty acids was not statistically higher at W2 compared to W0 but was significantly higher at W4 compared to W0 ($P<0.05$; Figure 2.0 A). There was an increase in % total n-3 PUFA/total fatty acids from W0 to W2 ($P<0.05$) that continued to rise at W4 ($P<0.05$; Figure 2.0 B). Percentage total n-6 PUFA/total fatty acids did not significantly alter over time (Figure 2.0 C). The AA (20:4n-6) /EPA (AA/EPA) ratio was lower at W1 ($P<0.05$) and declined further at W4 ($P<0.05$; Figure 2.0 D). Percentage n-3 highly unsaturated fatty acids (n-3 HUFA)/total HUFA increased from W0 to W2 ($P<0.05$; Figure 2.0 E) with no further detectable increase at W4.

Table 2.0 Muscle lipid composition at -2 week (W-2), 0 week (W0), 1 week (W1). 2 week (W2) and 4 week (W4) of fish oil supplementation. Values are percentage of total lipid composition presented as means ± SEM. Polyunsaturated fatty acids (PUFA), dimethyl aldehyde (DMA). Means that do not share a letter are significantly different.

Fatty acid	-2 week	0 week	1 week	2 Week	4 Week
14:0	0.95 ± 0.04	0.93 ± 0.05	0.95 ± 0.05	0.99 ± 0.08	0.85 ± 0.03
15:0	0.23 ± 0.01	0.21 ± 0.01	0.26 ± 0.01	0.20 ± 0.01	0.21 ± 0.01
16:0	17.49 ± 0.27	17.59 ± 0.31	18.03 ± 0.26	17.55 ± 0.37	17.71 ± 0.36
18:0	11.41 ± 0.27	11.19 ± 0.26	11.16 ± 0.17	10.98 ± 0.35	11.66 ± 0.16
20:0	0.13 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.13 ± 0.01
22:0	0.21 ± 0.02	0.17 ± 0.01	0.17 ± 0.02	0.16 ± 0.02	0.19 ± 0.02
24:0	0.18 ± 0.01	0.20 ± 0.02	0.15 ± 0.01	0.15 ± 0.02	0.17 ± 0.02
Total saturated	30.59 ± 0.26	30.42 ± 0.31	30.86 ± 0.21	30.15 ± 0.21	30.92 ± 0.34
16:1n-9	0.40 ± 0.02	0.39 ± 0.02	0.34 ± 0.01	0.42 ± 0.02	0.35 ± 0.01
16:1n-7	1.22 ± 0.14	1.40 ± 0.13	1.24 ± 0.11	1.49 ± 0.17	1.03 ± 0.10
18:1n-9	15.93 ± 1.06	16.58 ± 0.99	17.25 ± 0.90	17.69 ± 1.39	14.38 ± 0.76
18:1n-7	1.86 ± 0.03	1.91 ± 0.02	1.88 ± 0.03	1.89 ± 0.05	1.80 ± 0.03
20:1n-9	0.24 ± 0.02	0.24 ± 0.02	0.25 ± 0.02	0.25 ± 0.03	0.21 ± 0.02
24:1n-9	0.22 ± 0.01	0.25 ± 0.02	0.21 ± 0.02	0.23 ± 0.06	0.19 ± 0.01
Total monounsaturated	19.87 ± 1.15	20.76 ± 1.08	21.16 ± 0.96	21.97 ± 1.53	17.96 ± 0.81
18:2n-6	24.27 ± 0.47	23.75 ± 0.26	23.55 ± 0.56	22.91 ± 0.59	23.18 ± 0.40
18:3n-6	0.08 ± 0.00	0.08 ± 0.00	0.07 ± 0.00	0.07 ± 0.00	0.07 ± 0.00
20:2n-6	0.27 ± 0.01	0.27 ± 0.01	0.26 ± 0.01	0.24 ± 0.01	0.24 ± 0.01
20:3n-6	1.14 ± 0.06	1.14 ± 0.07	1.07 ± 0.05	1.04 ± 0.07	1.04 ± 0.06
20:4n-6	10.47 ± 0.50	10.46 ± 0.68	9.75 ± 0.54	9.69 ± 0.69	9.98 ± 0.63
22:4n-6	0.54 ± 0.04	0.53 ± 0.04	0.52 ± 0.05	0.50 ± 0.03	0.46 ± 0.04
22:5n-6	0.23 ± 0.02	0.22 ± 0.02	0.24 ± 0.01	0.20 ± 0.02	0.18 ± 0.02
Total n-6 PUFA	36.99 ± 0.83	36.45 ± 0.82	35.46 ± 0.71	34.64 ± 1.13	35.12 ± 0.56
18:3n-3	0.49 ± 0.03	0.51 ± 0.05	0.54 ± 0.02	0.58 ± 0.04	0.47 ± 0.03
20:5n-3	0.61 ± 0.05	0.59 ± 0.05	0.94 ± 0.08	1.36 ± 0.11	2.35 ± 0.22
22:5n-3	1.28 ± 0.04	1.24 ± 0.05	1.29 ± 0.04	1.46 ± 0.08	1.77 ± 0.09
22:6n-3	1.49 ± 0.17	1.47 ± 0.16	1.49 ± 0.16	1.69 ± 0.14	2.13 ± 0.21
Total n-3 PUFA	3.86 ± 0.23[a]	3.80 ± 0.22[a]	4.28 ± 0.24[a,b]	5.14 ± 0.28[b]	6.79 ± 0.46[c]
16:0DMA	5.37 ± 0.22	5.29 ± 0.21	5.10 ± 0.22	5.18 ± 0.29	5.68 ± 0.20
18:0DMA	1.77 ± 0.08	1.78 ± 0.07	1.68 ± 0.07	1.72 ± 0.08	1.90 ± 0.07
18:1DMA	1.55 ± 0.07	1.50 ± 0.06	1.46 ± 0.05	1.32 ± 0.13	1.63 ± 0.07
Total DMA	8.69 ± 0.34	8.57 ± 0.29	8.24 ± 0.31	8.22 ± 0.42	9.21 ± 0.29

2.3.3 Lipid profile changes in blood

Percentage EPA + DHA/total fatty acids significantly increased from W0 to W1 ($P<0.05$; Figure 2.1 A) with no further detectable increases at W2 or W4. Blood % total n-3 PUFA/total fatty acids was increased at W1 compared to W0 ($P<0.05$ Figure 2.1 B) and remained elevated for the remaining time-points. Percentage total n-6 PUFA/total fatty acids declined significantly from W0 to W2 ($P<0.05$ Figure 2.1 C). A decline from W0 in AA/EPA ratio was observed by W1 ($P<0.05$; Figure 2.1 D) with no further reduction at W4. Percentage n-3 HUFA/total HUFA increased from W0 to W1 ($P<0.05$), and was further elevated at W4 ($P<0.05$; Figure 2.1 E) A full breakdown of muscle fatty acid profiles is presented in Table 2.2.

2.3.4 Correlation analysis of muscle and blood

Correlation analysis revealed a significant association between blood and muscle n-3 PUFA composition at W0 ($P<0.05$, r^2 0.88; Figure 2.2 A) but not at W1 ($P>0.05$, r^2 0.23; Figure 2.2 B). However, the association was improved at W2 ($P>0.05$, r^2 0.42; Figure 2.2 C) and returned to a strong association at W4 ($P<0.05$, r^2 0.95; Figure 2.2 D), likely reflecting different time course changes in the blood and muscle n-3 PUFA composition.

2.3.5 Anabolic protein expression

Total FAK protein content increased from W0 to W4 ($P<0.05$; Figure 2.3 A). Total mTOR protein content significantly increased from W0 to W2 ($P<0.05$) with no further detectable increases at W4 ($P>0.05$; Figure 2.3 B). The largest fold change for total FAK and total mTOR protein content was 3.9 ± 1.5 (W0 to W4) and 3.2 ± 0.8 (W0 to W2), respectively. There was no effect of time on fold change in total p70S6K1 (Figure 2.3 C) or total 4EBP1

protein content (Figure 2.3 D). There was also no effect of time for fold change in the phosphorylation of FAK$^{tyr576/577}$, mTOR^{ser2448}, p70S6K1^{thr389} or 4EBP1$^{thr37/46}$ (data not shown).

Table 2.1 Lipid composition of muscle and blood at -2 and 0 wk.

Fatty acid	-2 Week	0 Week	*P* value
	Muscle		
% EPA + DHA/ total fatty acids	2.09 ± 0.23	2.05 ± 0.21	0.61
% n-3 PUFA/ total fatty acids	3.86 ± 0.24	3.80 ± 0.23	0.50
% n-6 PUFA/ total fatty acids	36.99 ± 0.87	36.45 ± 0.86	0.61
AA/EPA	18.68 ± 1.77	18.93 ± 1.75	0.35
% n-3 HUFA/ total HUFA	21.68 ± 1.70	21.43 ± 1.74	0.36
	Blood		
% EPA + DHA/ total fatty acids	3.43 ± 0.36	3.60 ± 0.32	0.36
% n-3 PUFA/ total fatty acids	5.42 ± 0.43	5.84 ± 0.33	0.19
% n-6 PUFA/ total fatty acids	33.17 ± 0.61	33.47 ± 0.62	0.58
AA/EPA	13.41 ± 1.26	13.07 ± 1.18	0.63
% n-3 HUFA/ total HUFA	25.89 ± 1.50	26.23 ± 1.40	0.53

Sum of eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA), n-3 polyunsaturated fatty acid (PUFA), n-6 PUFA, ratio of arachidonic acid (AA) to EPA (AA/EPA) and % n-3 highly unsaturated fatty acids to total highly unsaturated fatty acids (n-3 HUFA/total HUFA). EPA + DHA, n-3 PUFA and n-6 PUFA are presented as percentage of total lipid composition. AA/EPA as a ratio. Values are means ± SEM.

Table 2.2 Blood lipid composition at -2 week (W-2), 0 week (W0), 1 week (W1). 2 week (W2) and 4 week (W4) of fish oil supplementation. Values are percentage of total lipid composition presented as means ± SEM. Polyunsaturated fatty acids (PUFA), dimethyl aldehyde (DMA). Means that do not share a letter are significantly different.

Fatty acid	-2 week	0 week	1 week	2 Week	4 Week
14:0	0.54 ± 0.05	0.51 ± 0.05	0.55 ± 0.05	0.46 ± 0.06	0.51 ± 0.04
15:0	0.22 ± 0.03	0.18 ± 0.01	0.19 ± 0.01	0.18 ± 0.01	0.19 ± 0.01
16:0	20.78 ± 0.41	20.17 ± 0.28	19.90 ± 0.23	19.66 ± 0.37	20.40 ± 0.30
18:0	11.61 ± 0.25	11.53 ± 0.15	11.60 ± 0.11	11.83 ± 0.13	11.89 ± 0.22
20:0	0.23 ± 0.01	0.22 ± 0.01	0.22 ± 0.01	0.24 ± 0.01	0.22 ± 0.01
22:0	0.60 ± 0.03	0.56 ± 0.02	0.57 ± 0.02	0.59 ± 0.02	0.57 ± 0.02
24:0	1.19 ± 0.04	1.10 ± 0.04	1.12 ± 0.05	1.13 ± 0.06	1.10 ± 0.03
Total saturated	35.17 ± 0.56	34.27 ± 0.24	34.14 ± 0.23	34.07 ± 0.31	34.88 ± 0.32
16:1n-9	0.33 ± 0.02	0.35 ± 0.02	0.27 ± 0.02	0.27 ± 0.02	0.26 ± 0.01
16:1n-7	1.27 ± 0.11	1.47 ± 0.15	1.00 ± 0.08	0.93 ± 0.09	1.21 ± 0.20
18:1n-9	17.63 ± 0.34[a]	18.00 ± 0.41[a]	15.82 ± 0.28[b]	15.75 ± 0.46[b]	16.40 ± 0.43[a,b]
18:1n-7	1.66 ± 0.07	1.58 ± 0.07	1.46 ± 0.06	1.56 ± 0.07	1.56 ± 0.08
20:1n-9	0.28 ± 0.02	0.28 ± 0.01	0.25 ± 0.02	0.25 ± 0.02	0.25 ± 0.01
24:1n-9	1.70 ± 0.07	1.50 ± 0.08	1.49 ± 0.07	1.58 ± 0.09	1.43 ± 0.06
Total monounsaturated	22.87 ± 0.40[a,b]	23.19 ± 0.55[a]	20.30 ± 0.26[c]	20.34 ± 0.46[c]	21.11 ± 0.61[b,c]
18:2n-6	18.61 ± 0.37	18.28 ± 0.69	17.80 ± 0.52	17.19 ± 0.42	16.43 ± 0.33
18:3n-6	0.30 ± 0.04	0.32 ± 0.03	0.21 ± 0.02	0.15 ± 0.01	0.15 ± 0.03
20:2n-6	0.32 ± 0.01	0.37 ± 0.02	0.30 ± 0.01	0.27 ± 0.01	0.24 ± 0.01
20:3n-6	1.77 ± 0.06	1.88 ± 0.08	1.51 ± 0.07	1.32 ± 0.07	1.15 ± 0.07
20:4n-6	10.52 ± 0.44	10.88 ± 0.22	11.24 ± 0.26	10.88 ± 0.36	9.99 ± 0.24
22:4n-6	1.37 ± 0.09	1.44 ± 0.07	1.42 ± 0.07	1.33 ± 0.07	1.21 ± 0.07
22:5n-6	0.28 ± 0.03	0.30 ± 0.02	0.29 ± 0.02	0.28 ± 0.02	0.22 ± 0.02
Total n-6 PUFA	33.17 ± 0.58[a]	33.47 ± 0.60[a]	32.76 ± 0.44[a]	31.42 ± 0.71[a,b]	29.38 ± 0.42[b]
18:3n-3	0.47 ± 0.06	0.57 ± 0.06	0.63 ± 0.04	0.59 ± 0.06	0.52 ± 0.03
20:5n-3	0.86 ± 0.09	0.89 ± 0.07	3.62 ± 0.32[b]	4.54 ± 0.39[b,c]	4.81 ± 0.31[c]
22:5n-3	1.47 ± 0.07	1.58 ± 0.04	1.95 ± 0.07	2.09 ± 0.08	2.21 ± 0.09
22:6n-3	2.58 ± 0.26	2.72 ± 0.25	3.26 ± 0.19	3.52 ± 0.21	3.60 ± 0.21
Total n-3 PUFA	5.43 ± 0.41[a]	5.85 ± 0.31[a]	9.54 ± 0.43[b]	10.83 ± 0.60[b]	11.23 ± 0.53[b]
16:0DMA	1.18 ± 0.05	1.11 ± 0.03	1.16 ± 0.04	1.16 ± 0.05	1.22 ± 0.03
18:0DMA	1.64 ± 0.05	1.61 ± 0.04	1.61 ± 0.05	1.61 ± 0.07	1.65 ± 0.05
18:1DMA	0.55 ± 0.04	0.51 ± 0.04	0.50 ± 0.04	0.56 ± 0.03	0.54 ± 0.04
Total DMA	3.36 ± 0.13	3.22 ± 0.09	3.26 ± 0.12	3.33 ± 0.14	3.41 ± 0.10

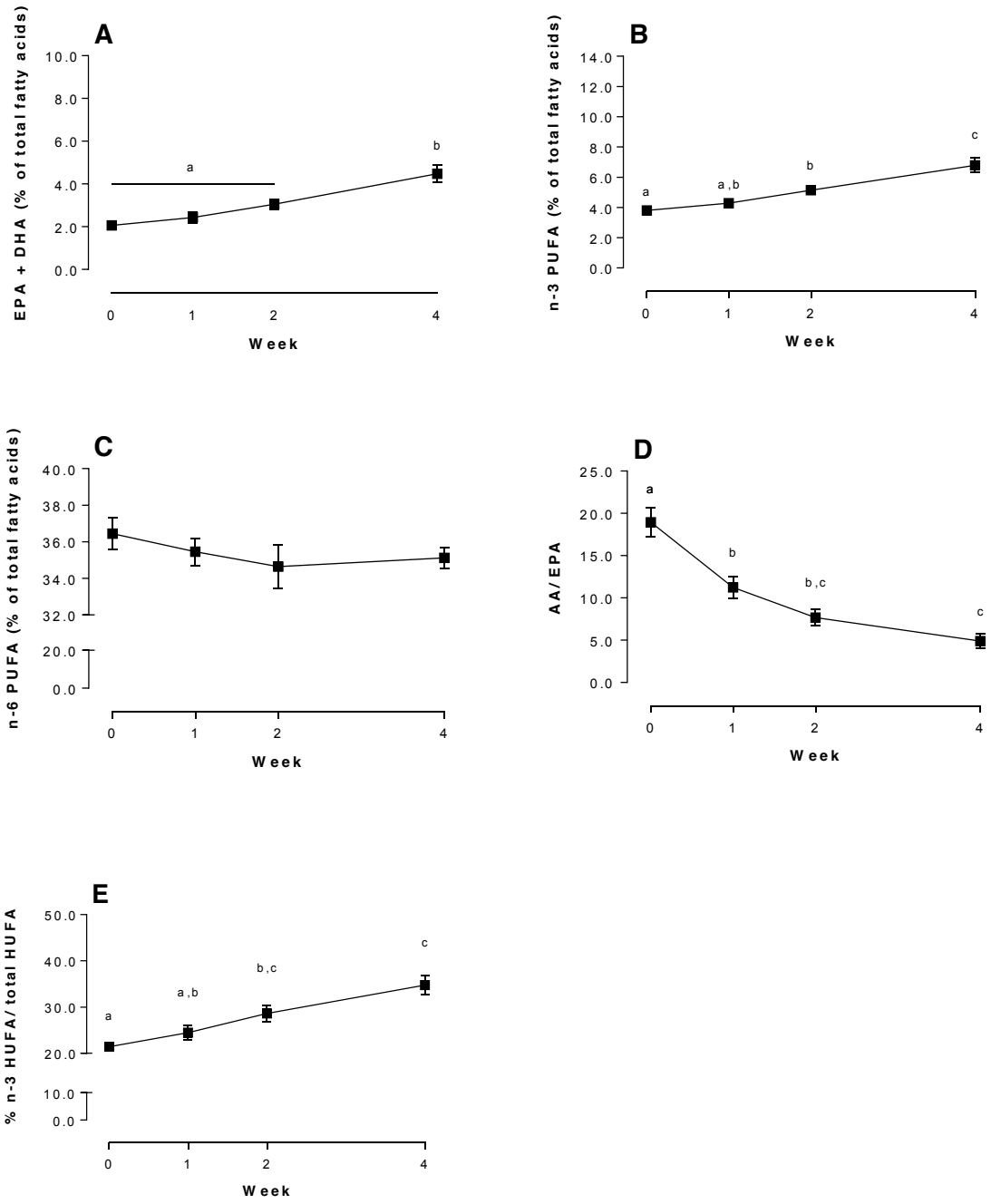

Figure 2.0 Skeletal muscle lipid composition changes of the sum of eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA), n-3 polyunsaturated fatty acid (PUFA), n-6 PUFA, ratio of arachidonic acid (AA) to EPA (AA/EPA) and % n-3 highly unsaturated fatty acids to total highly unsaturated fatty acids (n-3 HUFA/total HUFA). EPA + DHA, n-3 PUFA and n-6 PUFA are presented as percentage of total lipid composition. AA/EPA as a ratio. Values are means ± SEM. Means that do not share a letter are significantly different.

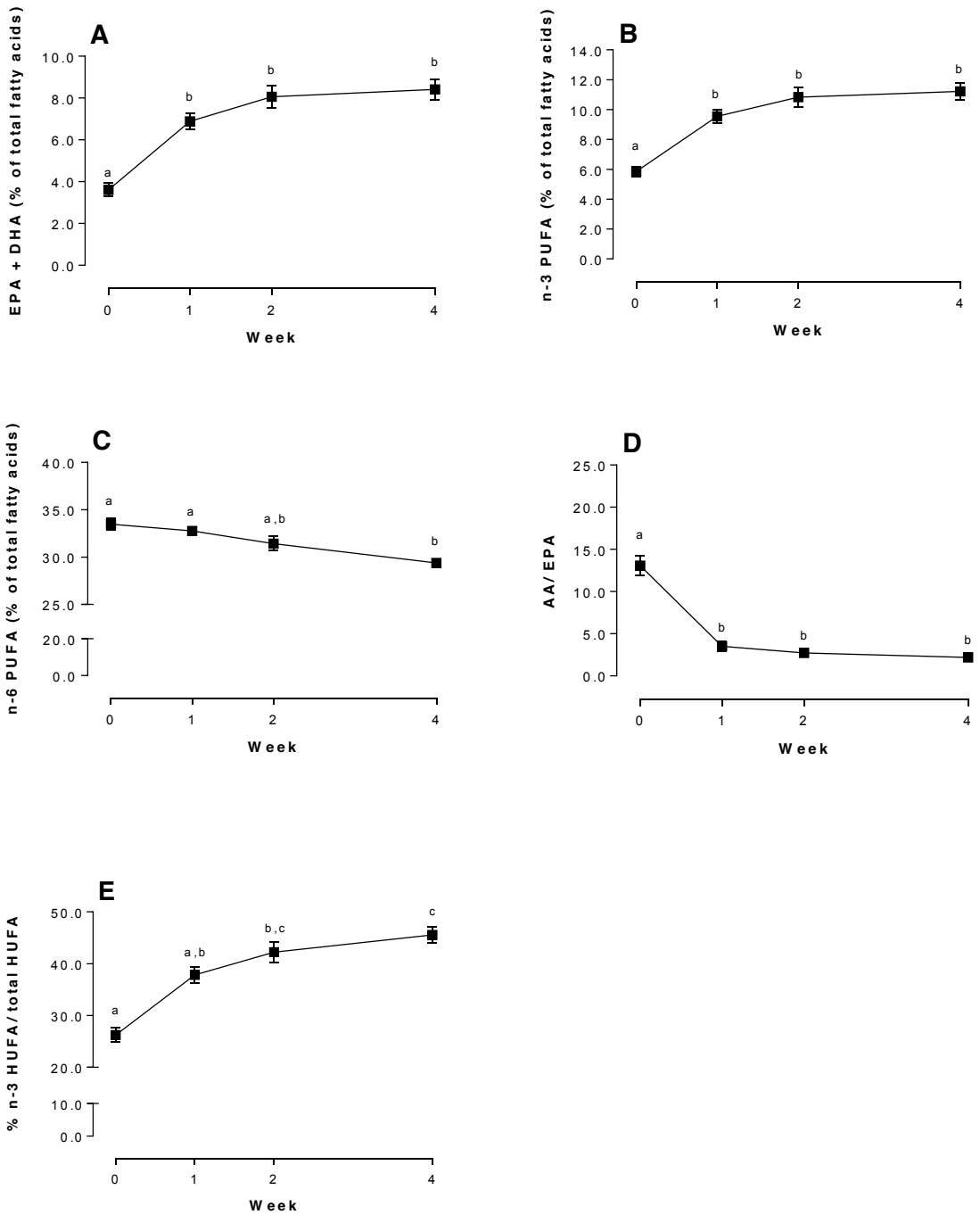

Figure 2.1 Blood lipid composition changes of the sum of eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA), n-3 polyunsaturated fatty acid (PUFA), n-6 PUFA, ratio of arachidonic acid (AA) to EPA (AA/EPA) and % n-3 highly unsaturated fatty acids to total highly unsaturated fatty acids (n-3 HUFA/total HUFA). EPA + DHA, n-3 PUFA and n-6 PUFA are presented as percentage of total lipid composition. AA/EPA as a ratio. Values are means ± SEM. Means that do not share a letter are significantly different.

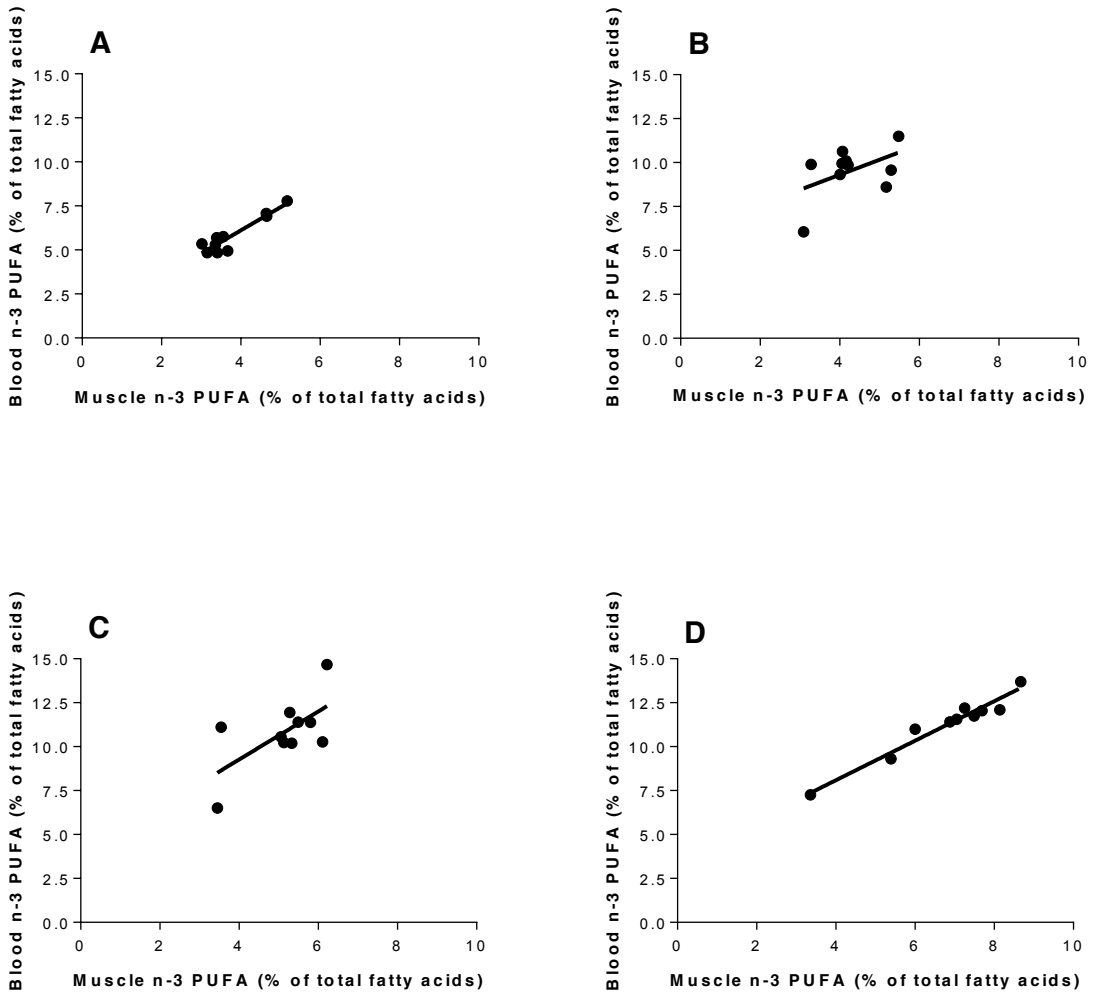

Figure 2.2 Correlation between muscle n-3 PUFA compared with blood n-3 PUFA at W0 (A), W1 (B), W2 (C) and W4 (D) of fish oil supplementation.

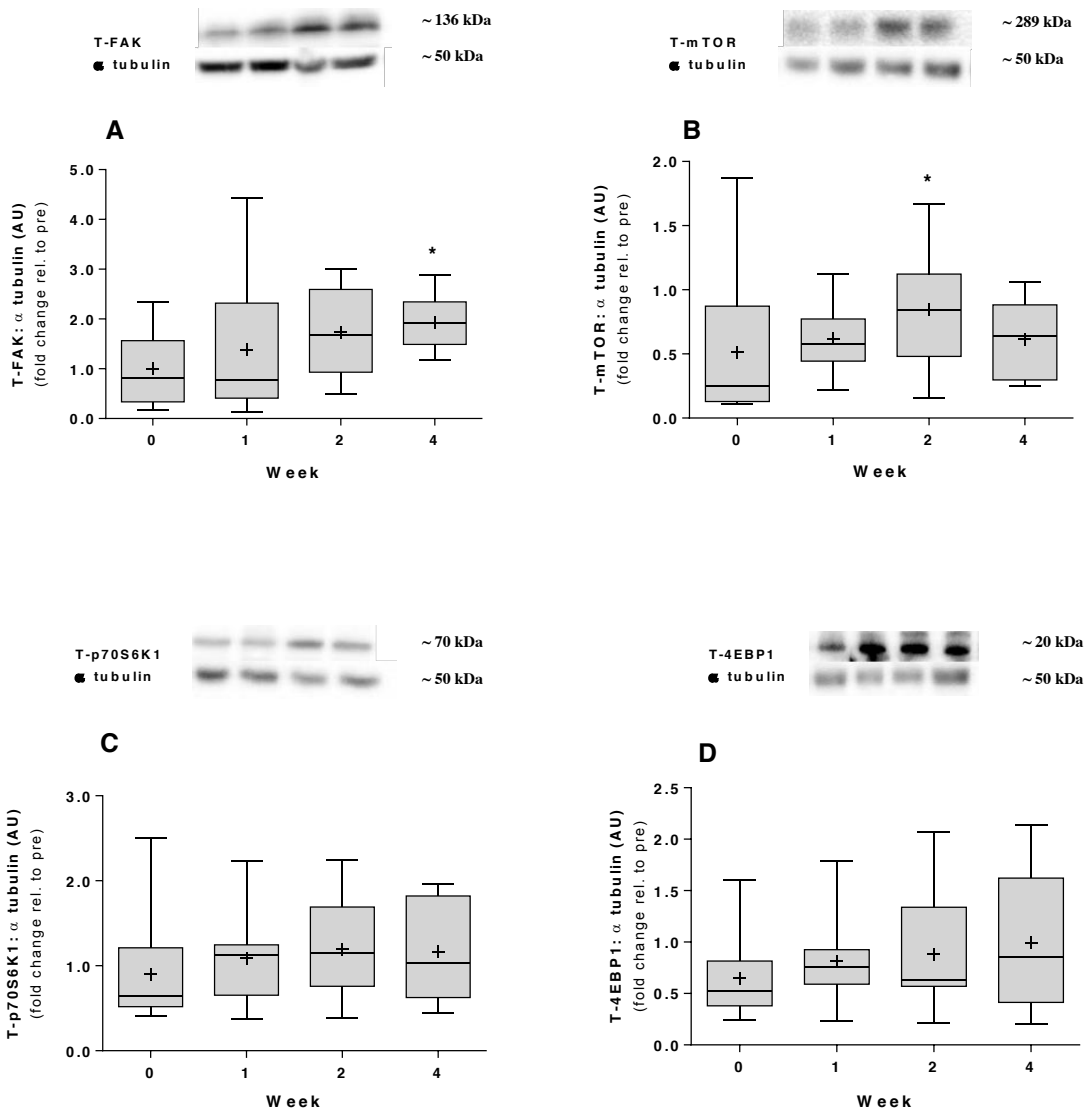

Figure 2.3. Signalling protein content of focal adhesion kinase (FAK) (A), mechanistic target of rapamycin (mTOR) (B), ribosomal protein S6 kinase (p70S6K1) (C) and eukaryotic initiation factor 4E-binding protein 1 (4EBP1) (D). Values expressed as arbitrary units relative to α tubulin and presented as mean (cross), median with interquartile-range and range. * denotes significantly higher than W0 (P<0.05).

2.4 Discussion

This study was designed to examine the changes in muscle and blood lipid composition as well as alterations in anabolic signalling expression during 4 weeks of n-3 PUFA-enriched fish oil supplementation. We report that 4 weeks of fish oil supplementation increased both

blood and skeletal muscle n-3 PUFA composition that was accompanied by an increase in intramuscular anabolic signalling protein content. In addition, we show that the increase in n-3 PUFA in blood occurred within 1 week, however in muscle significant increases in n-3 PUFA composition were not detected until 2 weeks of supplementation. Given the significance of increasing the n-3 PUFA composition of skeletal muscle on improved markers of metabolic health [145], these novel data therefore provide critical information for future studies in this field.

The time course of n-3 PUFA changes in blood and adipose tissue following n-3 PUFA supplementation has been examined previously [123,125,136]. These studies show that n-3 PUFA supplementation can induce detectable increases in the n-3 PUFA composition of blood within 1 week [125], whilst increases in adipose tissue n-3 PUFA composition may require >12 months of supplementation [123]. Herein, we add to the existing literature by demonstrating, for the first time, that a minimum of 2 weeks of fish oil supplementation at the dose used in our study is required in order to induce a detectable increase in n-3 PUFA composition in skeletal muscle. The delay in the response of the n-3 PUFA muscle composition compared with that of blood could be due to differing turnover rates between tissues. Indeed, although there was a strong correlation between blood and muscle n-3 PUFA composition at baseline, after 1 week and 2 weeks of supplementation this correlation was not significant. However, following 4 weeks of supplementation the correlation between blood and muscle n-3 PUFA composition was strengthened and returned to statistical significance. These data therefore highlight that during the initial stages of n-3 PUFA supplementation, changes in blood lipid composition do not accurately reflect those of skeletal muscle likely due to the differing turnover rates between tissues. Furthermore, unlike blood, there was no apparent saturation of muscle n-3 PUFA composition within the 4 week time course. Although, we did not assess muscle lipid composition after 4 weeks of supplementation and

therefore cannot fully discount the possibility of muscle n-3 PUFA saturation at this time point. As a result, a more prolonged period (>4 weeks) of fish oil supplementation may be required in order to reach a saturation in human skeletal muscle n-3 PUFA composition during fish oil supplementation.

Previous studies employing pre/post assessments of n-3 PUFA changes in muscle phospholipids following 8 weeks fish oil supplementation demonstrate a ~2-fold increase in the n-3 PUFA composition from baseline [21,22]. We observed similar ~2-fold increases in whole muscle n-3 PUFA composition, achieved within only 4 weeks of fish oil supplementation. Some of the differences between the present study and previous data may be due to differences in n-3 PUFA content between cellular compartments (i.e. whole muscle vs. membrane) or in the dose and/or composition of the supplements. Indeed, the rapid increase in n-3 PUFA composition in whole muscle observed in our investigation may relate to the high EPA content of the fish oil supplement. Indeed, our participants ingested nearly double the daily dose of EPA (3500 mg vs. 1860 mg daily) as used in previous studies, in which the supplementation protocol was twice as long as the current investigation [21,22]. These results suggest that the n-3 PUFA composition of skeletal muscle could be a function of the dose of n-3 PUFA consumed as well as the time course of supplementation. Unfortunately, to the author's knowledge, there are no data pertaining to changes in whole muscle vs. muscle membrane n-3 PUFA composition with fish oil supplementation. Thus, future studies employing differing n-3 PUFA supplementation protocols may elucidate a dose and time-dependant response of n-3 PUFA changes in skeletal muscle similar to those as established in other biological tissues [123,125,136]. Furthermore, identification of changes in n-3 PUFA composition of specific fractions of skeletal muscle to fish oil supplementation also would advance existing knowledge.

The impact of n-3 PUFA supplementation on skeletal muscle anabolism is becoming a topic of intense investigation. In humans, it is now known that n-3 PUFA supplementation potentiates MPS to a hyperaminoacidemic-hyperinsulinemic infusion [21,22]. Additionally, in rodents, n-3 PUFA supplementation has been shown to alleviate *soleus* atrophy during immobilization [17]. The findings of the present investigation add to these data as we show that 4 weeks of fish oil supplementation increases the total protein content of mTOR and FAK in free-living humans. Given that both FAK and mTOR play a key role in the molecular regulation of MPS [30,99,100], our data could be interpreted to suggest that the increases in total protein content provide an enhanced capacity of skeletal muscle to respond to anabolic stimulation. However, as a note of caution, this increase in mTOR was transient and a recent study in rodents has shown that during remobilisation from an immobilised state, dietary fish oil is associated with inhibition of myosin heavy chain content recovery [128]. This finding highlights a limitation of our investigation as our study design precludes us from demonstrating whether the increases in mTOR and FAK protein content translate to enhanced activation in response to stimulation. Given the sustained increase in FAK total protein content observed in our investigation, taken together with previous reports demonstrating the key role of FAK in mediating mechanically-induced changes in MPS [100], future work that identifies if n-3 PUFA supplementation enhances resistance exercise-induced rates of MPS in humans merits further investigation.

The physiological mechanisms by which fish oil supplementation influences anabolic signalling remain largely unclear. Increasing the n-3 PUFA composition of the muscle membrane may alter gene expression [122] and/or the regulation of lipid signals such as phosphatidic acid that subsequently impact anabolic protein signalling [146]. It is known that dietary fish oil inhibits the recovery of atrophied muscle in rodents that is associated with a reduction in prostaglandin F2α (PGF2α) [128]. A reduction of PGF2α in that study also was

associated with blunting of Akt-p70S6K1 signalling. These authors proposed that dietary fish oil could have reduced the bioavailability of arachidonic acid, a fatty acid from which PGF2α is derived. However, our data provide some evidence to counter the latter proposition in humans. We show that 4 weeks of fish oil supplementation increases the content of intramuscular anabolic signalling molecules, without influencing the n-6 PUFA or AA composition of the muscle. Thus, our data could suggest that there may be other, as yet unknown, mechanisms that account for the changes in skeletal muscle plasticity with fish oil supplementation. As a cautionary note, it is important to recognise that our data are indicative of whole muscle and not the phospholipid fraction. Therefore, research that characterises the impact of n-3 PUFA supplementation on lipid composition in specific cell fractions and lipid signalling using sophisticated techniques such as lipidomics and transcriptomics, are now required to expand our current understanding.

In summary, this study has characterised a time course of n-3 PUFA changes and anabolic signalling expression in human skeletal muscle during 4 weeks of fish oil supplementation. The primary conclusion of this investigation is that fish oil supplementation results in changes in muscle n-3 PUFA composition of skeletal muscle in weeks, rather than months as has been reported for adipose tissue [123]. To our knowledge, this study also is the first to show that fish oil supplementation increases the content of FAK that may suggest a 'priming' of the muscle to respond to mechanical stimulation. Future studies that identify how fish oil supplementation influences anabolic signalling in response to mechanical stimulation in a physiological setting are now warranted.

Author contributions

K.D.T, S.D.R.G and CMcG designed the study; C.McG, K.D.T and S.D.R.G conducted the study. C.McG, D.L.H, L.B, J.R.D and J.G.B performed the analysis.

CHAPTER 3 Application of the (γ-^{32}P) ATP kinase assay to study anabolic signalling in human skeletal muscle.

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3.0 Abstract

mTORC1/p70S6K1 signalling plays a crucial role in MPS. Understanding of this pathway has been advanced by the application of the WB technique. However, in some instances the semi-quantitative nature and poor dynamic range associated with the WB technique may lead to incongruence regarding the molecular response of skeletal muscle to anabolic stimulation. The aim of this study was to develop and apply a quantitative *in vitro* [γ-^{32}P] ATP kinase assay (KA) alongside a traditional WB methodology to assess p70S6K1 signalling responses in human skeletal muscle to RE and protein feeding. Following validation in tissue culture with rapamycin and optimization of the assay in human skeletal muscle, this methodology was tested in a physiologically relevant context. In this regard, six males performed unilateral RE followed by the consumption of 20 g of protein. Skeletal muscle biopsies were obtained at pre-RE, at 1 h and 3 h post-RE. In response to RE and protein consumption, p70S6K1 activity was significantly increased from pre-RE at 1 h and 3 h post-RE (8.84 ± 0.78 to 17.18 ± 2.62 and 15.62 ± 3.12 μU/mg). However, phosphorylated p70S6K1^{thr389} was not significantly elevated. Several reports have indicated that protein feeding alone or RE alone can down regulate AMPK phosphorylation. To assess if a combined stimulus of RE and feeding can influence AMPK activity we directly measured AMPK activity. AMPK activity was suppressed from pre-RE at 3 h post-RE (24.15 ± 1.6 to 15.64 ± 1.07 mU/mg), whereas phosphorylated ACC^{ser79} was unchanged. Total PKB activity also was unchanged after RE from pre-RE. Of the other markers we assessed by WB, 4EBP1$^{thr37/46}$ phosphorylation was the only significant responder being elevated at 3 h post-RE from pre-RE. These data highlight the utility of the KA to study skeletal muscle plasticity.

3.1 Introduction

In **Chapter 2** of this thesis it was identified that 4 weeks of n-3 PUFA-enriched fish oil supplementation increased the n-3 PUFA composition of skeletal muscle. This increase in n-3 PUFA composition of skeletal muscle was associated with the enhanced expression of the mechanically sensitive protein FAK. Given FAK has been shown to regulate contraction-induced changes in MPS via mTOR-p70S6K1 signalling [100], we therefore hypothesised that the increase in FAK expression associated with fish oil supplementation may serve to enhance anabolic signalling activity in response to a bout of RE. The traditional method to assess anabolic signalling activity in the exercise sciences is the WB technique. This technique assesses the phosphorylation of a kinase or a kinase target on serine, threonine and tyrosine residues, and infers the activity of the kinase based on the magnitude of phosphorylation as determined by densitometry. The WB technique is highly advantageous as it offers the capacity to measure phosphorylation changes in many targets in a financially appealing way. However, in some cases the WB technique possesses a limited dynamic range that can lead to type II statistical errors [147]. Furthermore, differences in methodological approaches to the WB are known to result in differential statistical outcomes for the same data sets [148]. Another consideration in the context of anabolic responses is that p70S6K1 has a constitutively low baseline phosphorylation. When changes in p70S6K1 phosphorylation to stimulation are represented as a fold or percentage change this low baseline phosphorylation results in an inflated response that is unlikely to be representative of a physiological change in activity [149,150]. Hence, our ability to detect changes in mTOR/p70S6K1 activity to RE and feeding following a period of n-3 PUFA supplementation is, in part, confined to both the limitations and assumptions of the WB technique.

In a recent commentary Murphy and Lamb [151] describe a fully quantitative approach to Western blotting that would appear to obviate these methodological considerations. These authors show that by using calibration curves for each gel, a quantitative assessment of changes in protein expression can be made. However, conducting calibration curves for the analysis of post translational modifications (PTM), such as phosphorylation, would be contingent upon 100% of the recombinant protein modified specifically at the specific PTM residue. Furthermore, the use of such calibration curves on every gel would prove costly when analysing numerous samples thus undermining the financial viability of the WB technique. As such, the use of the WB to assess changes in the phosphorylation of a kinase in the context of its activity for the assessment of anabolic signalling activity in response to RE remains limited.

The *in vitro* [γ-^{32}P] ATP kinase assay (KA) is the gold standard for assessing kinase function [152] and may represent a more practical alternative to quantitatively measure kinase activity in response to exercise. The original methodology involves immunoprecipitating the kinase of interest from homogenised tissue. The activity of the kinase is then assessed *in vitro* against a kinase-specific or kinase family-specific substrate. Gamma (γ)-^{32}P ATP is subsequently used to measure the incorporation of phosphate into the substrate via liquid scintillation counting thus enabling a quantitative assessment of kinase activity. This dual layer of specificity and quantitative approach may obviate some of the methodological shortcomings associated with the WB methodology [148]. Although, a semi-quantitative p70S6K1 KA has been described for use in rodent tissue [149] and a quantitative p70S6K1 KA has previously been used in cell culture studies [153]. However, no study has described a fully quantitative KA methodology for the assessment of p70S6K1 activity in human skeletal muscle. Additionally, a major drawback of the existing p70S6K1 KAs described in cell [154] and rodent [149] models is

that a large amount of muscle tissue is required. This demand would render these techniques redundant in many human experiments during in which muscle tissue availability is limited.

The aim of this methodological study was therefore three fold. Firstly, to develop and validate a quantitative p70S6K1 KA methodology to assess p70S6K1 activity in human skeletal muscle. Secondly, to validate a serial immunoprecipitation protocol for the assessment of multiple kinases from a single muscle sample thus economising on muscle tissue sample. Finally, to apply this methodology alongside a traditional WB methodology to detect changes in p70S6K1 activity and associated readouts of mTORC1 activity in response to RE and protein feeding in humans [25].

2.2 Methods

2.2.1 Materials

All materials, unless otherwise stated, were from Fisher Scientific (Loughborough, UK). All antibodies unless otherwise stated were used at a concentration of 1:1000 and were from New England Biolabs (Herts, UK). Selected primary antibodies were mTOR^{ser2448} (#2974), total mTOR (#2983), ACC^{ser79} (#3661), total ACC (#3676), total glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (#2118), Raptor^{ser792} (#2083), (#3702), p70S6K1^{thr389} (Santa Cruz; #9234), total p70S6K1 (#2708), AMPK^{thr172} (#2531), total AMPK (#2532), PKB^{thr308} (#2965), total PKB (#4691), PRAS40^{thr246} (#2997) and total PRAS40 (#2691), 4EBP1$^{thr37/46}$ (#2855) total 4EBP1 (#9644). Secondary HRP-conjugated antibody was purchased from ABCAM (#6721). Pre-poured gels for Western blotting were 4-20% Tris-Glycine Criterion gradient gels from BioRad (Herts, UK). AMPK α1 and α2 specific antibodies were produced by GL Biochem (Shanghai, China) against the following antigens; α1, CTSPPDSFLDDHHLTR and α2, CMDDSAMHIPPGLKPH [155].

2.2.2 Tissue Culture Experiments

C_2C_{12} myoblasts were grown to confluence on T75 plates in Growth Media [(GM) 20% FBS (Dundee Cell Products, Dundee, UK), 1% Penicillin/Streptomycin (Invitrogen, Paisley, UK) in high glucose DMEM (Invitrogen)]. Confluent myoblasts were then transferred to differentiation media [(DM) 2% Donor Horse Serum (Dundee Cell Products), 1% Penicillin/Streptomycin (Invitrogen) in high glucose DMEM (Invitrogen)]. Prior to the addition of inhibitors cells were serum and amino acid starved in PBS with 5 mM Glucose (Invitrogen) for 3 h. Starved cells were then pre-treated with an inhibitor [100 nM rapamycin (Sigma Aldrich), 10 μM LY294002 (Cell Signalling) or vehicle control (0.1% DMSO)] for 1 h prior to serum and amino acid stimulation by the addition of GM supplemented with or without inhibitors. After 30 min of stimulation cells were lysed on ice in 1 mL of radio immunoprecipitation assay (RIPA) buffer (50 mmol/L Tris/HCl, pH 7.5; 50 mmol/L NaF; 500 mmol/L NaCl; 1 mmol/L Na vanadate; 1 mmol/L EDTA; 1% [vol/vol] triton X-100; 5 mmol/L Na pyrophosphate; 0.27 mmol/L sucrose; and 0.1% [vol/vol] 2-mercaptoethanol) and then stored at -80 °C. HEK293 cell lysates over expressing either the α1 or the α2 subunit of AMPK were a gift from Professor Grahame Hardie (Division of Cell Signalling and Immunology, University of Dundee).

2.2.3 Mouse ex vivo and in vivo insulin stimulations

All animal experiments were approved by and conducted in accordance with the Animal Care Program at the University of California, San Diego for the *ex vivo* insulin stimulations and the Animal Care Program at the University of California, Davis for the *in vivo* insulin stimulations. *Ex vivo* insulin stimulations were carried out as follows: 6 male C57/Bl6 mice were fasted for 4 h and anaesthetised (150 mg/kg Nembutal) via intraperitoneal injection. Paired extensor *digitorum longus* (EDL) muscles were incubated at 35°C for 30 min in

oxygenated (95% O_2, 5% CO_2) flasks of Krebs-Henseleit buffer (KHB) containing 0.1% BSA, 2 mM Na-pyruvate and 6 mM mannitol. One muscle per pair was incubated in KHB without insulin, and the contralateral muscle was incubated in KHB with insulin (60 μU/mL [0.36 nM]; Humulin R, Eli Lilly and Company). After 50 min, muscles were blotted on ice-cold filter paper, trimmed, freeze clamped and then stored at -80°C (n=6). *In vivo* insulin stimulations were carried out as follows: 2 female C57/Bl6 mice were fasted for 4 h and anaesthetised with 2% isoflurane vaporised in 100% O_2. One mouse was intrapertioneally injected with 100 mU/kg of insulin (Humulin R, Eli Lilly and Company). Following 30 min the muscles from the lower limb were dissected and snap frozen in liquid N_2. The control mouse went through the same procedure except that it was injected with 0.9 % saline.

3.3 Human experimental study

3.3.1 Participants

Six healthy, moderately trained males (mean ± SD: age, 23 ± 2 yr; body mass, 76 ± 5 kg; height, 179 ± 5 cm; unilateral 1 repetition maximum [1 RM] leg press, 128 ± 8 kg; 1 RM leg extension, 54 ± 3 kg) were recruited to participate in this study. All participants engaged in resistance training ~2 times per week and played team sports recreationally. Prior to the commencement of the experiment each participant provided written informed consent after all procedures and risks were fully explained in lay terms. Participants also were required to satisfy a routine physical activity readiness questionnaire (PARQ). The study procedures were approved by Research Institute for Sport and Exercise Sciences Ethics Committee, Liverpool John Moores University and conformed to the standards as outlined in the 2008 version of the Declaration of Helsinki.

3.3.2 Study design

Seven days after confirmation of unilateral 1 RM for leg press and leg extension, six healthy, moderately trained males reported to the laboratory at ~0700 h in a 10 h post-absorptive state. Each participant's height and body mass were recorded after which they were rested (~30 min) in a semi-supine position on a bed and a resting biopsy was obtained. Immediately after the biopsy participants were transported by wheelchair to the resistance-training laboratory where they performed a bout of unilateral RE. Immediately following the bout of unilateral RE, participants were required to consume 20 g of pure egg white powder in a 500 mL solution. Participants were then transported back to the resting laboratory and rested again in a semi-supine position during which additional muscle biopsies were obtained at 1 h and 3 h post-RE.

3.3.3 Resistance exercise protocol

1 RM testing was conducted as previously described [156]. On the day of the experimental trial participants performed a bout of unilateral RE consisting of 4 sets of 10 repetitions at 70% 1 RM of leg press followed by leg extension performed at the same intensity with their dominant limb. Recovery time between exercises and sets was 3 min and 2 min, respectively. Participants were provided with verbal cues in order to ensure correct exercise technique. Each repetition consisted of a 1 s concentric action, 0 s pause then a 1 s eccentric action as previously reported [64].

3.3.4 Study controls

Participants were required to record dietary intake for 3 d prior to the initial one 1 RM testing session and to repeat this pattern of consumption for the 3 d preceding the day of the

experimental trial. For 3 d prior to both 1 RM testing and the experimental trial participants also were asked to refrain from any form of vigorous exercise. These controls were implemented in an attempt to prevent any nutritional or exercise-induced changes in protein activity adversely impacting the results of the study.

3.3.5 Skeletal muscle biopsies

Skeletal muscle biopsies were obtained on the exercising limb at pre-RE, 1 h post and 3 h post-RE using a Bard Monopty Disposable Core Biopsy Instrument 12 gauge × 10 cm length (Bard Biopsy Systems, Tempe, AZ). For each biopsy the lateral portion of the *vastus lateralis* was cleaned before an incision into the skin and fascia was made under local anaesthetic (0.5% Marcaine, without adrenaline: cat. no. MD92672). A sample of muscle (~30 mg) was extracted, rinsed with ice cold saline, blotted dry and any visible fat or connective tissue was removed. Muscle samples were then snap-frozen in liquid nitrogen and stored at – 80°C for further analysis.

3.3.6 Muscle Tissue Processing

Approximately 30 mg of human skeletal muscle tissue (~5 mg of mouse skeletal muscle tissue) was homogenised by scissor mincing on ice in RIPA buffer (50 mmol/l Tris/HCl, pH 7.5; 50 mmol/L NaF; 500 mmol/L NaCl; 1 mmol/L Na vanadate; 1 mmol/L EDTA; 1% [vol/vol] Triton X-100; 5 mmol/L Na pyrophosphate; 0.27 mmol/L sucrose; and 0.1% [vol/vol] 2-mercaptoethanol) followed by shaking at 1000 rpm on a shaking platform for 60 min at 4ºC. Debris was removed by centrifugation at 4°C for 15 min at 13,000 *g*. The supernatant was then removed and protein concentration determined using the BCA protein assay according to the manufacturers' instructions (Sigma Aldrich, UK).

3.3.7 Western blotting

For Western blots 300 µL of supernatant was made up in Lamelli Sample Buffer and 5-15 µg of total protein was loaded per well with the same amount of protein loaded in all wells for each gel and run at 150 V for 1 h 15 min. Proteins were then transferred onto Whatman Immobilon Nitrocellulose membranes (Fisher Scientific, Loughborough, UK) at 30 V overnight on ice. Membranes were blocked in 3% BSA- Tris-buffered saline (containing vol/vol 0.1% Tween 20) for 1 h at room temperature followed by incubation in primary antibodies at 4ºC overnight. Membranes underwent 3 × 5 min washes in TBST followed by incubation in the appropriate secondary antibodies for 1 h at room temperature. Membranes were again washed for 3 × 5 min followed by incubation in enhanced chemiluninescence (ECL) reagent (BioRad, Herts, UK). A BioRad ChemiDoc (Herts, UK) was used to visualise and quantify protein expression. All phospho-proteins were normalised to the corresponding total proteins after stripping the phospho antibody for 30 min at 50ºC in stripping buffer (65 mM Tris HCl, 2% SDS vol/vol, 0.8% Mercaptoethanol vol/vol) and re-probing with the primary antibody for the corresponding total protein. All phospho-proteins were normalised to the expression of the corresponding total with the exception of phosphorylated Raptor^{ser792} that was normalised to the expression of GAPDH.

3.3.8 [γ-^{32}P] ATP kinase assays

All KAs were carried out by immunoprecipitation (IP) either for 2 h at 4°C or overnight at 4°C in homogenisation buffer [AMPK (50 mM TrisHCl pH 7.25, 150 mM NaCl, 50 mM NaF, 5 mM NaPPi, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM benzamidine, 0.1 mM PMSF, 5 µg/mL soya bean trypsin inhibitor, 1% (v/v) TritonX-100) and p70S6K1/panPKB (50 mM TrisHCl pH 7.5, 0.1 mM EGTA, 1 mM EDTA, 1% (v/v) TritonX-100, 50 mM NaF, 5 mM NaPPi, 0.27 M sucrose, 0.1% β-mecertoehtanol, 1 mM $Na_3(OV)_4$ and 1 Complete

(Roche) protease inhibitor tablet per 10 mL)]. Protein G sepharose (70%, 2.5 µL per IP) was used to precipitate the immune-complexes. Immune-complexes were washed 2 x in assay specific high salt washes (homogenisation buffers as above with 0.5 M NaCl added) followed by 1 x wash in assay specific assay buffer (see below). Prior to carrying out the activity assay the immune-bead-complex was suspended in a total of 10 µl of assay buffer for p70S6K1 and panPKB assays and 20 µl of assay buffer for AMPK assays. All assays were carried out in a 50 µl reaction. Assays were started every 20 s by the addition of a hot assay mix that consisted of assay buffer [PKB/p70S6K1 (50 mM TrisHCl at pH 7.4, 0.03% Brij35, 0.1% β-mercaptoehtanol), AMPK (50 mM HEPES at pH 7.4, 1 mM DTT, 0.02% Brij35)], ATP-$MgCl_2$ (100 µM ATP + 10 mM $MgCl_2$ for p70S6K1/panPKB and 200 µM ATP + 50 µM $MgCl_2$ for AMPK), $^{32}\gamma$-ATP [specific activities as follows; panAMPK (0.25 x 10^6 cpm/nmol), panPKB (0.5 x 10^6 cpm/nmol), p70S6K1 (1 x 10^6 cpm/nmol)] and finally synthetic peptide substrates ["Crosstide" for panPKB (GRPRTSSFAEG at 30 µM), "S6tide" for p70S6K1 (KRRRLASLR at 30 µM) and "AMARA" for AMPK (AMARRAASAAALARRR at 200 µM)]. Assays were stopped at 20 s intervals by spotting onto squares of p81 chromatography paper (Whatman, GE Healthcare, UK) and immersing in 75 mM phosphoric acid. p81 papers were washed 3 x 5 min in 75 mM phosphoric acid and 1 x in acetone. They were then dried and immersed in Gold Star LT Quanta scintillation fluid (Meridian Biotechnologies Ltd, Chesterfield, UK) and counted in a United Technologies Packard 2200CA TriCarb scintillation counter. Assay results were quantified in nmol/min/mg (U/mg). Blanks for background subtractions were carried out with immunoprecipitated kinases with no peptide included in the assay reaction. For the AMPK antibody validation assays the AMPK α1 antibody (5 µg) was used to immunoprecipitate AMPK α1 complexes from 100 µg of lysate in duplicate whilst AMPK α2 antibody (5 µg) was used to immunoprecipitate AMPK α2 complexes from 100 µg of lysate. These lysates were from HEK cells overexpressing either AMPK α1 or AMPK α2 and were a kind gift from Professor Grahame Hardie (University of

Dundee). Assays were carried out for 15 min. panPKB and p70S6K1 activity assays were carried out on cell lysates by IP from 200 µg of cell lysate. The IP step was performed with 2 µg each of PKBα/β/γ antibodies (DSTT, Dundee University) or 2 µg of p70S6K1 antibody [#H-9 (Santa Cruz Biotechnology Inc, Heidelberg, Germany)] respectively. Antibodies were used with 2.5 µL of protein G sepharose per IP to immunoprecipitate for 2 h at 4°C. p70S6K1 and panPKB were assayed for 45 min and 20 min respectively.

3.3.9 Time dependant saturation assays

Three human skeletal muscle skeletal muscle biopsy samples were pooled and homogenised. Homogenate was aliquoted to 2.4 mg for panPKB assays, 6 mg for p70S6K1 assays and 0.6 mg for AMPK assays. 72 µg each of PKBα/β/γ antibodies were used to immunoprecipitate panPKB, 48 µg of p70S6K1 antibody was used to immunoprecipitate p70S6K1 and 60 µg each of AMPK α1 and α2 were used to immunoprecipitate panAMPK. Following IP each of these immune-complexes were aliquoted into 12 aliquots for activity assays, nine of the aliquots were used for activity assays for the time course of 7.5 min, 15 min and 30 min for AMPK, 15 min, 30 min and 60 min for panPKB and p70S6K1. The 3 remaining aliquots were used for no-peptide controls to generate assay specific blanks. Each assay represented an IP from 50 µg of lysate for panAMPK, 200 µg of lysate for panPKB and 500 µg of lysate for p70S6K1.

For the serial IP validation, lower limb muscles from a 4 h fasted (Con) and an insulin stimulated mouse [Ins (4 h fasted + 100 mU insulin/kg for 30 min)] were homogenised and aliquoted into 6 x 200 µg aliquots each. IP's were set up to IP panPKB (3.2 µg of each PKB antibody) from 3 Con and 3 Ins aliquots whilst the other aliquots had p70S6K1

immunoprecipitated (4 µg of p70S6K1 antibody) prior to immunoprecipitating with panPKB as before. panPKB activity assays were carried as described above following IP.

For p70S6K1/panPKB KA in human tissue, 500 µg of lysate was aliquoted and p70S6K1 was immunoprecipitated with 4 µg of p70S6K1 and 2.5 µl of protein G sepharose (GE Healthcare) for 2 h at 4 °C. The p70S6K1 KA was carried out for 45 min. 200 µg of the post IP supernatant was then used for panPKB IP. 2 µg each of PKBα/β/γ antibodies (DSTT, Dundee University) were used with 2.5 µl of protein G sepharose to immunoprecipitate PKB at 4 °C for 2 h. panPKB KA were carried out as previously described for a 30 min assay. Following homogenisation, 50 µg of lysate was aliquoted for AMPK activity assays. AMPK activity assays were carried out by IP with complexes in AMPK IP buffer (homogenisation buffer as above). Immunoprecipitates were then washed and AMPK activity was determined against AMARA peptide as previously described in a 20 min assay.

3.3.10 Statistical analysis

Data were analysed using GraphPad Prism Software version 6.0 (GraphPad, San Diego, CA, USA) unless otherwise stated. Differences in kinase signalling activity and phosphorylation (i.e., p70SK61^{thr389}, PKB^{thr308}, AMPK activity) were analysed using a one-way ANOVA and when appropriate a Tukey's post-hoc was employed for post-hoc analysis. Regression analysis was performed to identify correlation coefficients for time against kinase activity. Post-hoc sample size calculations were conducted using GPower 3.0.8 software based on an estimated effect size of 0.53, a 1-ß error probability of 0.8 and a significance level < 0.05. All data unless otherwise stated are presented as means ± SEM and P values < 0.05 indicate statistical significance.

3.4 Results

3.4.1 Antibody/Assay validation

panAMPK

Total (or pan) AMPK activity was measured by immunoprecipitating both catalytic subunits of AMPK (AMPK α1 and AMPK α2). We commissioned our own AMPK α1 and AMPK α2 antibodies (GL Biochem, China) against the following antigens; α1, CTSPPDSFLDDHHLTR and α2, CMDDSAMHIPPGLKPH [155]. To confirm that the AMPK antibodies were AMPK α1 and AMPK α2 specific and therefore capable of immunoprecipitating total AMPK when the antibodies are combined, we carried out a validation experiment (Figure 3.0 A). Cell lysates over expressing either AMPK α1 or AMPK α2 underwent an IP with either the AMPK α1 or AMPK α2 antibody. AMPK α1 immunoprecipitated substantial activity from the AMPK α1 overexpressing cell lysates, ~10 fold more activity than the AMPK α2 antibody immunoprecipitated. The reverse experiment demonstrated a similar result in that AMPK α2 immunoprecipitated ~10 fold more activity from the AMPK α2 overexpressing cell lysates than did the AMPK α1 antibody. These data demonstrate the specificity of our AMPK α1 and α2 antibodies. To further highlight that these antibodies are immunoprecipitating active endogenous AMPK complexes, a positive control experiment was conducted by treating C_2C_{12} myotubes with 100 μM 2,4-Dinitrophenol (DNP [a known AMPK activator [157]) for 30 min and followed this treatment with panAMPK activity assays. This treatment resulted in a ~4 fold increase in panAMPK activity (Figure 3.0 B) concurrent with a substantial increase in phosphorylation of AMPK at Thr172 (inset Figure 3.0 B).

panPKB

Total (or pan) PKB activity can be assessed by utilizing recombinant glycogen synthase kinase-3 (GSK3) as a substrate and then running a standard WB with a phosphorylated GSK3

antibody to determine phosphate incorporation [27]. However, this approach again relies upon densitometry analysis thus comparisons across large sample sets are problematic. Therefore, in this study a filter binding assay was employed that also allowed for quantitative scintillation counting. We used antibodies and a peptide substrate [158] that have been characterised previously [158,159]. However, to confirm that panPKB activity was being detected with the immune-complex a positive control experiment was carried out (Figure 3.0 C). We serum stimulated C_2C_{12} myotubes that had been treated with or without the PI3K inhibitor LY294002 [160]. Serum stimulation led to a ~5 fold increase in panPKB activity whilst the inhibition of PI3K with LY294002 significantly inhibited panPKB activity. The changes in activity were reflected by changes in phosphorylation (inset Figure 3.0 C).

p70S6K1

Traditionally p70S6K1 activity assays are carried out with recombinant S6 as a substrate [149] wherein the radioactively labelled substrate is run on a gel before being exposed to radiography film. This assay is more difficult to accurately quantify with large sample numbers due to the necessity to expose all samples to SDS-PAGE. Furthermore, this method still requires the use of densitometry analysis that can be subjective leading to variable outputs depending upon the method of quantification [148]. However, several laboratories have utilised a scintillation assay to quantitatively assess p70S6K1 activity [153,161]. In order to utilise a quantitative p70S6K1 activity assay that can be applied more easily to large sample numbers we employed a similar assay protocol with a peptide substrate analogue of S6 corresponding to amino acids 230-238 on human 40S ribosomal protein S6 (KRRRLASLR) [162]. This approach allowed for the use of filter paper capture of the labelled peptide that can then be analysed quantitatively via scintillation counting. In order to confirm that this method did not alter the results of the assay we carried out a validation experiment in C_2C_{12} myoblasts (Figure 3.0 D). We used serum and amino acid stimulation as

a positive control with rapamycin (specifically inhibits mTORC1 activity) as a control to confirm that serum and amino acid-induced activation of kinase activity was in fact, p70S6K1 specific. We show that serum and amino acid stimulation induces a ~10 fold increase in activity whilst rapamycin completely blocks this activation (Figure 3.0 D) and the phosphorylation of p70S6K1^{thr389} (inset Figure 3.0 D). These data demonstrate the mTORC1 dependence of the kinase activity we measured.

3.4.2 Time dependant saturation curves

In order to select the most appropriate duration for each assay in human biopsy samples we carried out a time dependant saturation curve for each assay from a pool of human muscle biopsies (Figure 3.2). We carried out the AMPK assays for 7.5, 15 and 30 min whilst PKB and p70S6K1 assays were carried out for 15, 30 and 60 min. These assays revealed linearity across the time course for each assay indicating that assays carried out for anywhere between 7.5 and 30 min for panAMPK and 15-60 min for panPKB and p70S6K1 would be within the linear range for time.

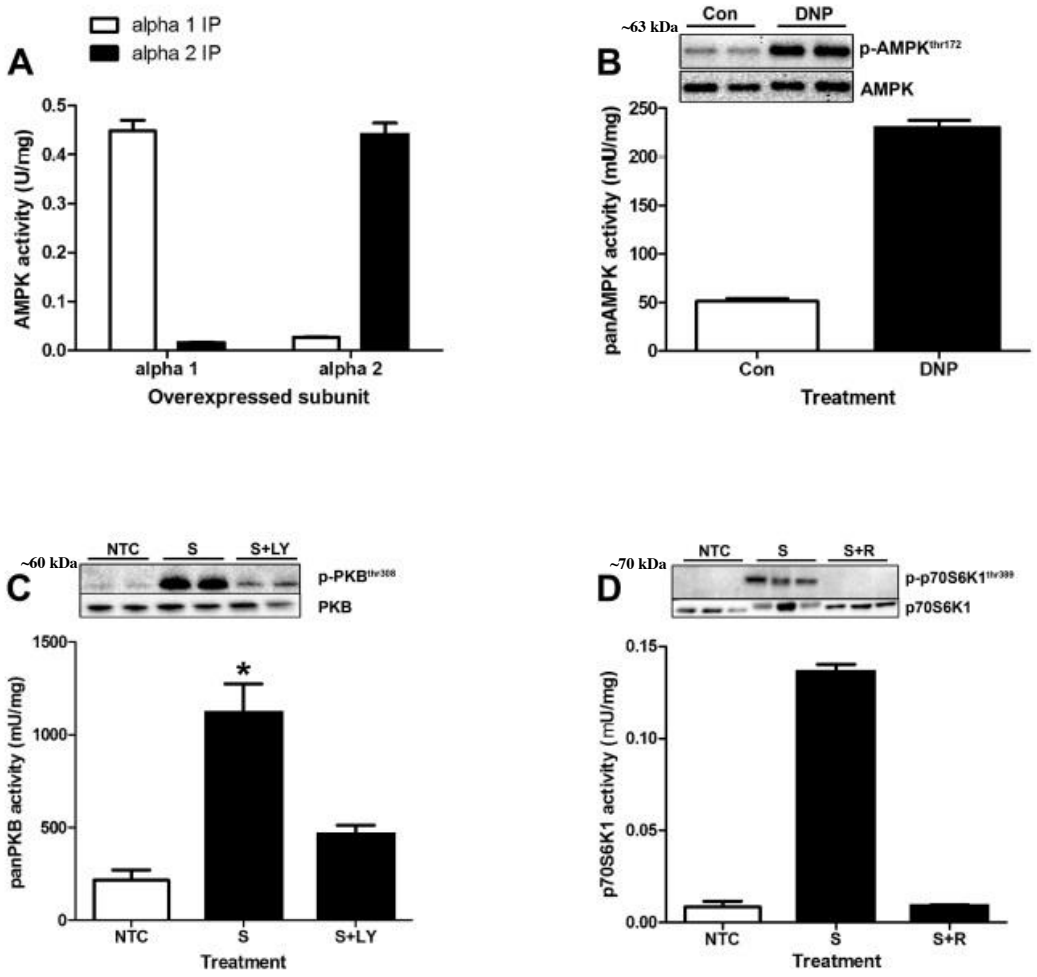

Figure 3.0 All data expressed as means ± SD Antibody and assay validation. **(A)** AMPK α1 and AMPK α2 activity assays derived from immune-complexes from cells overexpressing either AMPK α1 or AMPK α2. **(B)** panAMPK activation in response to energy stress in C_2C_{12} myotubes. C_2C_{12} myotubes were serum starved for 2 h prior to stimulation with DNP (100 μM) for 30 min (n=2 in duplicate). **(C)** Pan-PKB activation by serum stimulation and inhibition by LY294002 (10 μM). C_2C_{12} myotubes were serum starved for 3 h and pre-incubated with either vehicle (NTC – no treatment control) or LY294002 [10 μM (S+LY – stimulated + LY] for 1 h (n=3 in duplicate). Then they were stimulated for 30 min in 2 % FBS (S - stimulated). * indicates significantly different from NTC and S+LY. **(D)** p70S6K1 activation by serum + amino acid stimulation and inhibition by rapamycin (100 nM). C_2C_{12} myotubes were serum and amino acid starved for 3 h in PBS+5 mM glucose and pre-incubated with either vehicle (NTC) or rapamycin [100 nM (S+R – stimulated + rapamycin) for 1 h (n=2 in triplicate). Then they were stimulated for 30 min in 20% FBS+DMEM (S – stimulated). Insets on each graph are representative Western blots.

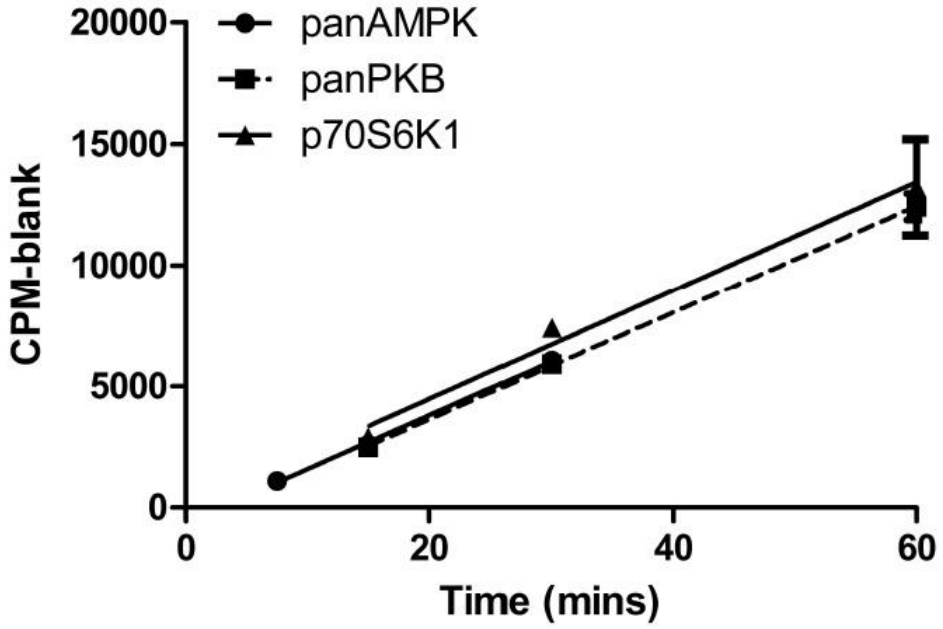

Figure 3.1 All data are expressed as means ± SD. Saturation time course of activity assays carried out from human skeletal muscle. r^2 values are as follows; AMPK = 0.969, panPKB = 0.982 and p70S6K1 = 0.856.

3.4.3 Validation of the serial IP

To economize on human muscle samples, panPKB and p70S6K1 activity assays were carried out via serial IP with p70S6K1 immunoprecipitated first. To confirm that this serial IP process did not impact PKB activity we performed a validation of this procedure in response to maximal insulin stimulation (Figure 3.2). Serially immunoprecipitating panPKB after p70S6K1 had no significant impact upon panPKB activity when compared to a standard IP (Figure 3).

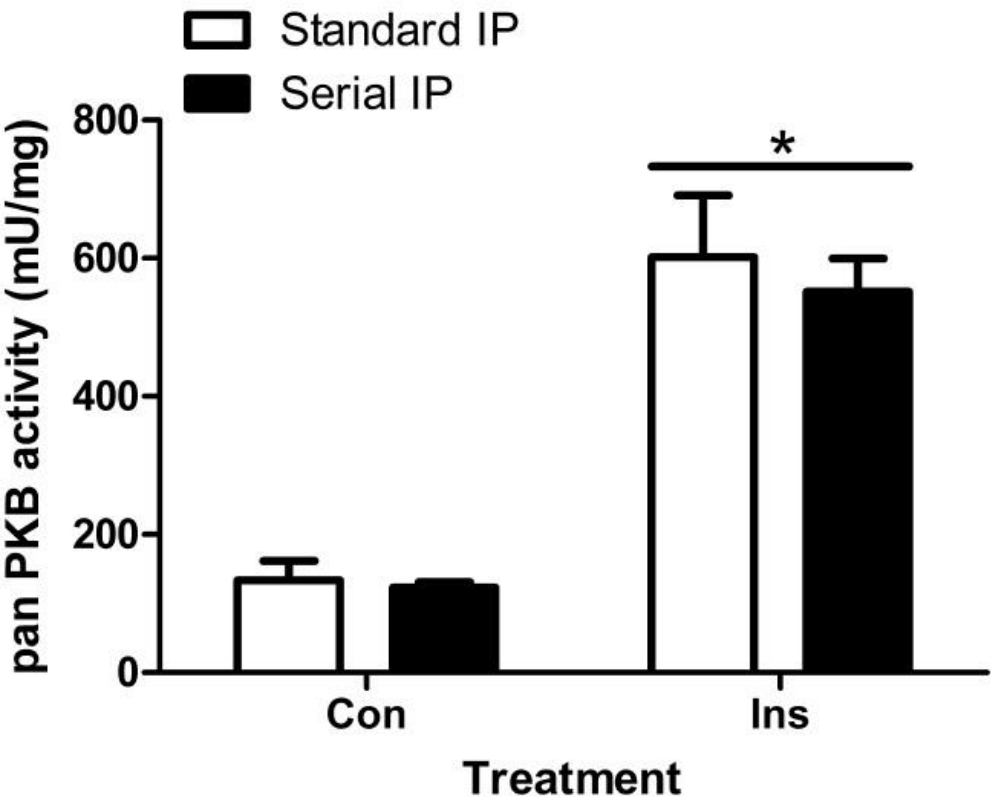

Figure 3.2 All data are expressed as means ± SEM. Serial IP validation. IPs were set up to immunoprecipitate panPKB alone or p70S6K1 immunoprecipitated prior to immunoprecipitating with panPKB. * indicates significantly different from both control (Con) conditions.

3.4.4 Application of the [γ-^{32}P] ATP kinase assay in a physiological context in human skeletal muscle

We next determined if we could measure the activity of panAMPK, panPKB and p70S6K1 from the same human skeletal muscle sample following RE and protein feeding in humans [25]. In our study we identified a significant increase in p70S6K1 activity from pre-RE at 1 h and 3 h post-RE ($P < 0.05$, Figure 3.3 C). However, there was no significant change in panPKB activity at any time point (Figure 3.3 B). Finally, panAMPK activity was significantly repressed ($P < 0.05$, Figure 3.3 A) at 3 h post-RE compared to pre-RE. To confirm that we are able to detect physiologically relevant changes in panPKB activity we assessed the activation of panPKB in response to a physiologically relevant (0.36 nM) insulin

stimulus in *ex vivo* mouse skeletal muscle (inset Figure 3.3 B). Indeed, we detected a significant increase in panPKB activity in response to 50 min of insulin stimulation thus confirming that this assay is capable of detecting changes in panPKB activity in a physiological context.

3.4.5 Western blotting

Following the assessment of kinase activity as markers of anabolic responses in humans we next measured the phosphorylation of proteins that are typically used as surrogate readouts of anabolic signalling activity. The responses of kinases, as determined by WB, are shown in Figure 3.4 (AMPK readouts), Figure 3.5 (PKB readouts) and Figure 3.6 (mTORC1 readouts). In response to RE and nutrition, there were no significant changes in phosphorylated $mTOR^{ser2448}$ (Figure 3.6 A), ACC^{ser79} (Figure 3.4 A), $Raptor^{ser792}$ (Figure 3.4 B), $p70S6K1^{thr389}$ (Figure 3.6 B), PKB^{thr308} (Figure 3.5 A) and $PRAS40^{thr246}$ (Figure 3.5 B). However, phosphorylated $4EBP1^{thr37/46}$ was significantly elevated at 3 h post-RE compared to pre-RE ($P < 0.05$; Figure 3.6 C). Representative WB images are inset above each graph.

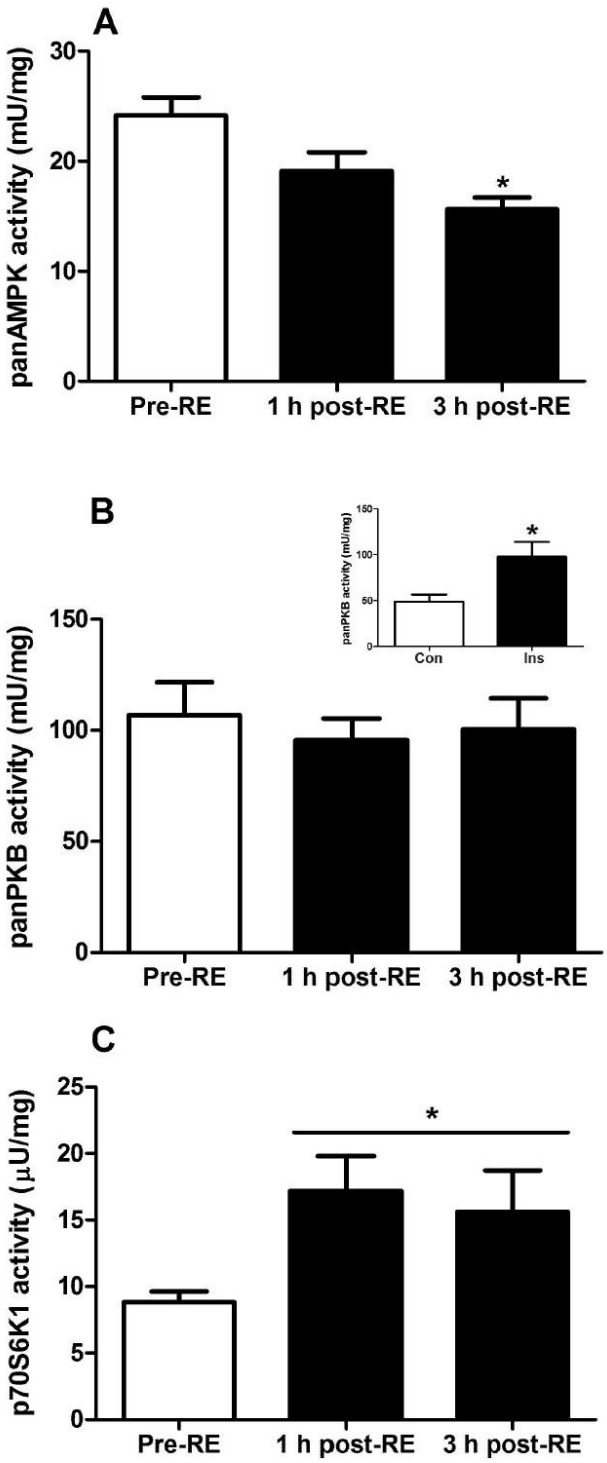

Figure 3.3 All data are expressed as means ± SEM. Application of 3 kinase assays in human skeletal muscle in response to a physiological anabolic stimulus of resistance exercise combined with feeding 20g of protein (n=6). **(A)** panAMPK activity. panAMPK activity was determined from 50 µg of lysate in a 20min reaction against the synthetic substrate AMARA. **(B)** panPKB activity. panPKB activity serially immunoprecipitated after p70S6K1 IP. Inset demonstrates the panPKB activity response to a physiological insulin stimulation of 0.36 nM for 50 min in *ex vivo* mouse skeletal muscle (n=6). panPKB activity was determined from 200 µg of lysate in a 30 min reaction against the synthetic peptide substrate Crosstide. **(C)** p70S6K1 activity. p70S6K1 activity was determined from 500 µg of lysate in a 45 min reaction against the synthetic peptide substrate S6K1tide. Pre-RE indicates biopsy taken prior to resistance exercise and feeding, 1 h post-RE indicates the biopsy taken 1 h following combined resistance exercise and feeding whilst 3 h post-RE indicates biopsy taken 3 h following combined resistance exercise and feeding. * indicates significantly different from Con or Pre-RE ($P < 0.05$).

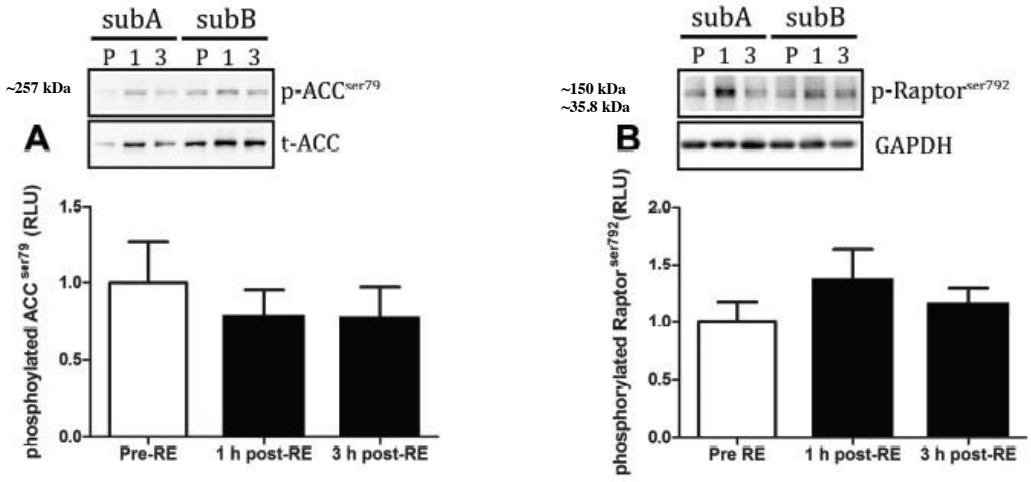

Figure 3.4 All data are expressed as means ± SEM. Markers of AMPK activity in response to a physiological anabolic stimulus of resistance exercise combined with feeding 20 g of protein. Protein phosphorylation of **(A)** ACC^{ser79}, and **(B)** Raptor^{ser792} obtained at pre, 1 h post and 3 h post-RE. .

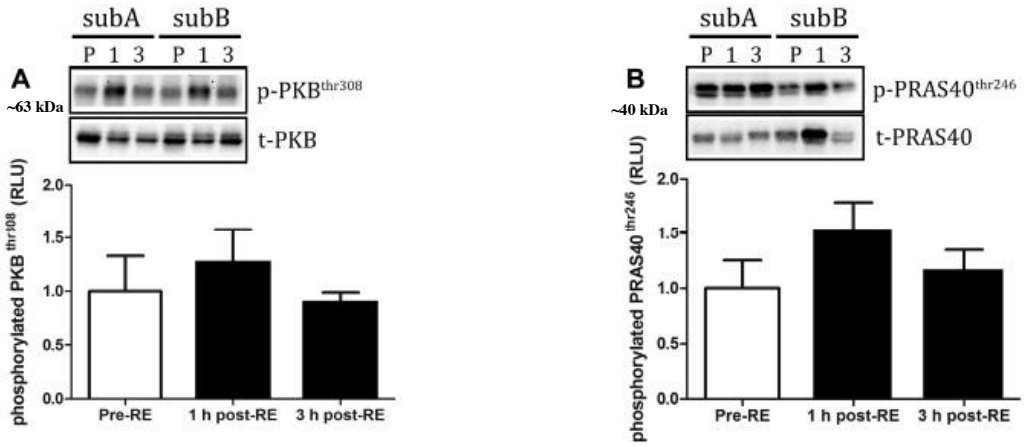

Figure 3.5 All data are expressed as means ± SEM. Markers of panPKB activity in response to a physiological anabolic stimulus of resistance exercise combined with feeding 20 g of protein. Protein phosphorylation of **(A)** PKB^{thr308} and **(B)** PRAS40^{thr246}.

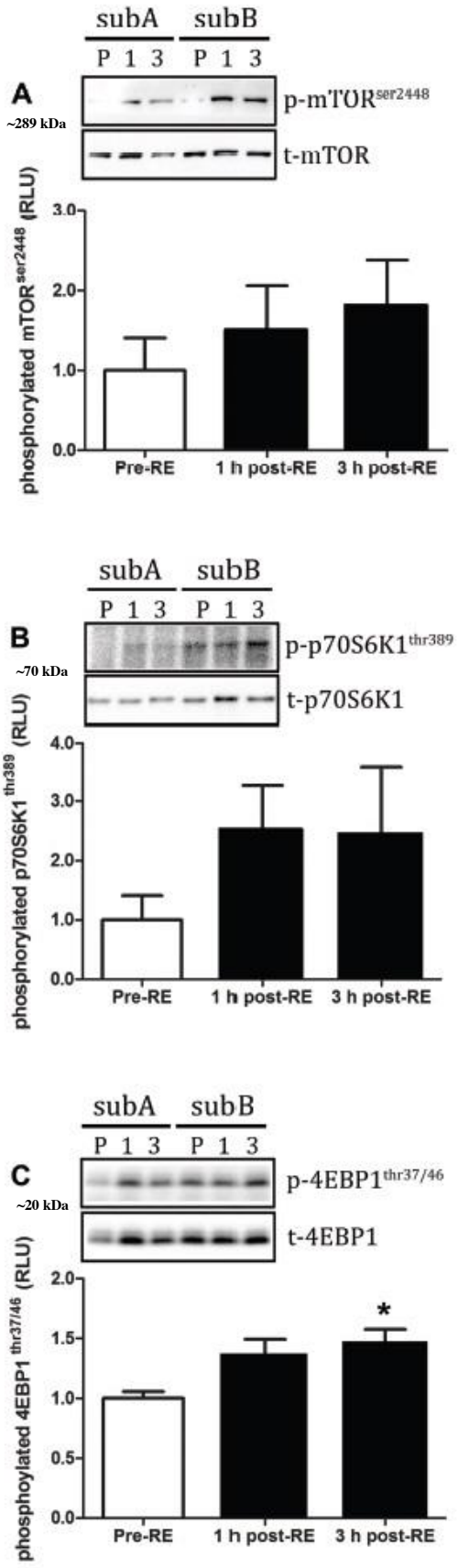

Figure 3.6. All data are expressed as means ± SEM. Markers of mTORC1 activation in response to a physiological anabolic stimulus of resistance exercise (RE) combined with feeding 20 g of protein. (A) $mTOR^{ser2448}$, (B) $p70S6K1^{thr389}$, and (C) $4EBP1^{thr37/46}$. * indicates significantly different from Pre-RE ($p<0.05$).

3.5 Discussion

There were three main aims of the present investigation. Firstly, to develop and validate a quantitative p70S6K1 KA for use in human skeletal muscle biopsy samples. Secondly, to validate a serial IP protocol for the assessment of the activity of multiple kinases from a single muscle sample. Lastly, to apply these novel methodologies alongside a traditional WB method to assess changes in human skeletal muscle kinase activity and phosphorylation in response to a physiologically relevant stimulus [25]. For the first time we demonstrate that combined RE and protein feeding significantly increases p70S6K1 activity by ~2 fold, as determined by the KA with a similar ~2 fold but non-significant change in p70S6K1^{thr389} phosphorylation. In addition, we demonstrate the capacity to achieve a dual measure of panPKB and p70S6K1 activity from the same muscle sample via a serial IP protocol. This study therefore highlights the potential application of the novel p70S6K1 KA described in this investigation to study the molecular signalling responses of skeletal muscle to RE and nutrition in humans.

Although we observed a significant increase in p70S6K1 activity to RE and protein feeding we detected no significant changes in the phosphorylation of p70S6K1^{thr389}. This finding was unexpected given previous reports of a significant ~2 fold [139] and ~12 fold [26] increase in phosphorylated p70S6K1^{thr389} to an acute bout of RE and protein feeding. However, the lack of detectable change in phosphorylated p70S6K1^{thr389} in our investigation appears to be related to low statistical power. Indeed, a post-hoc sample size calculation from the present study determined that a participant sample of 12 would have been necessary to detect a statistically significant difference in phosphorylated p70S6K1^{thr389} between pre-RE and 1 h post-RE and protein ingestion. However, by utilizing the KA we were able to detect a modest increase in p70S6K1 activity from pre-RE at 1 h and 3 h post-RE and feeding. Thus, these

data highlight not only the sensitivity but also the utility of the p70S6K1 KA as described in this investigation to assess p70S6K1 activity in response to anabolic stimulation.

Due to issues associated with ethical practice and participant compliance in human research, muscle tissue availability is often a limiting factor. In this investigation, we provide a validated serial IP protocol for the dual assessment of p70S6K1 and panPKB activity from a single muscle biopsy sample (30 mg). We show that this serial IP protocol has no impact on panPKB activity hence economising on muscle tissue requirements. When applying this protocol to study panPKB responses of human skeletal muscle to RE and feeding we show no change in panPKB activity at any time point, a finding that corroborates previous reports in human, rodent and cell culture models [70,146,163]. However, it is important to note that the panPKB KA described in this methodological investigation fails to provide information regarding PKB isoform-specific effects that could be useful in understanding the cell growth and metabolism [164]. The development of such a methodology is therefore a topic for future work.

In order to provide further evidence to highlight the quantitative nature of our KAs we conducted time-saturation curves for p70S6K1, panPKB and panAMPK KAs. We show that KAs carried out for anywhere between 15-60 min for panPKB and p70S6K1 and 7.5 and 30 min for panAMPK exhibit linearity for time in relation to activity. With regards to panAMPK activity we demonstrate a reduction in panAMPK activity 3 h post-RE and feeding, similar to the findings of others who demonstrate that RE [165] or feeding [104] also repress $AMPK^{thr172}$ phosphorylation. Interestingly, the significant reduction of AMPK activity in our study was not mirrored by a reduction in ACC^{ser79} phosphorylation, $P = 0.70$. We chose to assess the phosphorylation of ACC^{ser79} as a readout of AMPK activity as phosphorylated $AMPK^{thr172}$ possesses a low dynamic range that renders phosphorylated $AMPK^{thr172}$ on this

residue a poor surrogate of true AMPK activity in some instances [147]. Therefore, the decrease in AMPK activity paralleled with a non-significant change in ACC^{ser79} phosphorylation further emphasises the potential application of the KA described in this investigation, and others [166], to assess RE and nutrition-induced changes in cellular signalling.

As statistical power with regards to the p70S6K1^{thr389} is clearly a mitigating feature of this study, we decided to assess other readouts of mTORC1 activity using the traditional WB methodology. In response to RE and protein ingestion we detected no significant change in the phosphorylation status of mTOR^{ser2448} at any time point during the recovery period. However, there was a significant increase in the phosphorylation status of the mTOR substrate 4EBP1$^{thr37/46}$ at 3 h post-RE and protein feeding. The lack of change of mTOR^{ser2448} phosphorylation despite an increase in p70S6K1 activity and 4EBP1$^{thr37/46}$ was surprising as both p70S6K1 and 4EBP1 are known targets of mTORC1 [63]. Although, data do exist to suggest that the assessment of mTOR^{ser2448} phosphorylation may not be truly representative of mTORC1 activity. Indeed, one study shows no change in mTOR^{ser2448} phosphorylation in response to a 48 g whey bolus at both 1 h and 3 h post-feeding despite a profound increase in MPS as well as elevations in the phosphorylation of p70S6K1^{thr389} and 4EBP1$^{thr37/46}$ [27]. Moreover, it is known that mutation of the ser2448 residue on mTOR fails to significantly impact p70S6K1 activity in cell based systems [81]. Taken together with the findings of our investigation, these data suggest that mTOR^{ser2448} phosphorylation may not offer the most accurate readout of mTORC1 activity at the time points we measured. Hence, studies that employ the traditional WB technique as a means to assess mTORC1 activity may be better served by assessing changes in the phosphorylation of the mTOR substrates 4EBP1 and p70S6K1 in addition to mTOR^{ser2448} itself.

In summary, this study provides a novel, fully-quantitative methodology to assess p70S6K1 activity in human skeletal muscle. In addition, we provide a validated serial IP protocol that enables the dual assessment of PKB and p70S6K1 activity from a single skeletal muscle biopsy sample. Although, it is important to acknowledge that the KAs described in this investigation provide no information pertaining to the PTM of a protein such as phosphorylation. Indeed, phosphorylation is a critical regulatory step in protein function [167]. Nevertheless, this methodological study describes a bone fide methodology that can now be used to identify changes in p70S6K1 signalling activity in response to RE and feeding in humans. Therefore, this methodology may now be used in the final chapter of this thesis to identify if fish oil supplementation enhances anabolic signalling activity in response to a bout of RE and protein feeding.

Author contributions

Author contributions: C.M., and D.L.H. conception and design of research; C.M., C.T., I.T.C., and D.L.H. performed experiments; C.M., A.T.W., and D.L.H. analyzed data; C.M., A.T.W., A.P., J.P.M., and D.L.H. interpreted results of experiments; C.M. and D.L.H. prepared figures; C.M.wrote and D.L.H. drafted the chapter.

CHAPTER 4 Influence of 8 weeks of n-3 PUFA supplementation on human myotropic kinase activity in response to protein feeding and resistance exercise.

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4.0 Abstract

Previous studies have shown that ingestion of n-3 PUFA potentiates the phosphorylation of mTORC1 and associated kinases in response to nutrition. However, no study has identified whether n-3 PUFA supplementation potentiates anabolic kinase activity when RE is performed prior to nutrient provision. The aim of this investigation was to identify if 8 weeks of n-3 PUFA-enriched fish oil supplementation potentiates p70S6K1, panPKB, AMPK α1 and AMPK α2 activity to oral protein ingestion and the combined stimulus of unilateral RE and oral protein ingestion. Twenty healthy males consumed $5g \cdot d^{-1}$ of either fish oil (FO) or coconut oil (CO) capsules for 8 weeks. Venous blood and muscle biopsy samples were collected in the fasted, rested state before and after 8 weeks of supplementation for assessment of changes in lipid composition and kinase activity over time. Following 8 weeks of supplementation muscle samples also were obtained at rest (Rest), post RE in both the exercise leg (Post-RE) and the rested leg (Pre-FED) and also at 3 h post RE and protein feeding from both the exercise leg (3 h post-REF) and rested leg (3 h post-FED). There was a 2-fold increase in muscle (5.53 ± 0.3 to 11.16 ± 0.45 % of total fatty acids) and blood (6.74 ± 0.50 to 12.64 ± 0.73 % of total fatty acids) n-3 PUFA composition after supplementation in the FO group but no change in the CO group. Basal, fasted panPKB activity was significantly higher before compared to after supplementation in the FO group only (12.5 ± 2.6 to 8.2 ± 1.6 mU/mg). Following supplementation there was an increase in p70S6K1 activity at 3 h post-REF from Rest in the CO group (5.6 ± 1.4 to 12.2 ± 2.1 μU/mg) but no change in the FO group. In the CO group, AMPKα2 was significantly increased at Post-RE from Rest (3.7 ± 0.7 to 9.9 ± 2.0 mU/mg). These data show that 8 weeks of n-3 PUFA enriched fish oil supplementation suppresses the activity of p70S6K1 in response to RE and protein feeding.

4.1 Introduction

Chapter 2 of this thesis added to existing literature by demonstrating that 4 weeks of n-3 PUFA-enriched fish oil supplementation significantly increases the n-3 PUFA composition of skeletal muscle within 2 weeks. This time course increase in n-3 PUFA composition also was associated with increases in the content of anabolic signalling molecules, notably mTOR and FAK that could be interpreted to suggest n-3 PUFA ingestion may serve to 'prime' skeletal muscle to anabolic stimulation. Support for this contention is provided by two studies that show 8 weeks of n-3 PUFA-enriched fish oil supplementation enhances the phosphorylation of p70S6K1^{thr389}, a known readout of mTOR activity, in response to nutrition in the form of a hyperaminoacidemic-hyperinsulinemic clamp [21,22]. However, to date, no study has identified if 8 wk of n-3 PUFA supplementation potentiates p70S6K1 activity when RE is performed prior to nutrient provision. Given the potent synergistic impact of adding RE to protein feeding [91], such data would have relevance toward both the clinical and athletic settings.

However, it is important to recognise that in the clinical and athletic setting many people consume protein in a bolus form and previous reports of enhanced anabolic signalling in response to nutrition following n-3 PUFA ingestion are indicative of an intravenous amino acid and insulin infusion [21,22]. This method of nutrient administration may not reflect the temporal increase in blood amino acid concentrations [107], critical to stimulating muscle anabolism [139], as that of a practical protein bolus feeding strategy. Therefore, the primary aim of the present investigation was to use the KA methodology as outlined in **Chapter 3** to identify if 8 weeks of n-3 PUFA supplementation potentiates the activity of kinases known to regulate muscle anabolism (p70S6K1, panPKB, AMPKα1 and AMPKα2) to the combined stimulus of RE and oral protein ingestion. By employing a unilateral model of RE we also

aim to ascertain if 8 weeks of n-3 PUFA supplementation enhances the activity of these kinases in response to oral protein ingestion alone. This aim will be achieved by examining changes in kinase activity in the contralateral-rested limb.

4.2 Methods

4.2.1 Participants

Twenty moderately trained males were recruited from the University of Stirling and surrounding area to participate in the present investigation. Prior to the commencement of the experiment each participant provided written informed consent after all procedures and risks of the study were fully explained in lay terms. Participant characteristics can be seen in Table 4.0. Following health screening, participants were excluded if they were engaged in any form of dietary supplementation or were taking any prescribed medication. The East of Scotland Research Ethics Service (EoSRES, Rec No: FB/12/ES/0005) approved the study procedures.

Table 4.0 Characteristics of participants in each group.

Parameter	Fish oil (n=10)	Coconut oil (n=10)
Age (yrs)	24 ± 0.1*	21 ± 0.2
Body mass (kg)	87 ± 2.6*	80 ± 8.2
Lean body mass (%)	77 ± 1.3	76 ± 1.3
Body fat (%)	20 ± 1.5	20 ± 1.4
LP 1RM (kg)	143 ± 8.0*	134 ± 7.1
LP·kg^{-1}·BM^{-1}	2.13 ± 0.1	2.25 ± 0.1
LE 1RM (kg)	68 ± 2.5*	60 ± 2.5
LE·kg^{-1}·BM^{-1}	1.01 ± 0.1	1.01 ± 0.0

LP: leg press, LE: leg extension, 1RM: one repetition maximum, yrs: years, kg: kilogram: BM: body mass. Values expressed as mean ± SEM.* denotes significantly higher than coconut oil group (P< 0.05).

4.2.2 Study design

In a randomised, between-groups, repeated-measures design participants were assigned to either a fish oil (FO; n=10) or coconut oil (CO; n=10) condition. Coconut oil was chosen as a control as coconut oil does not contain any n-3 or n-6 PUFAs. As per Chapter 2, during each visit to the laboratory, participants were verbally requested to confirm the pattern of oily fish consumption in an attempt to ensure that changes in free-living oily fish consumption did not influence muscle and blood lipid profiles during the study. Following baseline testing for 1 RM on leg press and leg extension [156] participants reported to the Resting Laboratory of the Health and Exercise Sciences Research Group, Univ. of Stirling in the fasted state on two separate occasions. During the initial visit a resting muscle biopsy and venous blood sample were obtained for the assessment of muscle membrane and blood lipid profiles and also for baseline activity of muscle-specific anabolic signalling kinases (p70S6K1, panPKB, AMPKα1 and AMPKα2). Following baseline measurements participants consumed 5 $g \cdot d^{-1}$ of n-3 PUFA-rich fish oil capsules (providing 3500 mg EPA [20:5n-3], 900 mg DHA [22:6n-3], 100 mg DPA [22:5n-3] vitamin E 0.1 mg; Ideal Omega-3, Glasgow Health Solutions Ltd, UK) for 8 weeks. Participants were provided with a known quantity of capsules and a capsule count was conducted at the end of the 8 week period of supplementation in order to assess compliance to the supplementation protocol. The rationale to supplement for 8 weeks rather than the 4 week protocol of Chapter 2, was to enable a comparison with other published studies that also investigated the impact of 8 weeks of n-3 PUFA-enriched fish oil supplementation on kinase phosphorylation [21,22].

Participants were requested to complete a 3 d food diary questionnaire for the 3 d prior to baseline testing and to repeat this pattern of consumption for the 3 d leading up to the experimental trial. Participants also were verbally requested to confirm their pattern of oily

fish consumption over the 8 week supplementation period in an attempt to minimise the impact of changes in free-living oily fish consumption upon muscle and blood lipid profiles during the study. Following the 8 week period of supplementation, participants returned to the laboratory in the fasted state. During the second visit a venous blood sample and resting skeletal muscle biopsy (Rest) was obtained from the non-exercising leg. Following the resting muscle biopsy a bout of unilateral RE was performed and further muscle biopsies were conducted on the non-exercised (Pre-FED) and exercised (Post-RE) legs. A 30 g oral whey protein bolus was then consumed in 500 mL of water. Three hours later muscle biopsies were again obtained from the exercised leg (3 h post-REF) and non-exercised leg (3 h post-FED). The Rest muscle biopsy sample also was used to assess the impact of 8 wk of n-3 PUFA supplementation on muscle membrane lipid composition.

4.2.3 Resistance exercise trial

Following 8 weeks of supplementation participants reported to the laboratory at ~0700 in the fasted state. Participants rested in a semi-supine position on a bed for 2 h during which a venous blood sample was obtained from the anticubital vein of the forearm. Meanwhile, the lateral portion of the *vastus lateralis* from the non-exercising leg was cleaned before an incision into the skin and fascia was made under local anaesthetic (2 % Lidocaine). A 5 mm Bergstrom biopsy needle was inserted to extract ~60-100 mg of skeletal muscle tissue and the leg was subsequently bandaged. Following this 2 h resting period participants performed a bout of unilateral leg press and leg extension. This unilateral approach enabled us to identify if 8 weeks of fish oil supplementation enhanced kinase activity in response to protein feeding alone as well as the potentiating impact of RE. The bout of RE consisted of 4 sets of 10 repetitions for each exercise at 70% 1 RM. Between-set and between-exercise recovery was 2 min and 3 min respectively. Immediately post-RE muscle biopsies were obtained from both

the exercising and non-exercising legs after which participants consumed 30 g of whey protein in 500 mL of water. The participants' legs then were bandaged and the participants rested in the bed until further biopsies were obtained at 3 h post-RE on both the non-exercised and exercised legs. Muscle biopsy samples were rinsed in ice-cold saline, blotted to minimise blood saturation of the muscle sample and freed from any visible fat and/or connective tissue. Muscle samples then were frozen in liquid nitrogen and stored at -80°C until further analysis.

4.2.4 Skeletal muscle phospholipid extraction and analysis

The phospholipid fraction was prepared from 0·5 mg of total lipid (see section 2.2.4) applied to a 20 × 20 cm silica gel 60 TLC plate (VWR, Lutterworth, Leicestershire, UK) and developed in isohexane–diethyl ether–acetic acid (80:20:1, by vol.) before drying for a few minutes at room temperature. The plate was sprayed lightly with 2,7-dichlorofluorescein (0·1 %, w/v) in 97 % methanol (v/v) the phospholipid bands then were scraped from the plate and placed in a 15 mL test-tube. FAME were prepared by acid-catalysed transesterification in 2 mL of 1 % H_2SO_4 in methanol at 50°C overnight. The samples were neutralized with 2·5 mL of 2 % $KHCO_3$ and extracted with 5 mL isohexane–diethyl ether (1:1, v/v) þ BHT. The samples then were re-extracted with 5 mL isohexane–diethyl ether (1:1) and the combined extracts were dried and dissolved in 0·3 mL of isohexane prior to FAME analysis as previously described in section 2.2.6.

4.2.5 Statistical analyses

Data were analysed using GraphPad Prism Software version 6.0 (GraphPad, San Diego, CA, USA). An initial unpaired t-test was performed to detect differences between groups for anthropometric variables and composition of diet before the intervention. Changes in lipid composition and anabolic signalling activity over time, i.e., before-after supplementation as

well as during the experimental trial for protein kinase activity, were analysed using a between-participant repeated measures (group × time) ANOVA. When there was a significant main effect of time or interaction between-groups, data were subsequently analysed using a Tukey's post-hoc test to detect the time points at which differences between groups existed. Statistical significance was set at $P<0.05$. All lipid data are expressed as mean relative percentage of total fatty acids ± SEM unless otherwise stated. Protein activity data are expressed as μU/mg for p70S6K1 and mU/mg for panPKB, AMPKα1 and AMPKα2. All other data are presented as mean ± SEM unless otherwise stated.

4.3 Results

4.3.1 Dietary intake

Analysis of dietary intake demonstrates no difference in macronutrient composition and daily energy intake between the FO and CO groups (Table 4.1).

Table 4.1. Participants' daily energy intake and macronutrient composition.

	Fish oil (n=10)	**Coconut oil (n=10)**
Daily energy intake (kcal)	2427 ± 242*	2002 ± 96
Carbohydrate (%)	42 ± 4	44 ± 2
Fat (%)	32 ± 3	33 ± 2
Protein (%)	26 ± 3	23 ± 4

* denotes significantly higher than Coconut oil group

4.3.2 Phospholipid profile changes in muscle

All phospholipid profile changes in muscle are shown in Table 4.2 Percentage n-3 PUFA of total fatty acids was significantly higher before supplementation in the FO group compared to

the CO group (P<0.05). However, after supplementation there was a ~2-fold increase in the % n-3 PUFA of total fatty acids (P<0.05), whereas in the CO group % n-3 PUFA of total fatty acids remained unchanged (P>0.05). In contrast, % n-6 PUFA of total fatty acids was significantly lower before supplementation in the FO group compared to the CO group (P<0.05). Although, in the FO group % n-6 PUFA of total fatty acids was significantly lower after supplementation (P<0.05) but in the CO group % n-6 PUFA of total fatty acids remained unchanged (P>0.05). Percentage monounsaturated fatty acids of total fatty acids was significantly higher before supplementation in the CO group compared to the FO group (P<0.05). However, % monounsaturated fatty acids were reduced after supplementation in the CO group (P<0.05) only. There was no significant difference in % saturated fatty acids of total fatty acids between groups before the intervention although, % saturated fatty acids of total fatty acids was significantly decreased after supplementation for both groups (P<0.05).

4.3.3 Lipid profile changes in whole blood

All lipid changes in whole blood are shown in Table 4.3. There was no significant baseline difference between groups in any lipid species or group of lipid species. Percentage n-3 PUFA of total fatty acids was significantly increased after supplementation compared to before supplementation in the FO group (P<0.05) with no impact of supplementation on % n-3 PUFA of total fatty acids in the CO group. Similarly, % n-6 PUFA of total fatty acids was significantly reduced after supplementation compared to before supplementation in the FO group (P<0.05) but there was no impact of supplementation on % n-6 PUFA of total fatty acids in the CO group. There was no impact of supplementation on % monounsaturated fatty acids of total fatty acids or % saturated fatty acids of total fatty acids in either group.

Table 4.2 Phospholipid profile changes in muscle (% total fatty acids, mean ± SEM). Means that do not share a letter are significantly different.

	Fish oil		**Coconut oil**	
	Before	After	Before	After
		Saturated fatty acids		
14:0	0.37 ± 0.01	0.33 ± 0.02	0.32 ± 0.02	0.30 ± 0.02
15:0	0.18 ± 0.01	0.14 ± 0.00	0.15 ± 0.01	0.13 ± 0.01
16:0	18.96 ± 0.33	16.25 ± 0.10	18.87 ± 0.34	16.87 ± 0.38
18:0	14.16 ± 0.25	12.72 ± 0.13	14.10 ± 0.12	12.92 ± 0.17
20:0	0.08 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.09 ± 0.02
22:0	0.16 ± 0.01	0.16 ± 0.01	0.14 ± 0.01	0.15 ± 0.20
24:0	0.18 ± 0.02	0.17 ± 0.02	0.17 ± 0.02	0.18 ± 0.03
Total	**34.09 ± 0.45^{a}**	**29.83 ± 0.14^{b}**	**33.83 ± 0.36^{a}**	**30.61 ± 0.40^{b}**
		Monounsaturated fatty acids		
16:1n-9	0.17 ± 0.01	0.19 ± 0.01	0.15 ± 0.00	0.16 ± 0.01
16:1n-7	0.37 ± 0.02	0.33 ± 0.02	0.42 ± 0.01	0.39 ± 0.02
18:1n-9	6.05 ± 0.16	4.74 ± 0.20	6.25 ± 0.21	5.92 ± 0.29
18:1n-7	2.01 ± 0.06	1.86 ± 0.06	1.94 ± 0.06	1.89 ± 0.07
20:1n-9	0.09 ± 0.01	0.07 ± 0.01	0.09 ± 0.01	0.09 ± 0.01
24:1n-9	0.20 ± 0.01	0.20 ± 0.01	0.22 ± 0.02	0.24 ± 0.03
Total	**8.89 ± 0.14**	**7.35 ± 0.24**	**9.07 ± 0.22^{a}**	**8.69 ± 0.36**
		n-6 polyunsaturated fatty acids		
18:2n-6	26.87 ± 0.59	24.17 ± 0.65	29.19 ± 0.52	28.72 ± 0.60
18:3n-6	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.00	0.09 ± 0.01
20:2n-6	0.12 ± 0.01	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.01
20:3n-6	1.29 ± 0.04	1.14 ± 0.03	1.30 ± 0.09	1.48 ± 0.09
20:4n-6	13.55 ± 0.56	13.11 ± 0.38	12.67 ± 0.34	13.79 ± 0.47
22:4n-6	0.44 ± 0.03	0.30 ± 0.02	0.65 ± 0.03	0.75 ± 0.04
22:5n-6	0.35 ± 0.02	0.20 ± 0.01	0.39 ± 0.01	0.34 ± 0.02
Total	**42.69 ± 0.26^{a}**	**39.08 ± 0.43^{b}**	**44.39 ± 0.37^{c}**	**45.30 ± 0.45^{c}**
		n-3 polyunsaturated fatty acids		
18:3n-3	0.25 ± 0.02	0.21 ± 0.01	0.26 ± 0.01	0.24 ± 0.01
20:5n-3	1.16 ± 0.12	4.46 ± 0.22	0.65 ± 0.05	0.69 ± 0.06
22:5n-3	1.48 ± 0.06	2.27 ± 0.08	1.29 ± 0.06	1.47 ± 0.06
22:6n-3	2.64 ± 0.18	4.22 ± 0.23	1.55 ± 0.16	1.79 ± 0.21
Total	**5.53 ± 0.30^{a}**	**11.16 ± 0.45^{b}**	**3.74 ± 0.23^{c}**	**4.16 ± 0.31^{c}**

Table 4.3 Lipid profile changes in blood (% total fatty acids, mean ± SEM). Means that do not share a letter are significantly different.

	Fish oil		**Coconut oil**	
	Before	After	Before	After
		Saturated fatty acids		
14:0	0.75 ± 0.08	0.65 ± 0.05	0.55 ± 0.03	0.63 ± 0.04
15:0	0.21 ± 0.01	0.12 ± 0.01	0.18 ± 0.01	0.17 ± 0.01
16:0	21.56 ± 0.44	21.26 ± 0.26	21.39 ± 0.34	21.58 ± 0.21
18:0	11.92 ± 0.14	11.74 ± 0.14	11.76 ± 0.25	11.67 ± 0.16
20:0	0.25 ± 0.01	0.25 ± 0.01	0.25 ± 0.01	0.24 ± 0.01
22:0	0.71 ± 0.03	0.74 ± 0.02	0.70 ± 0.04	0.77 ± 0.04
24:0	1.19 ± 0.03	1.17 ± 0.04	1.18 ± 0.06	1.07 ± 0.03
Total	**36.60 ± 0.56**	**36.33 ± 0.25**	**36.00 ± 0.30**	**36.13 ± 0.23**
		Monounsaturated fatty acids		
16:1n-9	0.30 ± 0.02	0.26 ± 0.02	0.27 ± 0.02	0.28 ± 0.01
16:1n-7	0.94 ± 0.10	0.91 ± 0.10	1.14 ± 0.14	1.10 ± 0.10
18:1n-9	16.05 ± 0.47	15.21 ± 0.39	16.87 ± 0.76	16.82 ± 0.37
18:1n-7	1.45 ± 0.02	1.34 ± 0.07	1.62 ± 0.06	1.45 ± 0.03
20:1n-9	0.26 ± 0.02	0.22 ± 0.02	0.27 ± 0.01	0.24 ± 0.01
24:1n-9	1.47 ± 0.05	1.33 ± 0.07	1.59 ± 0.13	1.36 ± 0.05
Total	**20.46 ± 0.52**	**19.26 ± 0.48**	**21.76 ± 0.90**	**21.25 ± 0.47**
		n-6 polyunsaturated fatty acids		
18:2n-6	18.60 ± 0.61	16.45 ± 0.50	18.09 ± 0.49	18.97 ± 0.42
18:3n-6	0.24 ± 0.03	0.16 ± 0.03	0.20 ± 0.02	0.25 ± 0.03
20:2n-6	0.27 ± 0.02	0.21 ± 0.02	0.25 ± 0.01	0.25 ± 0.01
20:3n-6	1.63 ± 0.06	1.20 ± 0.06	1.66 ± 0.11	1.75 ± 0.09
20:4n-6	10.24 ± 0.57	9.04 ± 0.29	10.83 ± 0.45	10.44 ± 0.22
22:4n-6	1.19 ± 0.08	0.91 ± 0.04	1.59 ± 0.10	1.54 ± 0.07
22:5n-6	0.26 ± 0.02	0.17 ± 0.01	0.33 ± 0.01	0.32 ± 0.02
Total	**32.37 ± 0.71**	**28.15 ± 0.46**[a]	**32.95 ± 0.73**	**33.51 ± 0.39**
		n-3 polyunsaturated fatty acids		
18:3n-3	0.55 ± 0.04	0.46 ± 0.03	0.46 ± 0.02	0.52 ± 0.03
20:5n-3	1.09 ± 0.13	5.08 ± 0.49	0.69 ± 0.08	0.72 ± 0.07
22:5n-3	1.52 ± 0.07	2.48 ± 0.09	1.48 ± 0.06	1.48 ± 0.04
22:6n-3	3.50 ± 0.32	4.52 ± 0.21	2.68 ± 0.18	2.61 ± 0.17
Total	**6.74 ± 0.50**	**12.64 ± 0.73**[a]	**5.37 ± 0.26**	**5.42 ± 0.25**

4.3.4 Kinase activity in response to 8 weeks of n-3 PUFA supplementation

There were no differences between groups before supplementation in the activity of panPKB, AMPKα1, AMPKα2 or p70S6K1. panPKB activity was significantly supressed at rest compared to before supplementation in the FO group only, indicating that 8 weeks of fish oil supplementation supressed basal panPKB activity (Figure 4.0). However, there was no impact of supplementation in either group on the basal activity of AMPKα1, AMPKα2 or p70S6K1.

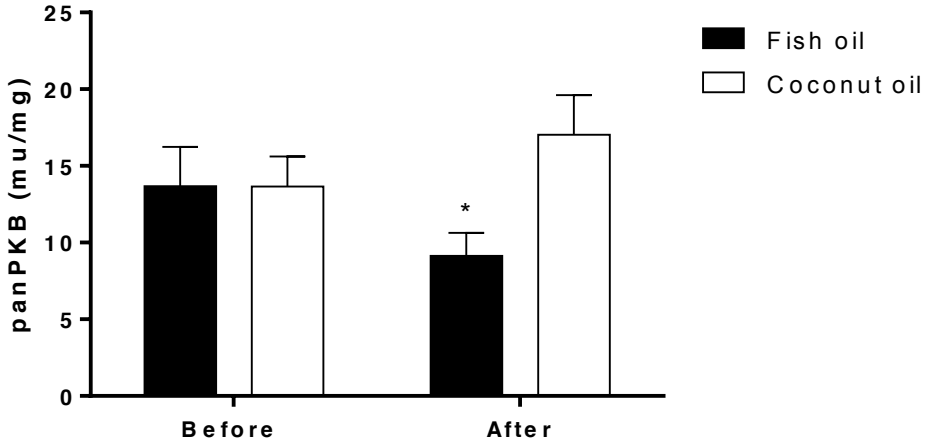

Figure 4.0 panPKB activity in response to 8 wk of either fish oil or coconut oil supplementation.* denotes significantly lower than before supplementation (P<0.05).

4.3.5 Kinase activity in response to protein feeding after 8 wk of n-3 PUFA supplementation

There was no impact of protein feeding on the activity of p70S6K1, AMPKα1, AMPKα2 or panPKB at 3 h post-FED in either group (Figure 4.2 A-D).

4.3.6 Protein kinase activity in response to RE and protein feeding after 8 wk of n-3 PUFA supplementation

There were no significant differences between groups in the activity of AMPKα1, AMPKα2 or p70S6K1 at rest (Figure 4.1 E, G and H). However, panPKB activity was significantly elevated in the CO group at rest compared to the FO group (Figure 4.1 F). In response to RE and protein feeding p70S6K1 activity was significantly elevated at 3 h post-REF from Pre-RE in the CO group. However, there was no impact of RE and protein feeding on p70S6K1 activity at Post-RE or 3 h post-REF in the FO group (Figure 4.1 E). There also was no impact of supplementation on the AMPKα1 response to RE at Post-RE or RE and protein feeding at 3 h post-REF (Figure 4.1 G). However, in the CO group, in response to RE, AMPKα2 was significantly increased at Post-RE from Rest. There was no impact of RE on AMPKα2 Post-RE or RE and protein feeding 3 h post-REF in the FO group (Figure 4.1 H).

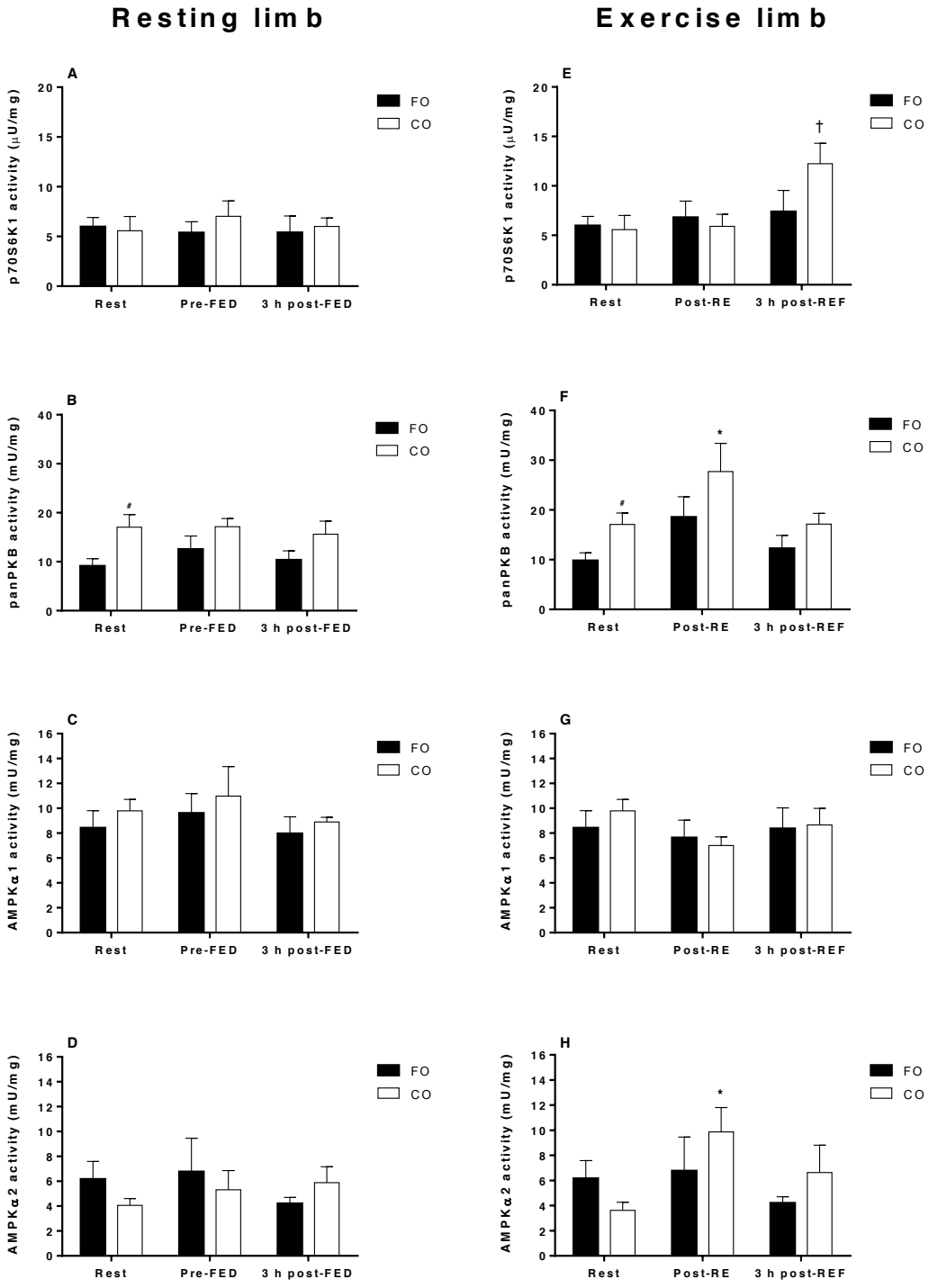

Figure 4.1 Activity of p70S6K1, panPKB, AMPKα1 and AMPKα2 in response to resistance exercise and protein feeding (E-H) and protein feeding alone (A-D) following 8 wk of supplementation with either fish oil (FO) or coconut oil (CO). †: significantly different from all other time points (P<0.05). #: significantly different compared with FO at REST (P< 0.05). *: significantly different from CO Rest and FO 3 h post-REF (P< 0.05). Protein activity data are expressed as mU/mg for panPKB, AMPKα1, AMPKα2 and μU/mg for p70S6K1. Values are means ± SEM. Rest= rest; Pre-Fed= immediately before protein feeding; 3 h post-FED= 3 h post protein feeding; post-RE= immediately post resistance exercise; 3 h post-REF=3 h post resistance exercise and protein feeding.

4.4 Discussion

The primary aim of this study was to examine the impact of 8 weeks of n-3 PUFA-enriched fish oil supplementation on p70S6K1, panPKB, AMPKα1 and AMPKα2 activity in response to RE and bolus protein feeding. This study provides novel data as we show that 8 weeks of n-3 PUFA supplementation increases the n-3 PUFA composition of skeletal muscle phospholipids by ~2 fold, which is associated with the suppression of basal panPKB activity and the blunted response of p70S6K1 activity to RE and protein feeding. By employing a unilateral RE model we also were able to ascertain the impact of 8 weeks of n-3 PUFA supplementation on kinase activity in response to the ingestion of a protein bolus alone. In this regard, we show no change in p70S6K1, panPKB, AMPKα1 or AMPKα2 activity in response to protein feeding at 3 h post feeding. Taken together, these data suggest that 8 weeks of n-3 PUFA supplementation exerts a suppressive effect on the activity of putative kinases that regulate human skeletal muscle anabolism in response to RE and protein feeding.

Our finding that 8 weeks of n-3 PUFA supplementation blunted p70S6K1 activity in response to anabolic stimulation is in contrast to previous reports. Indeed, two studies in humans showed that 8 weeks of n-3 PUFA supplementation enhanced p70S6K1^{thr389} phosphorylation to simulated feeding [21,22]. Moreover, in rodents, dietary fish oil has been shown to attenuate significant losses in skeletal muscle mass during a period of immobilisation, again via enhanced PKB-p70S6K1 signalling [17]. Reconciling the conflicting findings of our study and others may be considered challenging largely due to alternative experimental models (human vs. rodent) and mode of stimulation (RE + feeding vs. intravenous clamp vs. immobilisation). However, with respect to immobilisation it could be contended that contractile specificity, i.e. unloading vs. loading, may provide one explanation. Indeed, extant data show that dietary fish oil actually inhibits the recovery of skeletal muscle mass during reloading following immobilisation [128]. Somewhat in agreement with our data, this

inhibition of muscle mass recovery during increased loading also was associated with blunted PKB^{thr308} and $p70S6K1^{thr389}$ phosphorylation.

Mechanistically, we have no hypothesis as to how fish oil supplementation influences anabolic signalling activity. Although, in the aforementioned study, the reduction in PKB^{thr308} and $p70S6K1^{thr389}$ phosphorylation in response to loading was associated with lower expression of PGF2α. Increases in PGF2α expression have been shown to correlate with enhanced $p70S6K1^{thr389}$ phosphorylation in C2C12 cells [168] and a reduction in PGF2α with fish oil supplementation may provide one explanation for our observations. However, changes in PGF2α are thought only to occur when the content of its substrate, AA, in the membrane is altered. It has been proposed that the ingestion of n-3 PUFA results in an uptake of n-3 PUFA at the expense of n-6 PUFAs, such as AA, but that affect was not apparent in the present investigation. Although, no measurement of PGF2α was made in this investigation so we cannot substantiate or completely rule out a role of PGF2α in the regulation anabolic signalling with fish oil supplementation. Nevertheless, our data, taken together with that of others [128], could be interpreted to suggest that although n-3 PUFA supplementation promotes the retention of skeletal muscle mass during unloading [20,128], during times of muscle loading this effect may be abrogated.

A unique feature of exercise, particularly intense exercise, is the rapid hydrolysis of ATP [72,80]. The rapid hydrolysis of ATP increases the AMP:ATP ratio thus stimulating AMPK activity [73,76,80,169]. Our study provides novel data as we show that 8 weeks of n-3 PUFA supplementation suppresses the activity of the AMPKα2 domain on AMPK immediately Post-RE. Additionally, we show no change in AMPKα1 activity in either condition at any time point we assessed, a finding that supports the contention that it is AMPKα2 and not AMPKα1 that is sensitive to contractile perturbation in humans [166,170,171]. Although, it is important

to recognise that we do not have any information pertaining to phosphorylation of the Thr172 residue on AMPK or phosphorylation of the AMPK target ACC that would provide a more holistic insight into the impact of n-3 PUFA supplementation on AMPK function.

By employing a unilateral RE model we also were able to test the hypothesis that 8 weeks of n-3 PUFA supplementation augments kinase activity in response to protein feeding alone. We show no change in the activity of any kinase we measured to protein feeding. However, in the present study the timing of the muscle biopsies could be a mitigating feature. Indeed, muscle extraction was obtained immediately Post-RE and the protein bolus was consumed after this muscle biopsy. Thus, our only opportunity to detect an impact of protein feeding on kinase activity was at 3 h post-FED. It could be contended that this snapshot attempt to capture increases in anabolic signalling activity was too late and had the biopsy been obtained earlier, maybe at 1 h post feeding, then perhaps changes in kinase activity would have been detected. Indeed, a study from Moore and colleagues [88] provide evidence to support this supposition. In that study, the consumption of 25 g of whey protein stimulated an increase in both p70S6K1^{thr389} and PKB^{ser473} phosphorylation at 1 h but not at 3 h post feeding. In extending these findings, Atherton and colleagues [27] characterised a time course change in mTORC1 signalling with 48 g of whey protein feeding. They show that although the ingestion of a protein bolus stimulated panPKB activity, this increase was only significantly elevated at 45 min and 90 min post feeding and returned to baseline within 3 h. However, it is important to note that p70S6K1^{thr389} phosphorylation was still elevated at 3 h post-feeding but the protein bolus in that study was 48 g whereas in the present investigation only 30 g of whey protein was consumed. Thus, perhaps with a greater dose of whey protein we may have detected changes in p70S6K1 activity at 3 h post-FED similar to that of Atherton and colleagues [27].

In addition to the timing of skeletal muscle biopsies there are other limitations of the present investigation that are worthy of consideration. Firstly, no measurement of muscle function or MPS was made. We are therefore unable to examine if increases in the n-3 PUFA composition of skeletal muscle membrane influences the accrual of skeletal muscle mass that may impact the force producing capacity of skeletal muscle. Secondly, there was no assessment of kinase activity in response to RE and protein feeding before n-3 PUFA supplementation. As a result, we are unable to examine within-subject changes in the sensitivity of skeletal muscle to anabolic stimulation associated with n-3 PUFA supplementation. This limitation becomes more apparent as upon closer inspection of baseline n-3 PUFA muscle phospholipid composition between groups, the FO group possesses a small but significantly elevated n-3 PUFA composition compared to the CO group (5.53 ± 0.30 vs. 3.74 ± 0.23 % of total fatty acids). This difference between groups is apparent despite no obvious differences in dietary intake. However, when changes in the n-3 PUFA composition of muscle phospholipids are expressed as fold-change the FO group demonstrates a ~2-fold increase (Figure 4.2). As we detected no differences in baseline kinase activity between groups we believe that this small difference in baseline n-3 PUFA composition has little influence on our primary endpoint measures.

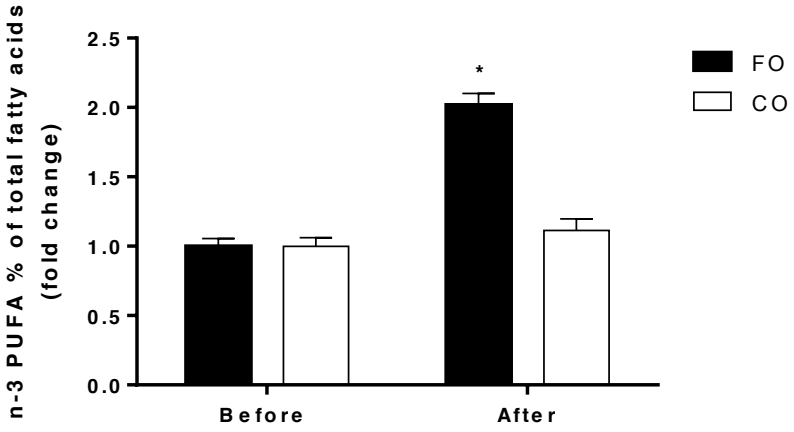

Figure 4.2 Change in n-3 PUFA muscle phospholipids following 8 wk supplementation with either fish oil (FO) or coconut oil (CO):* significantly higher from compared to before supplementation in both groups and after supplementation in the CO group (P<0.05).

To conclude, the present investigation shows that 8 weeks of n-3 PUFA-enriched fish oil supplementation suppresses the basal activity of panPKB as well as p70S6K1 activity in response to RE and protein feeding. Future work that directly assesses MPS and muscle function in response to RE and protein feeding before and following a period of n-3 PUFA supplementation may have valuable application in the clinical and athletic settings.

Author contributions

K.D.T, S.D.R.G and CMcG designed the study; C.McG, K.D.T and S.L.W., L.S.M., F.S., O.C.W and conducted the study. C.McG, D.L.H, L.B, J.R.D and J.G.B performed the analysis C.M, wrote and K.D.T and S.D.R.G edited the chapter.

CHAPTER 5 Synthesis of Findings

Recently, data have emerged to demonstrate that the consumption of n-3 PUFAs increases the n-3 PUFA composition of skeletal muscle that subsequently potentiates MPS to nutrition in both humans [21,22] and steers [124]. Mechanistically, these effects were shown to be associated with concomitant changes in mTOR-p70S6K1 phosphorylation [21,22]. However, assessment of changes in both muscle n-3 PUFA composition and mTOR-p70S6K1 phosphorylation with n-3 PUFA supplementation in those studies were limited to pre-post measurements with little temporal resolution. As a result, a time course change in the n-3 PUFA composition and expression of mTOR-p70S6K1 in skeletal muscle with n-3 PUFA supplementation had not been established. Moreover, whether n-3 PUFA supplementation enhanced mTOR-p70S6K1 activity to a bout of RE when combined with protein feeding remained unknown. Given the emerging role of n-3 PUFAs and the importance of mTOR-p70S6K1 signalling in the regulation of skeletal muscle mass, the overall aim of this thesis was to attempt to experimentally address these intriguing questions. This aim was to be achieved by the successful completion of the following objectives;

1. **To determine a time course change in muscle and blood lipid profiles in response to n-3 PUFA supplementation.**

2. **To determine a time course change in anabolic signalling protein phosphorylation and expression in response to n-3 PUFA supplementation.**

3. **a) To characterise the influence of n-3 PUFA supplementation on the molecular response of human skeletal muscle in response to the ingestion of a protein bolus and b) to the ingestion of a protein bolus following a bout of RE.**

The traditional method to assess protein/kinase activity in the exercise sciences is the WB technique. This technique enables the semi-quantitative assessment of protein/kinase phosphorylation that is often used as a proxy for a protein/kinase activity in response to exercise and nutrition [24-26,88,91]. Originally, it was intended that this technique would be employed to answer the third objective of this thesis **(Chapter 4)**. In this regard, for the purposes of conducting analysis for **Chapter 2** and training for the future analysis of **Chapter 4**, this author was afforded the opportunity to work in various domestic and international laboratories to learn the WB technique. However, several contrasting approaches to the WB technique between laboratories and researchers, specifically with regards to antibody sourcing, statistical analysis and general protocols were noted. These differences have been shown to result in contrasting statistical outcomes in both the published literature [148] and personal experience. Moreover, there is evidence that in some cases the WB technique possesses a limited dynamic range that can lead to type II statistical errors [147]. For example, undetectable changes in $AMPK^{Thr172}$ phosphorylation, despite increases in AMPK activity, can induce substantial changes in the phosphorylation in downs stream effector signalling (ACC). Another important consideration is that these pathways often operate in an amplification system where small changes in the activity of an upstream kinase results in large and significant alterations in functional endpoint measures that could be missed using semi-quantitative WB methods. As a result, following the successful completion of objectives 1 & 2 **(Chapter 2)**, this author elected to develop a more precise

method of assessing protein/kinase activity in order to address the final aim of this thesis **(Chapter 4)**. This method served as another objective to this thesis and was as follows;

4. **To develop a quantitative method to assess p70S6K1 activity in human skeletal muscle in response to resistance exercise and protein feeding.**

The following discussion will be subdivided into three parts. Part A will review the findings of **Chapters 2 and 4** both of which examined the impact of n-3 PUFA supplementation on skeletal muscle metabolism. Part B will focus upon how the development of the *in vitro* [γ-^{32}P] ATP KA in **Chapter 3** raises important questions for the field of molecular exercise physiology and in what way its application in future research may advance the study of human muscle metabolism. Part C will provide a brief summary of the limitations associated with this thesis as well as considerations for future philosophy.

5.1 Part A- Skeletal muscle metabolism and n-3 PUFA supplementation

The impact of n-3 PUFA supplementation on metabolic health is a now topic of great debate [122,123,172]. What is known is that n-3 PUFA supplementation increases the n-3 PUFA composition of skeletal muscle [21,22,123,125,173]. This increase in n-3 PUFA composition of skeletal muscle is thought to precipitate numerous beneficial health outcomes such as improved insulin sensitivity [121,124] as well as enhanced muscle sensitivity to anabolic stimulation [21,22]. Interestingly, although time course changes in the n-3 PUFA composition of blood [125], adipose tissue [123] and cholesterol esters [136] with n-3 PUFA supplementation have previously been established, no study had characterised such a time course in skeletal muscle. As

such, **Chapter 2** adds to existing literature as for the first time we show that 4 weeks of $5g \cdot d^{-1}$ n-3 PUFA-rich fish oil supplementation significantly increases skeletal muscle n-3 PUFA composition within 2 weeks. Moreover, this increase in muscle n-3 PUFA composition of skeletal muscle was delayed compared to that of blood, which was shown to occur within 1 week.

Although **Chapter 2** was the first study to identify a time course increase in skeletal muscle n-3 PUFA composition with fish oil supplementation, we were unable to confirm a saturation of n-3 PUFA muscle composition within the 4 week supplementation period. In **Chapter 4** of this thesis, using the same dose but this time for an 8 week period of supplementation, we also observed a similar ~2 fold increase in muscle n-3 PUFA composition. Taken together, these data would suggest that ingesting $5g \cdot d^{-1}$ of n-3 PUFA-enriched fish oil, a saturation of skeletal muscle n-3 PUFA composition might occur between 4 and 8 weeks of n-3 PUFA supplementation. Moreover, others also have observed a ~2 fold increase in muscle n-3 PUFA composition within 8 weeks of n-3 PUFA supplementation [21,22]. Importantly, in those studies, these changes were achieved using only half the dose of n-3 PUFA compared to that as used in **Chapters 2 and 4** of this thesis. Thus, it could be contended that the dose of n-3 PUFA provided to our participants exceeded the capacity of skeletal muscle to incorporate all of the available circulating n-3 PUFAs into muscle tissue. However, this thesis provides no data relating to markers of lipid peroxidation or evidence of a saturation of muscle n-3 PUFA composition with n-3 PUFA supplementation. As such, the impact of increasing doses of orally ingested n-3 PUFAs on muscle lipid composition and markers of lipid peroxidation are an area of future research.

Previously, it has been shown that 8 weeks of n-3 PUFA supplementation potentiates MPS to a hyperaminoacidemic-hyperinsulinemic infusion [21,22]. This potentiation of MPS was associated

with enhanced mTOR-p70S6K1 signalling [21,22]. In this regard, another aim of **Chapter 2** was to investigate whether 4 weeks of n-3 PUFA-rich fish oil impacted upon the expression of proteins associated with the mTOR-p70S6K1 signalling axis. For the first time we show that 4 weeks of $5g \cdot d^{-1}$ n-3 PUFA-enriched fish oil supplementation significantly increases the content of FAK and induces a transient increase in the content of mTOR. Thus, these data may provide a molecular mechanism by which n-3 PUFA supplementation confers anabolic influence on skeletal muscle [21,22]. Additionally, given its role in mediating contraction-induced changes in MPS [100] we hypothesised that the increase in FAK content with n-3 PUFA supplementation may serve to 'prime' skeletal muscle to respond favourably to anabolic stimulation in the form of RE. However, contrary to our hypothesis, in **Chapter 4** we demonstrated a suppression of p70S6K1 activity in response to RE and protein feeding following 8 weeks of $5g \cdot d^{-1}$ of n-3 PUFA-enriched fish oil supplementation.

Our finding of supressed p70S6K1 activity in response to RE and protein feeding following n-3 PUFA supplementation is in contrast to previous reports of enhanced mTOR-p70S6K1 signalling to n-3 PUFA treatment in humans [21,22] steers [124], rodent [17,18] and cell culture models [18]. A unique feature of **Chapter 4** was that it was the first study to examine the impact of n-3 PUFA supplementation on p70S6K1 activity in response to protein feeding combined with RE. It is the inclusion of this contraction protocol that may explain the incongruent findings between **Chapter 4** and the aforementioned studies. Indeed, in rodents, dietary fish oil is associated with inhibition of p70S6K1 signalling and muscle mass recovery following hind limb immobilisation, i.e. reloading [128]. However, the same authors demonstrate that during immobilisation, i.e. unloading, dietary fish oil alleviates muscle atrophy, again via p70S6K1 signalling [17]. Taken together with the findings of **Chapter 4**, it could therefore be suggested that the physiological

relevance of manipulating the n-3 PUFA composition of skeletal muscle may be contingent upon contraction specificity, i.e., unloading vs. loading or the mode of stimulation, i.e., contraction vs. nutrition.

Although an attractive hypothesis, this thesis provides no data to support the assertion that n-3 PUFA supplementation induces a differential response of mTOR-p70S6K1 signalling that is dependent upon mode of stimulation, i.e., nutrition vs. contraction or contraction specificity. It could be contended that increasing the n-3 PUFA composition of skeletal muscle improves membrane fluidity, thus potentiating nutrient transport into the intracellular space that subsequently enhances anabolic signalling [21,22,121]. However, this supposition does not explain the apparent suppression of p70S6K1 signalling in response to RE. One argument could be that changes in the n-3 PUFA composition of skeletal muscle somehow alters the capacity of skeletal muscle to transduce mechanical tension to a biochemical signal via an unknown mechanism. Another hypothesis is that changes in the n-3 PUFA composition of muscle may impact upon lipid raft formation [174] that also somehow negatively regulates contraction-mediated increases in mTOR-p70S6K1 signalling. However, little data exist in skeletal muscle with regards to lipid raft formation, although there is strong evidence that they do exist (REF). A key question is, if lipid rafts, which are comprised mainly of cholesterol are altered by n-3 PUFA supplementation, do the n-3 PUFAs actually influence the lipid composition of those structures? Moreover, what is the physiological relevance of manipulating lipid raft composition? Perhaps alterations in the muscle membrane lipid composition influence the architecture of the lipid raft that somehow impacts signal transduction mechanisms. All of these hypothesis are worthy of experimental testing.

5.2 Part B- Resistance exercise, protein feeding and p70S6K1 signalling

The mTOR-p70S6K1 signalling axis is known to be a key molecular pathway that regulates changes in MPS in response to RE [30] and nutrition [45]. The thesis adds to existing literature as in **Chapter 3** we provided a novel *in vitro* [γ-^{32}P] ATP KA technique to assess p70S6K1 activity in response to RE and protein feeding in human skeletal muscle. Moreover, we also validated a serial IP protocol that enabled the dual assessment of p70S6K1 and PKB activity from a single muscle homogenate. Using this technique we demonstrated that the combined stimulus of RE and protein feeding induced a significant ~2 fold increase in p70S6K1 activity at 3 h post-RE and feeding. However, despite a ~2 fold increase in p70S6K1 activity, concomitant increases in p70S6K1^{thr389} phosphorylation did not reach statistical significance. Thus, the findings of **Chapter 3** highlight not only the utility but also the precision of the *in vitro* [γ-^{32}P] ATP KA to study the regulation of p70S6K1 activity in response to exercise and nutrition in human skeletal muscle.

Although the *in vitro* [γ-^{32}P] ATP KA as described in **Chapter 3** offers a useful tool for future researchers to study skeletal muscle biology, it is important to reiterate that this technique was designed to be used analogous to the WB technique. Indeed, phosphorylation of specific kinase residues is known to encode for specific environmental cues that could have contrasting affects on the activity of that kinase [167]. For example, phosphorylation of AMPKα2 at Ser485 by IGF-1 results in a decrease in AMPK Thr172 phosphorylation [175] whereas increases in the AMP:ATP ratio induced by exercise actually increase AMPK activity via Thr172 phosphorylation [72,80,176]. Another example is that mTORC1 is known to phosphorylate p70S6K1 at Thr389 [82,177], but for full activation of p70S6K1 to occur, phosphorylation of p70S6K1 at Thr229 is required [53]. Thus, simply by assessing the phosphorylation of a kinase

alone it is difficult to appreciate how signalling cascades identify and then translate specific cellular perturbations to diverse functional outcomes. In this regard, application of the KA developed in **Chapter 3** alongside traditional methods of assessing PTMs, such as the WB, as well as direct measures of MPS in future studies would provide a more holistic approach to study the regulation of skeletal muscle plasticity. However, it is important to acknowledge that the KA developed in this Thesis does have other limitations in addition to its inability to asses PTMs. One is that it requires the use of radioactive ATP and therefore may not be practical for researchers who do not have radioactive clearance at their given institution. Moreover, the activity of the kinase is highly dependant upon freeze thaw cycles that often occurs during analytical analysis, especially when more than one assay (WB, enzyme-linked immunosorbent assay) is being performed. It is therefore always important to weigh up the benefit to cost ratio of using the KA as opposed to the WB in the context of a when considering sample analysis.

There is now a growing body of evidence to suggest discordance between increases in MPS and p70S6K1 phosphorylation in response to RE and/ or nutrition [26,27,139,178]. However, it is important to recognise that p70S6K1 may control ribosomal biogenesis but this supposition has yet to be experimentally corroborated in human skeletal muscle tissue [179]. Indeed, p70S6K1 has been shown to influence ribosomalSo, although the phosphorylation of p70S6K1 post stimulation does not coincide with the greatest MPS response, it may in fact be leading to greater levels of ribosomal transcription. Interestingly, phosphorylation of p70S6K1 following RE occurs in the nucleus where ribosomal biogenesis commences [180]. A caveat of the field is that no study has employed cellular fractionation techniques to reveal whether different RE and nutritional strategies alter the ratio of nuclear to cytoplasmic p70S6K1 activity in human skeletal muscle. Application of the p70S6K1 KA developed in **Chapter 3** of this thesis in studies that

answer these questions would assist in addressing this knowledge gap. Such findings also may help explain the apparent discordance between p70S6K1 phosphorylation and MPS in response to RE and nutrition [26,27,139,178], see Figure 5.0.

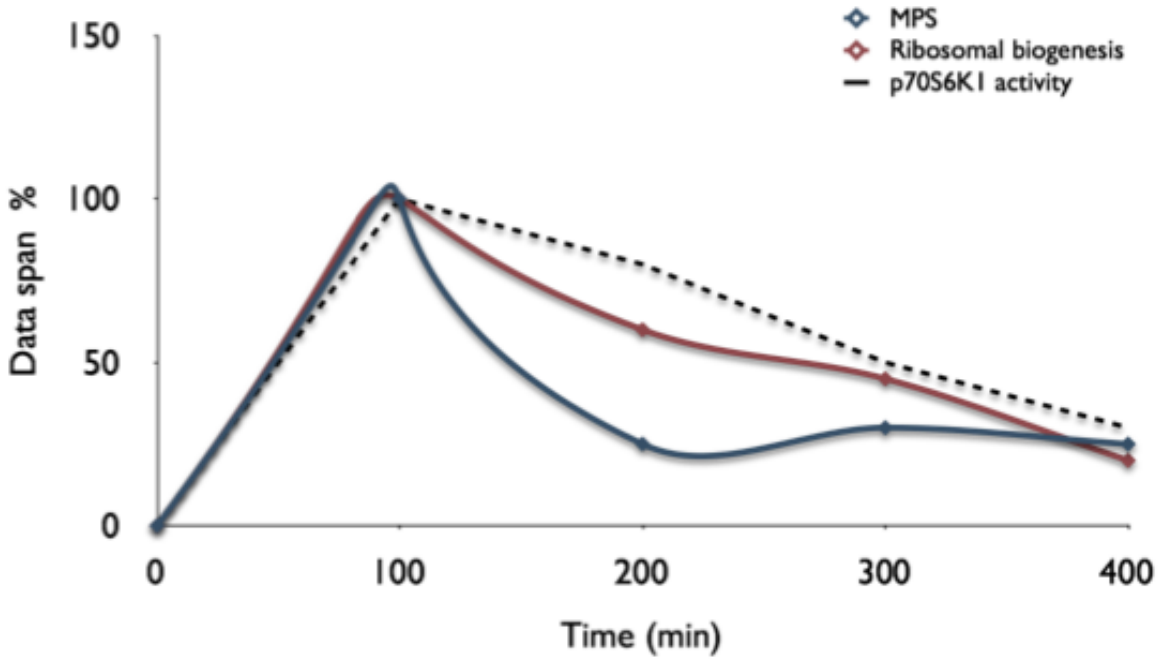

Figure 5.0 Theoretical construct of normalised MPS, ribosomal biogenesis and p70S6K1 activity in response to anabolic stimulation in human skeletal muscle. Adapted from Atherton and Colleagues [27].

5.3 Part C- Limitations of this thesis

This thesis contributes novel data to the literature, however, it is important to acknowledge that there are a number of limitations that should be considered. Firstly, no measurements of MPS or muscle function were made in the experimental studies. As a result, we are unable to identify if the increases in skeletal muscle n-3 PUFA composition and FAK content in **Chapter 2** with fish oil supplementation enhances the force producing capacity of the muscle. Moreover, we are unable to show whether increases in p70S6K1 activity in response to RE and protein feeding in **Chapters 3 and 4** are associated with changes in MPS. Thus, by employing direct measures of

muscle function and MPS to examine the impact of n-3 PUFA supplementation on skeletal muscle in response to stimulation, future researches may add to the findings of this thesis. Another important consideration is that in **Chapter 2** there was no control group. With regards to changes in the n-3 PUFA composition of skeletal muscle and blood this shortcoming has little impact as assessment of muscle and blood lipid profiles were made at -2 and 0 weeks of supplementation, thus each participant acted as their own internal control. However, the lack of repeated baseline sampling for anabolic signalling protein content taken together with the large variability associated with gene expression between people [181], suggests that in this instance, a lack of control group could be a confounding factor. Although, it is important to reiterate that the primary focus of this study was the time course change in muscle lipid profiles with n-3 PUFA supplementation and analysis of changes in anabolic signalling expression was a secondary objective. Nevertheless, given the importance placed on changes in anabolic signalling with n-3 PUFA supplementation in this thesis, the absence of a control group in **Chapter 2** remains a major limitation of this programme of work.

Another limiting feature of this thesis is that all the human participants were active, young males. Given the apparent sex difference in the composition of blood lipid profiles [125], it could be contended that the time course change in muscle lipid profiles with n-3 PUFA supplementation as described in **Chapter 2** may differ between men and women. Moreover, elderly individuals are known to display differential molecular responses to anabolic simulation compared to their younger counterparts [182,183]. As such, the findings of this thesis may have less relevance to those populations. Future work that investigates the impact of n-3 PUFA-enriched fish oil supplementation on lipid composition changes and kinase activity in response to RE and protein feeding would further advance existing knowledge. Finally, the present thesis provides little

information as to how altering the lipid composition of the skeletal muscle membrane influences the molecular regulation of skeletal muscle anabolism. There is speculation that the incorporation of n-3 PUFA into skeletal muscle may alter lipid raft formation that somehow alters gene expression [122,184]. Similarly, alterations in the lipid composition of skeletal muscle also have been purported to impact upon the expression of eicosanoids that subsequently influence muscle anabolic processes [128]. The impact of n-3 PUFA supplementation of both the formation of lipid rafts and eicosanoids is therefore an interesting area of future research.

5.4 Future philosophy

As a result of this programme of work, a time course change in skeletal muscle lipid composition with fish oil supplementation and a novel protocol to assess p70S6K1 activity in skeletal muscle has been established. Moreover, for the first time, this thesis shows that 8 wk of fish oil supplementation suppresses p70S6K1 activity in response to RE and protein feeding in humans. However there are still a number of questions that remain unanswered. These questions are listed below and it is hoped that they will be addressed in the near future.

1. Is there a dose-response relationship between n-3 PUFA ingestion and changes in the n-3 PUFA composition of skeletal muscle?

2. What is the washout period of n-3 PUFA composition of skeletal muscle associated with the cessation of n-3 PUFA supplementation?

3. Is there a correlation between the degree of p70S6K1 activity/phosphorylation and the rate of ribosomal biogenesis following exercise and protein feeding?

4. Can n-3 PUFA supplementation potentiate MPS to a bout of RE and protein feeding?

5. What mediates increases in the n-3 PUFA composition of skeletal muscle with changes in anabolic signalling expression and activity in response to stimulation?

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APPENDICES

Prostaglandins, Leukotrienes and Essential Fatty Acids ■ (■■■■) ■■■–■■■

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Temporal changes in human skeletal muscle and blood lipid composition with fish oil supplementation

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ABSTRACT

The aim of this study was to examine changes in the lipid profile of red blood cells and muscle tissue along with the expression of anabolic signalling proteins in human skeletal muscle. Following a 2-week control period, 10 healthy male participants consumed 5 g d^{-1} of fish oil (FO) for 4 weeks. Muscle biopsies and venous blood samples were collected in the fasted state 2 weeks prior (W-2) and immediately before (W0) the initiation of FO supplementation for internal control. Muscle biopsies and venous blood samples were again obtained at week 1 (W1), 2 (W2) and 4 (W4) during FO supplementation for assessment of changes in lipid composition and expression of anabolic signalling proteins. There was no change in the composition of any lipid class between W-2 and W0 confirming control. Following FO supplementation n-3 polyunsaturated fatty acid (n-3 PUFA) muscle lipid composition was increased from W0 to W2 and continued to rise at W4. n-3 PUFA blood lipid composition was increased from W0 to W1 and remained elevated for the remaining time points. Total protein content of focal adhesion kinase (FAK) increased from W0 to W4 whereas total mechanistic target of rapamycin (mTOR) was increased from W0 at W1 with no further significant increases at W2 and W4. These data show that FO supplementation results in discordant changes in the n-3 PUFA composition of skeletal muscle compared to blood that is associated with increases in total FAK content.

1. Introduction

The consumption of food rich in Omega n-3 polyunsaturated fatty acids (n-3 PUFA) is thought to be beneficial for many cardiovascular disease risk factors, including blood pressure and [1] immune function [2]. There are also other clinically relevant health claims associated with the consumption of n-3 PUFA [3–5] that are concomitant with increases in the n-3 PUFA composition of the associated biological tissues [3,6–10]. However, whilst time course increases in the n-3 PUFA composition of erythrocytes [11], platelets, buccal cells, mononuclear cells and adipose tissue with n-3 PUFA supplementation have recently been established [10], to date, no study has characterised such a time course in human skeletal muscle.

Whilst much is known about other tissues, far less is known about the impact of n-3 PUFA supplementation on human skeletal muscle. Some data exist to show that n-3 PUFA supplementation may alter respiration kinetics and render skeletal muscle more sensitive to the effects of insulin [7,12]. Moreover, work in rodents has demonstrated that dietary fish oil alleviates *soleus* muscle atrophy during a period of enforced immobilization [13]. In humans, supplementation with n-3 PUFA-rich fish oil for 8 weeks is reported to enhance muscle protein synthesis rates (MPS) in response to a hyperaminoacidemic–hyperinsulinemic infusion in both the young and elderly [8,9]. Moreover, there is evidence that n-3 PUFA supplementation augments strength gains in response to resistance training in elderly humans [14]. Mechanistically, the anabolic influence of n-3 PUFA supplementation is purported to be mediated by enhanced mechanistic target of rapamycin (mTOR)-p70 ribosomal protein S6 kinase (p70S6K) signalling [8,9]. Indeed, changes in mTOR-p70S6K signalling with fish oil supplementation in those studies were also shown to be accompanied by significant increases in the n-3 PUFA composition of skeletal muscle [8,9]. Thus, it appears that n-3 PUFA supplementation increases the n-3 PUFA composition of skeletal muscle, which may confer an anabolic influence in part, via mTOR-p70S6K signalling. However, whether fish oil supplementation alters the expression of these proteins and or other mechanically sensitive proteins remains uncertain.

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Although there are now a growing number of studies that characterise changes in the n-3 PUFA composition of skeletal muscle with n-3 PUFA supplementation [8,9,12,15,16], many of these studies are limited to pre- and post-supplementation measurements with little temporal resolution. Data are available on the time course of n-3 PUFA changes in blood [11] and adipose tissue with [10] n-3 PUFA supplementation; however, to our knowledge, no study has established the time course of n-3 PUFA changes in human skeletal muscle. Given the potential beneficial impact of increasing the skeletal muscle n-3 PUFA composition on metabolic health [3,7], data that demonstrate a time course increase in skeletal muscle n-3 PUFA composition with fish oil supplementation could therefore provide critical data for clinical and athletic practice. Thus, the primary aim of the present investigation was to identify the time course of n-3 PUFA change in skeletal muscle over 4 weeks of n-3 PUFA-enriched fish oil supplementation. In addition, as previous reports demonstrate improved strength gains during resistance training [14] and changes in mTOR-p70S6K signalling with n-3 PUFA supplementation [8,9,13,17], a secondary aim was to determine whether 4 weeks of fish oil supplementation modified the expression and or phosphorylation of key anabolic intramuscular signalling proteins (FAK, mTOR, p70S6K, and 4E-BP1).

2. Materials and methods

2.1. Participants

Ten healthy, moderately active males who participated in team sports recreationally (aged 21 ± 3 yrs; body mass 76 ± 4 kg, mean ± SEM) from the University of Stirling and the surrounding area volunteered to participate in the present investigation. Following health screening, participants were excluded if they were engaged in any form of dietary supplementation or were taking any prescribed medication. This study was conducted according to the guidelines laid down in the Declaration of Helsinki (2008) and the Local Ethics Committee, University of Stirling, approved all procedures. Written, informed consent was obtained prior to the commencement of the experiment.

2.2. Experimental design

In a one-way, repeated measures design, participants reported to the laboratory on five separate occasions. Initial baseline assessment of muscle and blood lipid profiles was conducted at −2 (W-2) and 0 (W0) week, to determine muscle and blood lipid profiles over a period of habitual diet and physical activity; thus, participants served as their own internal control (Tables 1 and 2). By employing a one-way, repeated measures design, we were able to circumvent issues such as genetic variability between participants and statistical power associated with a between-groups approach. Following this baseline control period, participants consumed 5 g d^{-1} of fish oil capsules (providing 3500 mg EPA [20:5n-3]; 900 mg DHA [22:6n-3] and vitamin E 0.1 mg, Ideal Omega-3, Glasgow Health Solutions Ltd, UK; see Supplementary Table 1 for full fatty acid profile) for 4 weeks. The supplemental fish oil dose and participant number were chosen based on previous work showing that a similar dose in 10 males can induce significant changes in the lipid profile of human blood over a

Table 1
Full muscle lipid profiles.

Fatty acid	−2 week	0 week	1 week	2 week	4 week
14:0	0.95 ± 0.04	0.93 ± 0.05	0.95 ± 0.05	0.99 ± 0.08	0.85 ± 0.03
15:0	0.23 ± 0.01	0.21 ± 0.01	0.26 ± 0.01	0.20 ± 0.01	0.21 ± 0.01
16:0	17.49 ± 0.27	17.59 ± 0.31	18.03 ± 0.26	17.55 ± 0.37	17.71 ± 0.36
18:0	11.41 ± 0.27	11.19 ± 0.26	11.16 ± 0.17	10.98 ± 0.35	11.66 ± 0.16
20:0	0.13 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.13 ± 0.01
22:0	0.21 ± 0.02	0.17 ± 0.01	0.17 ± 0.02	0.16 ± 0.02	0.19 ± 0.02
24:0	0.18 ± 0.01	0.20 ± 0.02	0.15 ± 0.01	0.15 ± 0.02	0.17 ± 0.02
Total saturated	**30.59 ± 0.26**	**30.42 ± 0.31**	**30.86 ± 0.21**	**30.15 ± 0.21**	**30.92 ± 0.34**
16:1n-9	0.40 ± 0.02	0.39 ± 0.02	0.34 ± 0.01	0.42 ± 0.02	0.35 ± 0.01
16:1n-7	1.22 ± 0.14	1.40 ± 0.13	1.24 ± 0.11	1.49 ± 0.17	1.03 ± 0.10
18:1n-9	15.93 ± 1.06	16.58 ± 0.99	17.25 ± 0.90	17.69 ± 1.39	14.38 ± 0.76
18:1n-7	1.86 ± 0.03	1.91 ± 0.02	1.88 ± 0.03	1.89 ± 0.05	1.80 ± 0.03
20:1n-9	0.24 ± 0.02	0.24 ± 0.02	0.25 ± 0.02	0.25 ± 0.03	0.21 ± 0.02
24:1n-9	0.22 ± 0.01	0.25 ± 0.02	0.21 ± 0.02	0.23 ± 0.06	0.19 ± 0.01
Total monounsaturated	**19.87 ± 1.15**	**20.76 ± 1.08**	**21.16 ± 0.96**	**21.97 ± 1.53**	**17.96 ± 0.81**
18:2n-6	24.27 ± 0.47	23.75 ± 0.26	23.55 ± 0.56	22.91 ± 0.59	23.18 ± 0.40
18:3n-6	0.08 ± 0.00	0.08 ± 0.00	0.07 ± 0.00	0.07 ± 0.00	0.07 ± 0.00
20:2n-6	0.27 ± 0.01	0.27 ± 0.01	0.26 ± 0.01	0.24 ± 0.01	0.24 ± 0.01
20:3n-6	1.14 ± 0.06	1.14 ± 0.07	1.07 ± 0.05	1.04 ± 0.07	1.04 ± 0.06
20:4n-6	10.47 ± 0.50	10.46 ± 0.68	9.75 ± 0.54	9.69 ± 0.69	9.98 ± 0.63
22:4n-6	0.54 ± 0.04	0.53 ± 0.04	0.52 ± 0.05	0.50 ± 0.03	0.46 ± 0.04
22:5n-6	0.23 ± 0.02	0.22 ± 0.02	0.24 ± 0.01	0.20 ± 0.02	0.18 ± 0.02
Total n-6 PUFA	**36.99 ± 0.83**	**36.45 ± 0.82**	**35.46 ± 0.71**	**34.64 ± 1.13**	**35.12 ± 0.56**
18:3n-3	0.49 ± 0.03	0.51 ± 0.05	0.54 ± 0.02	0.58 ± 0.04	0.47 ± 0.03
20:5n-3	0.61 ± 0.05	0.59 ± 0.05	0.94 ± 0.08	1.36 ± 0.11	2.35 ± 0.22
22:5n-3	1.28 ± 0.04	1.24 ± 0.05	1.29 ± 0.04	1.46 ± 0.08	1.77 ± 0.09
22:6n-3	1.49 ± 0.17	1.47 ± 0.16	1.49 ± 0.16	1.69 ± 0.14	2.13 ± 0.21
Total n-3 PUFA	**3.86 ± 0.23[a]**	**3.80 ± 0.22[a]**	**4.28 ± 0.24[a,b]**	**5.14 ± 0.28[b]**	**6.79 ± 0.46[c]**
16:0DMA	5.37 ± 0.22	5.29 ± 0.21	5.10 ± 0.22	5.18 ± 0.29	5.68 ± 0.20
18:0DMA	1.77 ± 0.08	1.78 ± 0.07	1.68 ± 0.07	1.72 ± 0.08	1.90 ± 0.07
18:1DMA	1.55 ± 0.07	1.50 ± 0.06	1.46 ± 0.05	1.32 ± 0.13	1.63 ± 0.07
Total DMA	**8.69 ± 0.34**	**8.57 ± 0.29**	**8.24 ± 0.31**	**8.22 ± 0.42**	**9.21 ± 0.29**

Total saturated fatty acids, total monounsaturated fatty acids, total n-6 polyunsaturated fatty acids (PUFA), total n-3 PUFA and total dimethyl aldehyde (DMA). Values are % total fatty acids mean ± SEM. Means that do not share a letter are significantly different from one another ($p < 0.05$).

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Table 2
Full blood lipid profiles.

Fatty acid	−2 week	0 week	1 week	2 week	4 week
14:0	0.54 ± 0.05	0.51 ± 0.05	0.55 ± 0.05	0.46 ± 0.06	0.51 ± 0.04
15:0	0.22 ± 0.03	0.18 ± 0.01	0.19 ± 0.01	0.18 ± 0.01	0.19 ± 0.01
16:0	20.78 ± 0.41	20.17 ± 0.28	19.90 ± 0.23	19.66 ± 0.37	20.40 ± 0.30
18:0	11.61 ± 0.25	11.53 ± 0.15	11.60 ± 0.11	11.83 ± 0.13	11.89 ± 0.22
20:0	0.23 ± 0.01	0.22 ± 0.01	0.22 ± 0.01	0.24 ± 0.01	0.22 ± 0.01
22:0	0.60 ± 0.03	0.56 ± 0.02	0.57 ± 0.02	0.59 ± 0.02	0.57 ± 0.02
24:0	1.19 ± 0.04	1.10 ± 0.04	1.12 ± 0.05	1.13 ± 0.06	1.10 ± 0.03
Total saturated	**35.17 ± 0.56[a]**	**34.27 ± 0.24[a,b]**	**34.14 ± 0.23[b]**	**34.07 ± 0.31[b]**	**34.88 ± 0.32[a]**
16:1n-9	0.33 ± 0.02	0.35 ± 0.02	0.27 ± 0.02	0.27 ± 0.02	0.26 ± 0.01
16:1n-7	1.27 ± 0.11	1.47 ± 0.15	1.00 ± 0.08	0.93 ± 0.09	1.21 ± 0.20
18:1n-9	17.63 ± 0.34	18.00 ± 0.41	15.82 ± 0.28	15.75 ± 0.46	16.40 ± 0.43
18:1n-7	1.66 ± 0.07	1.58 ± 0.07	1.46 ± 0.06	1.56 ± 0.07	1.56 ± 0.08
20:1n-9	0.28 ± 0.02	0.28 ± 0.01	0.25 ± 0.02	0.25 ± 0.02	0.25 ± 0.01
24:1n-9	1.70 ± 0.07	1.50 ± 0.08	1.49 ± 0.07	1.58 ± 0.09	1.43 ± 0.06
Total monounsaturated	**22.87 ± 0.40[a]**	**23.19 ± 0.55[a]**	**20.30 ± 0.26[b]**	**20.34 ± 0.46[b]**	**21.11 ± 0.61[b]**
18:2n-6	18.61 ± 0.37	18.28 ± 0.69	17.80 ± 0.52	17.19 ± 0.42	16.43 ± 0.33
18:3n-6	0.30 ± 0.04	0.32 ± 0.03	0.21 ± 0.02	0.15 ± 0.01	0.15 ± 0.03
20:2n-6	0.32 ± 0.01	0.37 ± 0.02	0.30 ± 0.01	0.27 ± 0.01	0.24 ± 0.01
20:3n-6	1.77 ± 0.06	1.88 ± 0.08	1.51 ± 0.07	1.32 ± 0.07	1.15 ± 0.07
20:4n-6	10.52 ± 0.44	10.88 ± 0.22	11.24 ± 0.26	10.88 ± 0.36	9.99 ± 0.24
22:4n-6	1.37 ± 0.09	1.44 ± 0.07	1.42 ± 0.07	1.33 ± 0.07	1.21 ± 0.07
22:5n-6	0.28 ± 0.03	0.30 ± 0.02	0.29 ± 0.02	0.28 ± 0.02	0.22 ± 0.02
Total n-6 PUFA	**33.17 ± 0.58[a]**	**33.47 ± 0.60[a]**	**32.76 ± 0.44[a,b]**	**31.42 ± 0.71[b]**	**29.38 ± 0.42[c]**
18:3n-3	0.47 ± 0.06	0.57 ± 0.06	0.63 ± 0.04	0.59 ± 0.06	0.52 ± 0.03
20:5n-3	0.86 ± 0.09	0.89 ± 0.07	3.62 ± 0.32	4.54 ± 0.39	4.81 ± 0.31
22:5n-3	1.47 ± 0.07	1.58 ± 0.04	1.95 ± 0.07	2.09 ± 0.08	2.21 ± 0.09
22:6n-3	2.58 ± 0.26	2.72 ± 0.25	3.26 ± 0.19	3.52 ± 0.21	3.60 ± 0.21
Total n-3 PUFA	**5.43 ± 0.41[a]**	**5.85 ± 0.31[a]**	**9.54 ± 0.43[b]**	**10.83 ± 0.60[b]**	**11.23 ± 0.53[b]**
16:0DMA	1.18 ± 0.05	1.11 ± 0.03	1.16 ± 0.04	1.16 ± 0.05	1.22 ± 0.03
18:0DMA	1.64 ± 0.05	1.61 ± 0.04	1.61 ± 0.05	1.61 ± 0.07	1.65 ± 0.05
18:1DMA	0.55 ± 0.04	0.51 ± 0.04	0.50 ± 0.04	0.56 ± 0.03	0.54 ± 0.04
Total DMA	**3.36 ± 0.13**	**3.22 ± 0.09**	**3.26 ± 0.12**	**3.33 ± 0.14**	**3.41 ± 0.10**

Total saturated fatty acids, total monounsaturated fatty acids, total n-6 polyunsaturated fatty acids (PUFA), and total n-3 PUFA and total dimethyl aldehyde (DMA). Values are % total fatty acids mean ± SEM. Means that do not share a letter are significantly different from one another ($p < 0.05$).

4-week period [11]. Moreover, by prescribing a high dose of fish oil along with multiple skeletal muscle biopsies within a 4-week time course we are able to provide a degree of resolution to the anticipated changes in the n-3 PUFA composition of skeletal muscle during the 4-week experiment. Participants were required to complete a 7 d food and physical activity diary prior to baseline testing (W-2). This diary was presented back to the participants who were then asked to replicate a similar pattern of food consumption and physical activity for the remainder of the experiment. Dietary analysis was conducted using the software program Microdiet (Downlee Systems, Ltd., UK). During each visit to the laboratory, participants were verbally requested to confirm the pattern of oily fish consumption in an attempt to ensure that changes in free-living oily fish consumption did not influence muscle and blood lipid profiles during the study. Each participant's height, nude body mass, resting skeletal muscle samples and duplicate 5 mL venous blood samples were obtained at 0700 following a 10 h overnight fast at W-2, W0, 1 week (W1), 2 weeks (W2) and 4 weeks (W4) of supplementation. Supplementation compliance was assessed via a blind capsule count. Resting, fasting venous blood samples were analysed for glucose concentration to ensure compliance with the overnight fast.

2.3. Experimental procedures

2.3.1. Venous blood and muscle biopsy procedures

Blood samples were obtained from an antecubital forearm vein. All samples were drawn into evacuated 5 mL vacutainers containing ethylenediametetraacetic acid (EDTA) (Vacutainer Systems, Becton, Dickinson and Company, UK). An aliquot of blood was removed and centrifuged at 3000 rpm min^{-1} for 15 min and the plasma was stored at −80 °C until further analysis. Plasma glucose was determined using an ILAB automated analyser (Instrumentation laboratory, Cheshire, UK).

Muscle biopsies were obtained from the lateral portion of the *Vastus lateralis*. Initially the site was cleaned before an incision into the skin and fascia was made under local anaesthetic (2% Lidocaine). A 5 mm Bergstrom biopsy needle was inserted to extract ~60–100 mg of skeletal muscle tissue. Muscle samples were rinsed with ice-cold saline, blotted dry and any visible fat or connective tissue was removed. Muscle samples were separated into two eppendorf tubes, before being snap-frozen in liquid nitrogen and stored at −80 °C pending further analysis. All subsequent muscle biopsies, i.e. 0, 1, 2 and 4 weeks, were obtained from the contralateral limb to the previous sample.

2.3.2. Lipid extraction of muscle biopsy samples

Total lipid was extracted from the muscle by a modification of a method previously described [18]. The frozen muscle biopsy samples (20–60 mg) were placed in a pre-weighed reacti-vial and an accurate tissue mass recorded. The reacti-vials were capped and placed on ice and then 1 mL of chloroform/methanol (C:M, 2:1 v/v) was added to each vial. Each muscle sample was homogenised using a hand-held IKA-Werke Ultra-turrax T8 homogeniser (Fisher, Loughborough, UK). The probe was rinsed with 3 mL of C:M, 2:1, and added to the reacti-vial before being placed on ice for 1 h. Then 1 mL 0.88% KCl was added, shaken and allowed

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to stand for 10 min to remove non-lipid impurities. The vials were then centrifuged at 400*g* for 5 min before removal of the aqueous layer. The lower solvent layer was removed using a Pasteur pipette and filtered through a 5.5 mm Whatman No. 1 filter, pre-washed with C:M, 2:1, into a 7 mL bottle, and dried under N_2. The lipid was then re-dissolved in 1 mL of C:M, 2:1, and transferred to a pre-weighed 1.7 mL bottle. The 1.7 mL bottle was then rinsed with 0.5 mL C:M, 2:1, and this was added to the 1.7 mL bottle. The lipid was dried under N_2 and desiccated overnight in a vacuum desiccator after which the lipid was reweighed and dissolved in C:M, 2:1+0.01% butylated hydroxyl toluene (BHT) (v/v), at a concentration of 2 mg mL^{-1}.

2.3.3. Lipid extraction of whole blood samples

Samples of whole blood were placed onto two circular collection spots on Whatman 903 blood collection cards (GE Healthcare Ltd., Forest Farm Industrial Estate, Cardiff, CF 14 7YT, UK). The cards were left open and allowed to dry for 3 h after which the dried whole blood sample was detached from the collection device using forceps and placed into a screw-cap vial containing 1 mL of methylating solution (1.25 M methanol/HCl). The vials were placed in a hot block at 70 °C for 1 h. The vials were allowed to cool to room temperature before 2 mL of distilled water and 2 mL of saturated KCl solution were added. Fatty acid methyl esters (FAME) were then extracted using 1 × 2 mL of iso-hexane+BHT followed by a second extraction using 2 mL of isohexane alone. This extraction method has been previously validated as a reliable measure of whole blood fatty acid composition in our own laboratories [19].

2.3.4. Analysis of fatty acid methyl esters (FAME)

FAME were separated and quantified by gas–liquid chromatography (ThermoFisher Trace, Hemel Hempstead, England) using a 60 m × 0.32 mm × 0.25 μm film thickness capillary column (ZB Wax, Phenomenex, Macclesfield, UK). Hydrogen was used as carrier gas at a flow rate of 4.0 mL min^{-1} and the temperature program was from 50 to 150 °C at 40 °C min^{-1}, then to 195 °C at 2 °C · min^{-1} and finally to 215 °C at 0.5 °C min^{-1}. Individual FAME were identified and compared to well-characterised in house standards as well as commercial FAME mixtures (Supelco™ 37 FAME mix, Sigma-Aldrich Ltd., Gillingham, England).

2.3.5. Western Blot

Muscle tissue (20–40 mg) was homogenised in ice-cold homogenization buffer containing 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 10 mM ß-glycerophosphate, 50 mM NaF, 0.5 mM activated sodium orthovanadate (all SigmaAldrich, St Louis, MO, USA) and a complete protease inhibitor cocktail tablet (Roche, Indianapolis, IN, USA). Homogenates were centrifuged at 2200*g* for 10 min at 4 °C before recovery of supernatants representing the sarcoplasmic protein pool for analysis by Western Blot. Protein concentration was determined using a BCA protein assay (Thermo Fisher Scientific, Ontario, Canada). Equal aliquots of protein (20 μg) were boiled in Laemmli sample buffer (250 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue, and 5% β-mercaptoethanol) and separated on SDS polyacrylamide gels (range 10–15%) for 1.5 h at 140 V. Following electrophoresis, proteins were transferred to a PVDF membrane at 100 V for 1 h. Following 1 h of blocking in 5% milk powder in TBST (Tris Buffered Saline and 0.1% Tween-20; both Sigma-Aldrich, Poole, UK), membranes were incubated overnight at 4 °C with the appropriate primary antibody diluted in TBST. Primary antibodies were $mTOR^{Ser2448}$ (Cell Signalling, #2971, 1:1000), total mTOR (Cell Signalling, #2972, 1:1000), $p70S6K^{Thr389}$ (Cell Signalling, #9234, 1:1000), total p70S6K (Cell Signalling, #9202, 1:1000), initiation factor 4E binding protein $(4E\text{-}BP1)^{Thr37/46}$ (Cell Signalling, #2855, 1:1000), total 4E-BP1 (Cell Signalling, #9452, 1:2000), focal adhesion kinase $(FAK)^{Tyr576/577}$ (Santa Cruz Biotechnology, #21831, 1:5000), total FAK (Santa Cruz, #558, 1:5000) and α-tubulin (Sigma-Aldrich, #T6074, 1:2000). The following morning the membrane was rinsed three times for 5 min in TBST. The membrane was then incubated for 1 h at room temperature in HRP-conjugated anti-rabbit secondary antibody diluted in TBST (New England Biolabs, UK, 1:10000). The membrane was then cleared three times for 5 min in TBST. Antibody binding was detected using enhanced chemiluminescence (Millipore, Billerica, MA). Band visualization was carried out using a Chemidoc XRS system (Bio-Rad, Hemel Hempstead, UK) and quantification using densitometry (ImageJ v1.34s 281 software, rsbweb.nih.gov/ij/). Molecular signalling proteins were determined with $n=10$. Phosphoproteins were expressed relative to total protein by stripping the membrane using Restore Western Blot Stripping Buffer (Thermo Scientific, FL, USA, #21059) and re-probing for total protein. Phosphorylated antibodies were removed from all membranes before using Restore Stripping Buffer (Thermo Fisher Scientific, Ontario, Canada) prior to probing for total protein. Total protein was expressed relative to α-tubulin as a protein loading control.

2.3.6. Data presentation and statistical analysis

All statistical analyses were performed using Minitab 17 statistical software (Minitab Ltd., Coventry, UK). Paired *t*-tests were used to detect differences in lipid profiles of blood and muscle between W-2 and W0. As no differences were detected between baseline measurements (W-2 and W0) for lipid profiles of the blood or muscle, all further statistical analyses were performed using only W0 as the baseline measurement. In a single-factor (week of supplementation), repeated measures ANOVA was employed to evaluate changes in lipid composition of both the blood and muscle as well as changes in protein signalling from W0 to W4. In order to comply with the assumptions of the ANOVA, when required, protein signalling data were initially log transformed. If a main effect of time was observed a Tukey's post-hoc analysis was applied to detect differences between weeks. Regression analysis was performed to identify correlation coefficients between blood and muscle lipids. Statistical significance was set at $p<0.05$. Protein signalling data are presented as mean (cross), median with interquartile-range and range. All lipid profiling data are expressed as mean relative percentage of total fatty acids ± SEM unless otherwise stated.

3. Results

3.1. Dietary analysis

Daily energy intake was 2244 ± 132 kcal (55% ± 5% carbohydrate, 15% ± 2% protein, 30% ± 4% fat, and n-3 PUFA 0.7% ± 0.2%). Participants' mean fasted, plasma glucose samples across all trials were 4.81 ± 0.12 mM (range 4.1–5.5 mM).

3.2. Lipid profile changes in muscle

There was no significant change ($p=0.099$) in the total lipid content of muscle (1.38 ± 0.04 [W-2] to 1.25 ± 0.05 [W0] to 1.22 ± 0.02 [W1] to 1.36 ± 0.08 [W2] to 1.20 ± 0.05 [W4], mg/100 mg of muscle) at any time point. Thus, in order to enable meaningful comparisons between changes in blood and muscle n-3 PUFA composition as well with previously published studies [8–11] changes in muscle and blood lipids are presented as changes in % composition of total fatty acids. Full muscle lipid profiles can be seen in Table 1. Lipid composition of muscle for any lipid species or group of lipid species did not differ between W-2 and W0. The difference in mean % EPA+DHA/total fatty acids was statistically higher at W2 compared to W0 ($p=0.001$) and continued to increase at W4 ($p<0.001$). There was an increase in % total n-3 PUFA/total

fatty acids from W0 to W2 ($p < 0.001$) that continued to rise at W4 ($p < 0.001$). There was no change in percentage total n-6 polyunsaturated fatty acids (n-6 PUFA)/total fatty acids, ($p = 0.218$), % total saturated fatty acids/total fatty acids ($p = 0.076$), % monounsaturated fatty acids/total fatty acids ($p = 0.432$) or % dimethyl aldehyde (DMA)/total fatty acids ($p = 0.418$) over time.

3.3. Lipid profile changes in blood

Full blood lipid profiles can be seen in Table 2. Lipid composition of blood for any lipid species or group of lipid species did not differ between W-2 and W0. Percentage EPA+DHA/total fatty acids significantly increased from W0 to W1 ($p < 0.001$; Fig. 1B) and from W1 to W2 ($p = 0.041$; Fig. 1B) with no further detectable increases at W4 ($p = 0.831$; Fig. 1B). Blood % total n-3 PUFA/total fatty acids was increased at W1 compared to W0 ($p < 0.001$) and was further increased at W2 ($p = 0.047$) with no further detectable increases at W4 ($p = 0.824$). Percentage total n-6 PUFA/total fatty acids declined significantly from W0 to W2 ($p = 0.009$) and was further decreased at W4 ($p = 0.010$). Percentage monounsaturated fatty acids/total fatty acids were significantly higher at W0 ($p < 0.001$) compared to W1, W2 and W4. There was a significant increase in percentage total saturated fatty acids/total fatty acids ($p = 0.076$) at W4 compared to W1 and W2. There was no change in percentage dimethyl aldehyde (DMA)/total fatty acids ($p = 0.249$) over time.

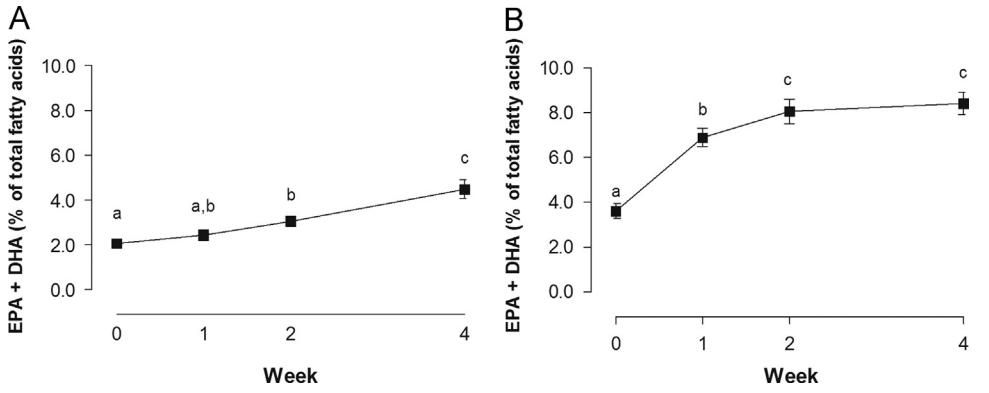

Fig. 1. Skeletal muscle (A) and blood (B) lipid composition changes of the sum of EPA+DHA values mean ± SEM. Means that do not share a letter are significantly different from one another ($p < 0.05$).

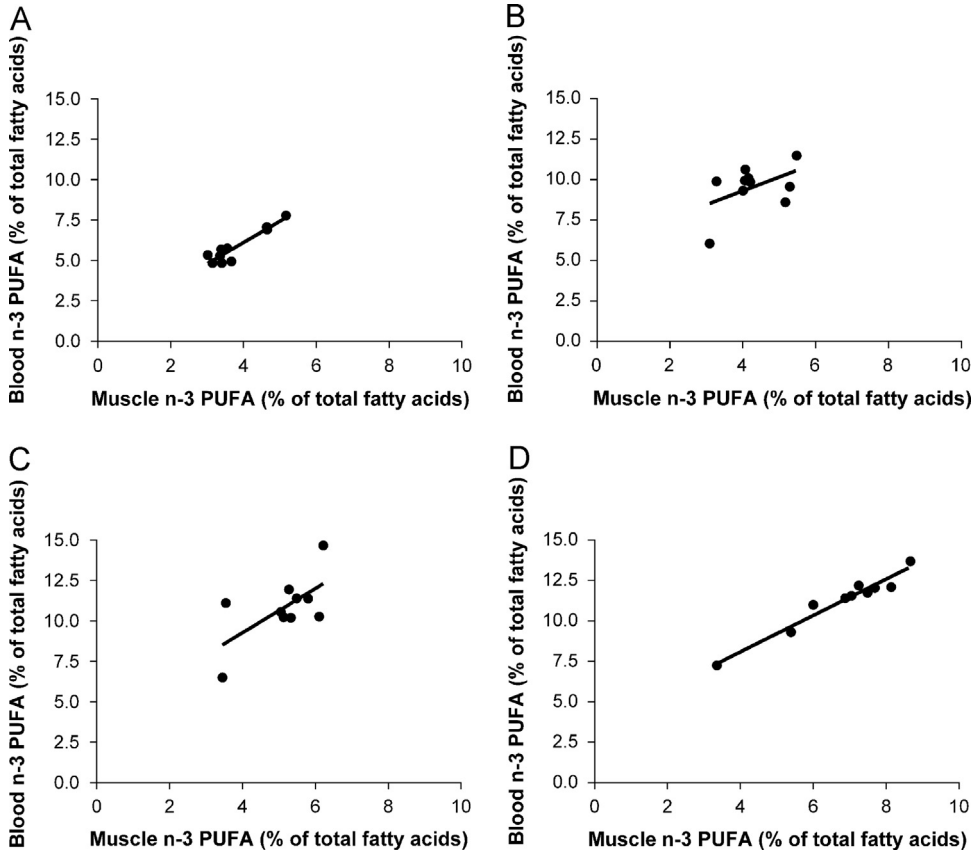

Fig. 2. Correlation between muscle n-3 PUFA composition compared with blood n-3 PUFA composition at W0 (A) ($p < 0.001$, r^2 0.93), W1 (B) ($p = 0.163$, r^2 0.48), W2 (C) ($p = 0.045$, r^2 0.64) and W4 (D) ($p < 0.001$, r^2 0.98) of fish oil supplementation.

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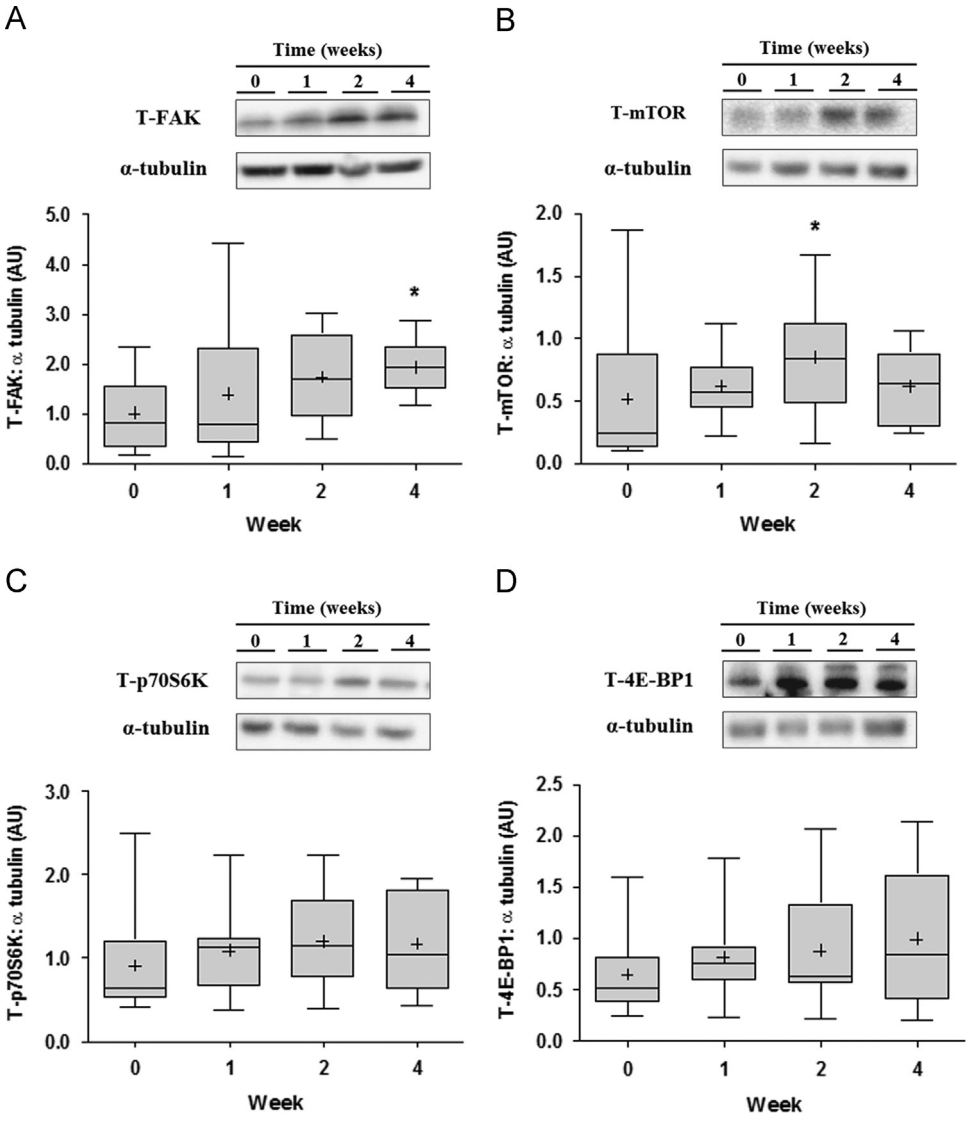

Fig. 3. Signalling protein content of focal adhesion kinase (FAK) (A), mechanistic target of rapamycin (mTOR) (B), ribosomal protein S6 kinase (p70S6K) (C) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) (D). Values expressed as arbitrary units relative to α tubulin and presented as mean (cross), median with interquartile-range and range; * denotes significantly higher than W0 ($p < 0.05$).

3.4. Correlation analysis of muscle and blood

Correlation analysis revealed a significant association between blood and muscle n-3 PUFA composition at W0 ($p < 0.001$, r^2 0.93; Fig. 2A) but not at W1 ($p = 0.163$, r^2 0.48; Fig. 2B). However, the association was increased at W2 ($p = 0.045$, r^2 0.64; Fig. 2C) and returned to a strong association at W4 ($p < 0.001$, r^2 0.98; Fig. 2D).

3.5. Anabolic protein expression

Total FAK protein content increased from W0 to W4 ($p = 0.013$; Fig. 3A). Total mTOR protein content significantly increased from W0 to W2 ($p = 0.008$) with no further detectable increases at W4 ($p = 0.166$; Fig. 3B). The largest fold change for total FAK and total mTOR protein content was 3.9 ± 1.5 (W0 to W4) and 3.2 ± 0.8 (W0 to W2), respectively. There was no effect of time on fold change in total p70S6K ($p = 0.295$; Fig. 3C) or total 4E-BP1 protein content ($p = 0.444$; Fig. 3D). There was also no effect of time for fold change in the phosphorylation of $FAK^{Tyr576/577}$, $mTOR^{Ser2448}$, $p70S6K^{Thr389}$ or $4E\text{-}BP1^{Thr37/46}$ (data not shown).

4. Discussion and conclusions

This study was designed to examine the changes in muscle and blood lipid composition as well as alterations in anabolic signalling expression during 4 weeks of n-3 fish oil supplementation. We report that 4 weeks of fish oil supplementation increased both blood and skeletal muscle n-3 PUFA composition that was accompanied by an increase in intramuscular anabolic signalling protein content. In addition, we show that the increase in n-3 PUFA in blood occurred within 1 week; however in muscle, significant increases in n-3 PUFA composition were not detected until 2 weeks of supplementation. Given the potential significance of increasing the n-3 PUFA composition of skeletal muscle on metabolic health [20], these novel data therefore provide relevant information pertaining to fish oil prescription for future studies in this field.

The time course of n-3 PUFA changes in blood and adipose tissue following n-3 PUFA supplementation has been examined previously [10,11,21]. These studies show that n-3 PUFA supplementation can induce detectable increases in the n-3 PUFA composition of blood within 1 week [11], whilst increases in adipose tissue PUFA may require >12 months of supplementation [10]. Herein,

our novel data add to the existing literature by demonstrating that a minimum of 2 weeks of fish oil supplementation at the dose used in our study is required in order to induce a detectable increase in n-3 PUFA composition in skeletal muscle. The delay in the response of the n-3 PUFA muscle composition compared with that of blood could be due to differing turnover rates between tissues. By employing a within-subject, repeated measures design, for the first time we are able to directly compare changes in blood and muscle n-3 PUFA composition with fish oil supplementation. There was a strong correlation between blood and muscle n-3 PUFA composition at baseline but after 1 week of supplementation this correlation was not significant. However, after 2 and 4 weeks of supplementation the correlation between blood and muscle n-3 PUFA composition was strengthened and returned to statistical significance. These data therefore highlight that during the initial stages of n-3 PUFA supplementation, changes in blood lipid composition do not accurately reflect that of skeletal muscle. Furthermore, unlike blood, there was no apparent saturation of muscle n-3 PUFA composition within the 4-week time course. As a result, a more prolonged period (>4 weeks) of fish oil supplementation may be required in order to reach a saturation of n-3 PUFA composition in human skeletal muscle during fish oil supplementation.

Previous studies employing pre/post-assessments of n-3 PUFA changes in muscle phospholipids following 8 weeks of fish oil supplementation demonstrate a ~2-fold increase in the n-3 PUFA composition from baseline [8,9]. We observed similar ~2-fold increases in whole muscle n-3 PUFA composition, achieved within only 4 weeks of fish oil supplementation. Some of the differences between the present study and previous data may be due to differences in n-3 PUFA content between cellular compartments (i.e. whole muscle vs. membrane) or in the dose and/or composition of the supplements. The rapid increase in n-3 PUFA in whole muscle observed in our investigation may relate to the high EPA content of the fish oil supplement. Indeed, our participants ingested nearly double the daily dose of EPA (3500 mg vs. 1860 mg daily) as used in previous studies, in which the supplementation protocol was twice as long as that of the current investigation [8,9]. Taken together with our data, it appears that the n-3 PUFA composition of skeletal muscle could be a function of the dose of n-3 PUFA consumed as well as the time course of supplementation. However, it is important to note that the dose of DHA as used in our investigation was approximately 900 mg lower than in previous reports [9,11]. In this regard, perhaps had we increased the daily dose of DHA prescribed to the participants it is possible that we may have observed a different rate of change in the n-3 PUFA composition of skeletal muscle over time. Thus, future studies employing differing n-3 PUFA supplementation protocols may elucidate a dose and time-dependant response of n-3 PUFA changes in skeletal muscle similar to those established in other biological tissues [10,11,21].

The impact of n-3 PUFA supplementation on skeletal muscle metabolism is now becoming a topic of intense investigation. In humans, n-3 PUFA supplementation has recently been shown to alter respiration kinetics [12] as well as potentiate MPS to a hyperaminoacidemic–hyperinsulinemic infusion [8,9]. In addition, in rodents, n-3 PUFA supplementation has been demonstrated to alleviate *soleus* atrophy during a period of immobilization [13] whilst in pigs 21 d of fish oil supplementation increases muscle protein mass [22]. The findings of the present investigation add to these data as we show that 4 weeks of fish oil supplementation significantly increases the total protein content of mTOR and the mechanically sensitive kinase, FAK, in free-living humans. Given that both FAK and mTOR play a key role in the molecular regulation of MPS [23–25], our data could be interpreted to suggest that the increases in total protein content may provide an enhanced capacity of skeletal muscle to respond to anabolic stimulation. However, as a note of caution, unlike the pre supplementation, repeated measures assessment of changes in blood and muscle n-3 PUFA composition, we have no control group to statistically compare against for the increases in the expression of mTOR and FAK. Furthermore, the increase in mTOR content was transient and a recent study in rodents has shown that during remobilisation from an immobilised state, dietary fish oil was associated with inhibition of myosin heavy chain content recovery [17]. In the context of anabolic signalling molecules, this finding highlights a limitation of our descriptive investigation as our study design precludes us from demonstrating whether the increases in mTOR and FAK protein content translate to enhance kinase activation in response to stimulation. Nevertheless, given previous reports demonstrating an associative role of FAK in mediating mechanically-induced changes in MPS [23], future work that identifies if n-3 PUFA supplementation enhances resistance exercise-induced rates of MPS in humans could substantiate our findings.

In summary, we have characterised a time course of n-3 PUFA changes and anabolic signalling expression in human skeletal muscle during 4 weeks of fish oil supplementation. The primary conclusion of this investigation is that fish oil supplementation results in changes in muscle n-3 PUFA composition of skeletal muscle within 2 weeks, compared to months as has been reported for adipose tissue. In addition, we demonstrate that during the initial stages of fish oil supplementation, changes in muscle n-3 PUFA composition are slower than that of blood. This finding provides novel information for future researches who wish to employ fish oil supplementation as a means to manipulate the n-3 PUFA composition of skeletal muscle for experimental purposes. To our knowledge, this study is also the first to suggest that fish oil supplementation increases the content of FAK that may indicate a 'priming' of muscle to respond to mechanical stimulation in humans. As such, future studies that identify how fish oil supplementation influences MPS in response to mechanical stimulation in a physiological setting are now warranted.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.plefa.2014.03.001.

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Application of the [γ-^{32}P] ATP kinase assay to study anabolic signaling in human skeletal muscle

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McGlory C, White A, Treins C, Drust B, Close GL, MacLaren DP, Campbell IT, Philp A, Schenk S, Morton JP, Hamilton DL. Application of the [γ-^{32}P] ATP kinase assay to study anabolic signaling in human skeletal muscle. *J Appl Physiol* 116: 504–513, 2014. First published January 16, 2014; doi:10.1152/japplphysiol.01072.2013.—AMPK (AMP-dependant protein kinase)-mTORC1 (mechanistic target of rapamycin in complex 1)-p70S6K1 (ribosomal protein S6 kinase 1 of 70 kDa) signaling plays a crucial role in muscle protein synthesis (MPS). Understanding this pathway has been advanced by the application of the Western blot (WB) technique. However, because many components of the mTORC1 pathway undergo numerous, multisite posttranslational modifications, solely studying the phosphorylation changes of mTORC1 and its substrates may not adequately represent the true metabolic signaling processes. The aim of this study was to develop and apply a quantitative in vitro [γ-^{32}P] ATP kinase assay (KA) for p70S6K1 to assess kinase activity in human skeletal muscle to resistance exercise (RE) and protein feeding. In an initial series of experiments the assay was validated in tissue culture and in p70S6K1-knockout tissues. Following these experiments, the methodology was applied to assess p70S6K1 signaling responses to a physiologically relevant stimulus. Six men performed unilateral RE followed by the consumption of 20 g of protein. Muscle biopsies were obtained at pre-RE, and 1 and 3 h post-RE. In response to RE and protein consumption, p70S6K1 activity as assessed by the KA was significantly increased from pre-RE at 1 and 3 h post-RE. However, phosphorylated p70S6K1^{thr389} was not significantly elevated. AMPK activity was suppressed from pre-RE at 3 h post-RE, whereas phosphorylated ACC^{ser79} was unchanged. Total protein kinase B activity also was unchanged after RE from pre-RE levels. Of the other markers we assessed by WB, 4EBP1$^{thr37/46}$ phosphorylation was the only significant responder, being elevated at 3 h post-RE from pre-RE. These data highlight the utility of the KA to study skeletal muscle plasticity.

mTORC1; p70S6K1; AMPK; resistance exercise

THE AMPK (AMP-DEPENDANT PROTEIN KINASE)-mTORC1 (mechanistic target of rapamycin in complex 1)-p70S6K1 (ribosomal protein S6 kinase 1 of 70 kDa) cascade is a key regulatory signaling axis controlling a plethora of human metabolic events such as skeletal muscle protein synthesis (MPS) (11), glucose disposal (18, 29), and fatty acid metabolism (26). Our understanding of how the AMPK-mTORC1-p70S6K1 pathway responds to physiological perturbation such as exercise (11) and nutrition (9) has been advanced by the application of the phosphorylation-specific Western blot (WB) technique. This technique assesses the phosphorylation of a kinase or a kinase target on serine, threonine, and tyrosine residues, and infers the activity of a kinase on the basis of the magnitude of phosphorylation as determined by densitometry. The WB technique is highly advantageous because it offers the capacity to measure phosphorylation changes in many targets in a cost-effective way. However, in some cases, the WB technique possesses a limited dynamic range that can lead to type II statistical errors (18). Furthermore, differences in methodological approaches to the WB are known to lead to different statistical outcomes for the same data sets (14). Another consideration is that p70S6K1 has a constitutively low baseline phosphorylation. As such, when changes in p70S6K1 phosphorylation to anabolic stimulation are represented as a fold or percentage change, this low baseline phosphorylation results in an inflated response that is not representative of a physiological change in activity (21, 27). Hence, our understanding of how various stimuli such as exercise and nutrition affect p70S6K1 signaling is in part confined to both the limitations and assumptions of the WB technique.

In a recent commentary, Murphy and Lamb (24) describe a fully quantitative approach to WB. These authors show that by using calibration curves for each gel, a quantitative assessment of changes in protein expression can be made. However, conducting calibration curves for the analysis of posttranslational modifications (PTM) such as phosphorylation would be contingent upon 100% of the recombinant protein modified specifically at the specific PTM residue. Furthermore, the use of such calibration curves on every gel would prove costly when analyzing numerous samples, thus undermining the financial viability of the WB technique. As such, the use of the WB to assess changes in the phosphorylation of a kinase as a proxy of kinase activity remains a challenge.

The in vitro [γ-^{32}P] ATP kinase assay (KA) is the gold standard for assessing kinase activity (16). This methodology involves immunoprecipitating the kinase of interest from homogenized tissue. The activity of the kinase is then assessed in vitro against a kinase-specific or kinase family-specific substrate. Gamma (γ)-^{32}P ATP is subsequently used to measure the incorporation of phosphate into the substrate via liquid scintillation counting, thus enabling a quantitative assessment of activity. The dual layer of specificity and quantitative nature of the KA may obviate some of the methodological shortcom-

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ings associated with using the WB (14) and its use to assess AMPK activity in response to exercise is now a feature in the human exercise sciences (10, 37, 40). A semiquantitative p70S6K1 KA does exist for use in rodent tissue (21), and a quantitative p70S6K1 KA has previously been used in cell culture studies (33). However, no study has described a fully quantitative KA methodology for the assessment of p70S6K1 activity in human skeletal muscle.

Therefore, the primary aim of this methodological study was to develop and validate a fully quantitative p70S6K1 KA methodology to assess p70S6K1 activity in human skeletal muscle in response to resistance exercise (RE) and protein feeding. Because muscle tissue availability is often a major limitation to routine analytical procedures, a secondary aim was to simultaneously assess AMPK activity and another regulator of mTOR (mechanistic target of rapamycin), protein kinase B (PKB), from the same muscle biopsy sample as p70S6K1. In this regard, we also aimed to validate a serial immunoprecipitation (IP) protocol to enable the dual assessment of p70S6K1 and PKB, from the same muscle homogenate. It is hoped that these methodological developments will enhance our capacity to accurately delineate the molecular mechanisms that regulate human skeletal muscle plasticity.

METHODS

Materials

Unless otherwise stated, all materials were from Fisher Scientific (Loughborough, UK). All antibodies, unless otherwise stated, were used at a concentration of 1:1,000, and were from New England Biolabs (Herts, UK). Selected primary antibodies were mTOR^{ser2448} (#2974), total mTOR (#2983), acetyl-CoA carboxylase (ACC)^{ser79} (#3661), total ACC (#3676), total glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (#2118), regulatory-associated protein of mTOR (Raptor)^{ser792} (#2083), total GRB10 (#3702), p70S6K1^{thr389} (#11759; Santa Cruz Biotechnology), total p70S6K1 (#2708), PKB^{thr308} (#2965), total PKB (#4691), proline-rich Akt/PKB substrate 40 kDa (PRAS40)^{thr246} (#2997) and total PRAS40 (#2691), 4EBP1$^{thr37/46}$ (#2855), and total 4EBP1 (#9644). Secondary horseradish peroxidase-conjugated antibody was purchased from ABCAM (#6721). Prepoured gels for WB were 4–20% Tris-Glyc Criterion gradient gels from BioRad (Herts, UK). AMPK α1- and α2-specific antibodies were produced by GL Biochem (Shanghai, China) against the following antigens: α1, CTSPPDSFLDDHHLTR; and α2, CMDDSAMHIPPGLKPH (38).

Tissue Culture Experiments

C_2C_{12} myoblasts were grown to confluence on T75 plates in growth media [(GM) 20% fetal bovine serum (FBS) (Dundee Cell Products, Dundee, UK), 1% penicillin/streptomycin (Invitrogen, Paisley, UK) in high-glucose DMEM (Invitrogen)]. Confluent myoblasts were then transferred to differentiation media [(DM) 2% donor horse serum (Dundee Cell Products), 1% penicillin/streptomycin (Invitrogen) in high-glucose DMEM (Invitrogen)]. Prior to the addition of inhibitors cells were serum- and amino acid-starved in PBS with 5 mM glucose (Invitrogen) for 3 h. Starved cells were then pretreated with an inhibitor [100 nM rapamycin (Sigma Aldrich), 10 μM LY294002 (Cell Signaling) or vehicle control (0.1% DMSO)] for 1 h prior to serum and amino acid stimulation by the addition of GM supplemented with or without inhibitors. After 30 min of stimulation cells were lysed on ice in 1 ml of radio immunoprecipitation assay (RIPA) buffer [50 mmol/l Tris·HCl pH 7.5, 50 mmol/l NaF, 500 mmol/l NaCl, 1 mmol/l sodium vanadate, 1 mmol/l EDTA, 1% (vol/vol) Triton X-100, 5 mmol/l sodium pyrophosphate, 0.27 mmol/l sucrose, and 0.1% (vol/vol) 2-mercaptoethanol and Complete protease inhibitor cocktail (Roche)] and then stored at −80°C. HEK293 cell lysates overexpressing either the α1 or the α2 subunit of AMPK were a gift from Professor Grahame Hardie (Division of Cell Signaling and Immunology, University of Dundee).

p70S6K1$^{-/-}$ tissues. All animal experiments on p70S6K1$^{-/-}$ and littermate controls (wild type; WT) were approved by and conducted in accordance with the Direction Départementale des Services Vétérinaires, Préfecture de Police, Paris, France (authorization 75–1313). Mice (p70S6K1$^{-/-}$) were generated as previously described (26a). The mice were housed in plastic cages and maintained at 22°C with a 12-h dark/12-h light cycle and had free access to food. Starved mice were WT; p70S6K1$^{-/-}$ mice had food withdrawn overnight and were then refed standard chow for 4 h. Animals were killed by cervical dislocation, and tibialis anterior muscles were rapidly dissected, blotted dry, and snap-frozen in liquid N_2.

Mouse ex vivo and in vivo insulin stimulations. All animal experiments were approved by and conducted in accordance with the Animal Care Program at the University of California, San Diego, for the ex vivo insulin stimulations; and the Animal Care Program at the University of California, Davis, for the in vivo insulin stimulations. Ex vivo insulin stimulations were carried out as follows: 6 male C57/Bl6 mice were fasted for 4 h and anesthetized (150 mg/kg nembutal) via ip injection. Paired extensor digitorum longus muscles were excised and incubated at 35°C for 30 min in oxygenated (95% O_2, 5% CO_2) flasks of Krebs-Henseleit buffer (KHB) containing 0.1% BSA, 2 mM sodium pyruvate, and 6 mM mannitol. One muscle per pair was incubated in KHB without insulin, and the contralateral muscle was incubated in KHB with insulin [60 μU/ml (0.36 nM); Humulin R, Eli Lilly]. After 50 min, muscles were blotted on ice-cold filter paper, trimmed, freeze-clamped, and then stored at −80°C (n = 6). In vivo insulin stimulations were carried out as follows: 2 female C57/Bl6 mice were fasted for 4 h and anesthetized with 2% isoflourane vaporized in 100% O_2. One mouse was ip injected with 100 mU/kg of insulin (Humulin R, Eli Lilly). After 30 min the muscles from the lower limb were dissected and snap-frozen in liquid N_2. The control mouse went through the same procedure except that it was injected with 0.9% saline.

Human Experimental Study

Participants. Six healthy, moderately trained men [mean ± SD: age, 23 ± 2 yr; body mass, 76 ± 5 kg; height, 179 ± 5 cm; unilateral 1 repetition maximum (1 RM) leg press, 128 ± 8 kg; 1 RM leg extension, 54 ± 3 kg] were recruited to participate in this study. All participants engaged in resistance training approximately two times per week and played team sports recreationally. Prior to the commencement of the experiment each participant provided written informed consent after all procedures and risks were fully explained in lay terms. Participants also were required to satisfy a routine physical activity readiness questionnaire. The study procedures were approved by the Research Institute for Sport and Exercise Sciences Ethics Committee, Liverpool John Moores University, and conformed to the standards as outlined in the most recent version of the Declaration of Helsinki.

Study design. Seven days after confirmation of unilateral 1 RM for leg press and leg extension, six healthy, moderately trained men reported to the laboratory at ~7:00 a.m. in a 10-h postabsorptive state. Each participant's height and body mass were recorded, after which they rested (~30 min) in a semisupine position on a bed, and a resting biopsy was obtained. Immediately after the biopsy participants were transported by wheelchair to the resistance-training laboratory where they performed a bout of unilateral RE. Immediately following the bout of unilateral RE, participants were required to consume 20 g of pure egg white powder in a 500-ml solution. Participants were then transported back to the resting laboratory and rested again in a

semisupine position during which additional muscle biopsies were obtained at 1 and 3 h post-RE.

Resistance exercise protocol. Testing (1 RM) was conducted as previously described (34). On the day of the experimental trial participants performed a bout of unilateral RE consisting of four sets of 10 repetitions at 70% 1 RM of leg press, followed by leg extension performed at the same intensity with their dominant limb. Recovery time between exercises and sets was 3 min and 2 min, respectively. Participants were provided with verbal cues to ensure correct exercise technique. Each repetition consisted of a 1-s concentric action, 0-s pause, then a 1-s eccentric action as previously reported (4).

Study controls. Participants were required to record dietary intake for 3 days prior to the initial single 1 RM testing session, and to repeat this pattern of consumption for the 3 days preceding the day of the experimental trial. For 3 days prior to both 1 RM testing and the experimental trial, participants also were asked to refrain from any form of vigorous exercise. These controls were implemented in an attempt to prevent any nutritional or exercise-induced changes in protein activity that might adversely affect the results of the study.

Skeletal muscle biopsies. Skeletal muscle biopsies were obtained on the exercising limb at pre-RE, 1 h post-RE, and 3 h post-RE using a Bard Monopty Disposable Core Biopsy Instrument (12 gauge × 10 cm length; Bard Biopsy Systems, Tempe, AZ). For each biopsy, the lateral portion of the vastus lateralis was cleaned before an incision into the skin and fascia was made under local anesthetic (MD92672; 0.5% marcaine without adrenaline). A sample of muscle (~30 mg) was extracted, rinsed with ice-cold saline, blotted dry, and any visible fat or connective tissue was removed. Muscle samples were then snap-frozen in liquid nitrogen and stored at −80°C for further analysis.

Muscle tissue processing. Approximately 30 mg of human skeletal muscle tissue (~5 mg of mouse skeletal muscle tissue) was homogenized by scissor mincing on ice in RIPA buffer [50 mmol/l Tris·HCl pH 7.5, 50 mmol/l NaF, 500 mmol/l NaCl, 1 mmol/l sodium vanadate, 1 mmol/l EDTA, 1% (vol/vol) Triton X-100, 5 mmol/l sodium pyrophosphate, 0.27 mmol/l sucrose, and 0.1% (vol/vol) 2-mercaptoethanol and Complete protease inhibitor cocktail (Roche)] followed by shaking at 1,000 rpm on a shaking platform for 60 min at 4°C. Debris was removed by centrifugation at 4°C for 15 min at 13,000 *g*. The supernatant was then removed, and protein concentration was determined using the bicinchoninic acid protein assay according to the manufacturer's instructions (Sigma Aldrich, UK).

Western blotting. For WB, 300 μg of supernatant was made up in Lamelli sample buffer, and 5–15 μg of total protein was loaded per well with the same amount of protein loaded in all wells for each gel, and run at 150 V for 1 h 15 min. Proteins were then transferred onto Whatman Immunobilon Nitrocellulose membranes (Fisher Scientific, Loughborough, UK) at 30 V overnight on ice. Membranes were blocked in 3% BSA-Tris-buffered saline (containing vol/vol 0.1% Tween 20) for 1 h at room temperature, followed by incubation in primary antibodies at 4°C overnight. Membranes underwent three 5-min washes in TBST followed by incubation in the appropriate secondary antibodies for 1 h at room temperature. Membranes were again washed three times for 5 min followed by incubation in enhanced chemiluninescence reagent (BioRad, Herts, UK). A BioRad ChemiDoc (Herts, UK) was used to visualize and quantify protein expression. All phospho proteins were normalized to the corresponding total proteins after stripping the phospho antibody for 30 min at 50°C in stripping buffer (65 mM Tris·HCl, 2% SDS vol/vol, 0.8% mercaptoethanol vol/vol) and reprobing with the primary antibody for the corresponding total protein. All phospho proteins were normalized to the expression of the corresponding total with the exception of phosphorylated Raptor^{ser792}, which was normalized to the expression of GAPDH.

[γ-^{32}P] ATP kinase assays. All KA were carried out by IP either for 2 h at 4°C or overnight at 4°C in homogenization buffer {AMPK [50 mM Tris·HCl pH 7.25, 150 mM NaCl, 50 mM NaF, 5 mM NaPPi, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml soybean trypsin inhibitor, 1% (vol/vol) Triton X-100] and p70S6K1/panPKB [50 mM Tris·HCl pH 7.5, 0.1 mM EGTA, 1 mM EDTA, 1% (vol/vol) Triton X-100, 50 mM NaF, 5 mM NaPPi, 0.27 M sucrose, 0.1% β-mercaptoethanol, 1 mM $Na_3(OV)_4$, and 1 Complete (Roche) protease inhibitor tablet per 10 ml]}. Protein G sepharose (2.5 μl per IP) was used to precipitate the immune complexes. Immune complexes were washed twice in assay-specific high-salt washes (homogenization buffers as above with 0.5 M NaCl added) followed by one wash in assay-specific assay buffer (see below). Prior to carrying out the activity assay the immune-bead-complex was suspended in a total of 10 μl of assay buffer for p70S6K1 and panPKB assays, and 20 μl of assay buffer for AMPK assays. All assays were carried out in a 50-μl reaction. Assays were started every 20 s by the addition of a hot assay mix, which consisted of assay buffer [PKB/p70S6K1 (50 mM Tris·HCl pH 7.4, 0.03% Brij35, and 0.1% β-mercaptoethanol), AMPK (50 mM HEPES pH 7.4, 1 mM DTT, and 0.02% Brij35)], ATP-$MgCl_2$ (100 μM ATP + 10 mM $MgCl_2$ for p70S6K1/panPKB, and 200 μM ATP + 50 μM $MgCl_2$ for AMPK), ^{32}γ-ATP [specific activities as follows; panAMPK (0.25×10^6 cpm/nmol), panPKB (0.5×10^6 cpm/nmol), p70S6K1 (1×10^6 cpm/nmol)], and finally synthetic peptide substrates [Crosstide for panPKB (GRPRTSSFAEG at 30 μM), S6tide for p70S6K1 (KRRRLASLR at 30 μM), and AMARA for AMPK (AMARRAASAAALARRR at 200 μM)]. Assays were stopped at 20-s intervals by spotting onto squares of p81 chromatography paper (Whatman; GE Healthcare, UK) and immersing in 75 mM phosphoric acid. Papers (p81) were washed three times for 5 min in 75 mM phosphoric acid and once in acetone. They were then dried and immersed in Gold Star LT Quanta scintillation fluid (Meridian Biotechnologies, Chesterfield, UK) and counted in a Packard 2200CA TriCarb scintillation counter (United Technologies). Assay results were quantified in $nmol \cdot min^{-1} \cdot mg^{-1}$ (U/mg). Blanks for background subtractions were carried out with immunoprecipitated kinases with no peptide included in the assay reaction. For the AMPK antibody validation assays the AMPK α1 antibody (5 μg) was used to immunoprecipitate AMPK α1 complexes from 100 μg of lysate in duplicate, whereas AMPK α2 antibody (5 μg) was used to immunoprecipitate AMPK α2 complexes from 100 μg of lysate. These lysates were from HEK cells overexpressing either AMPK α1 or AMPK α2, and were a kind gift from Prof. Grahame Hardie (University of Dundee). Assays were carried out for 15 min. For p70S6K1 antibody validation, 2 μg of p70S6K1 antibody was used to immunoprecipitate p70S6K1 from 250 μg of muscle lysate from WT starved/refed and p70S6K1$^{-/-}$ refed mice. Activities assays for panPKB and p70S6K1 were carried out on cell lysates by IP from 200 μg of cell lysate. The IP step was performed with 2 μg each of PKBα/β/γ antibodies (DSTT, Dundee University) or 2 μg of p70S6K1 antibody (H-9; Santa Cruz Biotechnology, Heidelberg, Germany), respectively. Antibodies were used with 2.5 μl of protein G sepharose per IP to immunoprecipitate for 2 h at 4°C. p70S6K1 and panPKB were assayed for 45 min and 20 min, respectively.

Time-dependent saturation assays. Three human skeletal muscle biopsy samples were pooled and homogenized. Homogenate was aliquoted to 2.4 mg for panPKB assays, 6 mg for p70S6K1 assays, and 0.6 mg for AMPK assays. Antibodies of PKBα/β/γ (72 μg each) were used to immunoprecipitate panPKB, 48 μg of p70S6K1 antibody was used to immunoprecipitate p70S6K1, and 60 μg each of AMPK α1 and α2 were used to immunoprecipitate panAMPK. Following IP, each of these immune complexes were aliquoted into 12 aliquots for activity assays; 9 of the aliquots were used for activity assays for the time course of 7.5, 15, and 30 min for AMPK; 15, 30, and 60 min for panPKB and p70S6K1. The three remaining aliquots were used for no-peptide controls to generate assay-specific blanks. Each assay represented an IP from 50 μg of lysate for panAMPK, 200 μg of lysate for panPKB, and 500 μg of lysate for p70S6K1.

For the serial IP validation, lower limb muscles from a 4-h fasted (Con) and an insulin-stimulated mouse [Ins (4-hr fasted + 100 mU insulin/kg for 30 min)] were homogenized and aliquoted into 6 × 200-μg aliquots each. IPs were set up to immunoprecipitate panPKB (3.2 μg of each PKB antibody) from three Con and three Ins aliquots, whereas the other aliquots had p70S6K1 immunoprecipitated (4 μg of p70S6K1 antibody) prior to immunoprecipitating with panPKB as before. Activity assays for panPKB were carried out as before following IP.

For p70S6K1/panPKB KA in human tissue, 500 μg of lysate was aliquoted, and p70S6K1 was immunoprecipitated with 4 μg of p70S6K1 and 2.5 μl of protein G sepharose (GE Healthcare) for 2 h at 4°C. The p70S6K1 KA was carried out for 45 min. Two hundred micrograms of the post-IP supernatant was then used for PKB IP. Two micrograms each of PKBα/β/γ antibodies (DSTT, Dundee University) were used with 2.5 μl of protein G sepharose to immunoprecipitate PKB at 4°C for 2 h. KA for panPKB were carried out as previously described for a 30-min assay. Following homogenization, 50 μg of lysate was aliquoted for AMPK activity assays. AMPK activity assays were carried out by IP with complexes in AMPK IP buffer (homogenization buffer as above). Immunoprecipitates were then washed, and AMPK activity was determined against AMARA peptide as previously described in a 20-min assay.

Statistical Analysis

Data were analyzed using GraphPad Prism Software version 6.0 (GraphPad, San Diego, CA). Differences in kinase signaling activity and phosphorylation (i.e., p70SK61^{thr389}, PKB^{thr308}, AMPK activity) were analyzed using a one-way ANOVA and, when appropriate, a Tukey's post hoc analysis. Post hoc sample size calculations were conducted using GPower 3.0.8 software on the basis of an estimated effect size of 0.53, a 1-β error probability of 0.8, and a significance level < 0.05. All data unless otherwise stated are presented as means ± SE, and $P < 0.05$ indicates statistical significance.

RESULTS

Antibody/Assay Validation

panAMPK. Total (or pan) AMPK activity is measured by immunoprecipitating both catalytic subunits of AMPK (AMPK α1 and AMPK α2). We commissioned our own AMPK α1 and AMPK α2 antibodies (GL Biochem, China) against the following antigens: α1, CTSPPDSFLDDHHLTR; and α2, CMDDSAMHIPPGLKPH (38). To confirm that our AMPK antibodies were specific for AMPK α1 and AMPK α2 and therefore capable of immunoprecipitating total AMPK when the antibodies are combined, we carried out a validation experiment (Fig. 1*A*). Cell lysates overexpressing either AMPK α1 or AMPK α2 underwent an IP with either the AMPK α1 or AMPK α2 antibody. AMPK α1 immunoprecipitated substantial activity from the AMPK α1 overexpressing cell lysates—approximately 10-fold more activity than the AMPK α2 antibody immunoprecipitated. The reverse experiment demonstrated a similar result, in that AMPK α2 immunoprecipitated approximately 10-fold more activity from the AMPK α2 overexpressing cell lysates than did the AMPK α1 antibody. These data demonstrate the specificity of our AMPK α1 and α2 antibodies. To further prove that these antibodies are immunoprecipitating active endogenous AMPK complexes, we carried out a positive control experiment by treating C_2C_{12} myotubes with 100 μM 2,4-dinitrophenol [a known AMPK activator (39)] for 30 min, and followed this with panAMPK activity assays. This treatment resulted in a an approximately fourfold increase in panAMPK activity (Fig. 1*B*), concurrent with a substantial increase in phosphorylation of AMPK at Thr172 (Fig. 1*B*, *inset*).

panPKB. Total (or pan) PKB activity can be assessed by utilizing recombinant glycogen synthase kinase-3 (GSK3) as a substrate and then running a standard WB with a phosphorylated GSK3 antibody to determine phosphate incorporation (3). However, this approach again relies upon densitometry analysis and makes comparisons across large sample sets difficult. Therefore, we utilized a filter binding assay that also allowed for quantitative scintillation counting. We used antibodies and a peptide substrate (6) that have been previously well characterized (6, 22). However, to confirm that we were detecting panPKB activity with the immune complex we carried out a positive control experiment (Fig. 1*C*). We serum-stimulated C_2C_{12} myotubes that had been treated with or without the PI3K inhibitor LY294002 (35). Serum stimulation led to an approximate fivefold increase in panPKB activity, whereas the inhibition of PI3K with LY294002 significantly inhibited panPKB activity. The changes in activity were reflected by changes in phosphorylation (Fig. 1*C*, *inset*).

p70S6K1. Traditionally, p70S6K1 activity assays are carried out with recombinant S6 as a substrate (21) wherein the radioactively labeled substrate is run on a gel before being exposed to radiography film. This assay is more difficult to accurately quantitate with large sample numbers due to the necessity to expose all samples to SDS-PAGE. Furthermore, this method still requires the use of densitometry analysis that can be subjective, leading to variable outputs depending upon the method of quantification (14). However, several laboratories have utilized a scintillation assay to quantitatively assess p70S6K1 activity (7, 33). To utilize a quantitative p70S6K1 activity assay that can be applied more easily to large sample numbers we employed a similar assay protocol with a peptide substrate analog of S6 corresponding to amino acids 230–238 on human 40S ribosomal protein S6 (KRRRLASLR) (12). This approach allowed for the use of filter paper capture of the labeled peptide that can then be quantitatively analyzed via scintillation counting. To confirm that this method did not alter the output of the assay we carried out a validation experiment in C_2C_{12} myoblasts (Fig. 1*D*). We used serum and amino acid stimulation as a positive control with rapamycin (specifically inhibits mTORC1 activity) as a control to confirm that serum and amino acid-induced activation of kinase activity was in fact p70S6K1-specific. We showed that serum and amino acid stimulation induces an approximately 10-fold increase in activity, whereas rapamycin completely blocks this activation (Fig. 1*D*) and the phosphorylation of p70S6K1^{thr389} (Fig. 1*D*, *inset*). These data demonstrate the mTORC1 dependence of the kinase activity we measured. To further validate that no other contaminating kinases could be contributing activity in our assay we also ran the assay from starved/refed WT mice and refed p70S6K1$^{-/-}$ mice. We found approximately 19-fold more activity in refed mouse muscle vs. starved mouse muscle, and we could not detect any activity in the p70S6K1$^{-/-}$ mice. These data highlight the specificity of our assay to p70S6K1. Prior to moving the assay into human tissue we first needed to define the amount of antibody required to saturate p70S6K1 in human skeletal muscle. This would ensure that all the p70S6K1 in the lysate was immunoprecipitated, thus improving consistency across sample sets. We used increasing amounts of

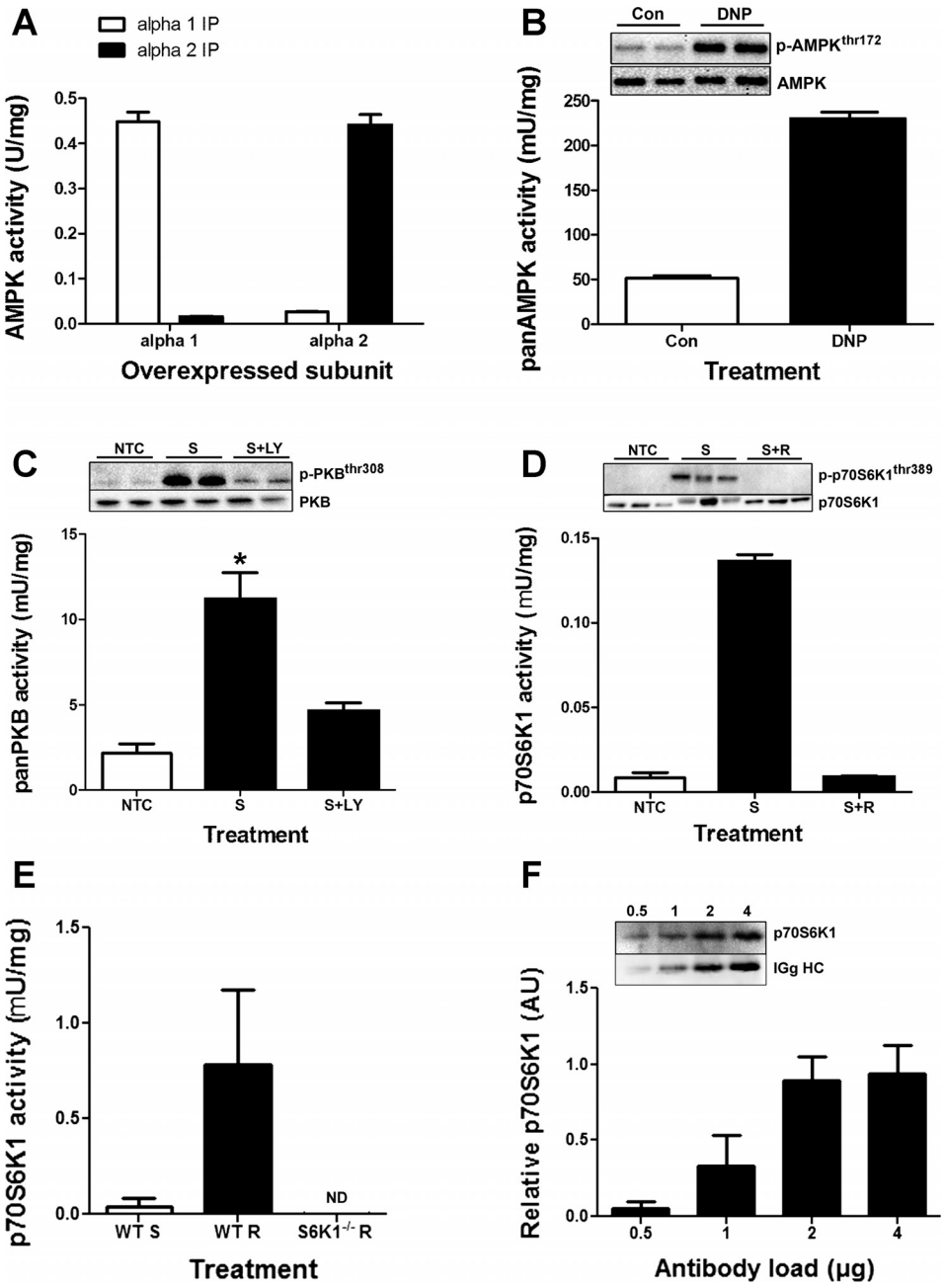

Fig. 1. Antibody and assay validation. *A*: AMPK α1 and AMPK α2 activity assays derived from immune complexes from cells overexpressing either AMPK α1 or AMPK α2. *B*: panAMPK activation in response to energy stress in C_2C_{12} myotubes. C_2C_{12} myotubes were serum-starved for 2 h prior to stimulation with 2,4-dinitrophenol (DNP) (100 μM) for 30 min ($n = 2$ in duplicate). *C*: panPKB activation by serum stimulation and inhibition by LY294002 (10 μM). C_2C_{12} myotubes were serum-starved for 3 h and preincubated with either vehicle (no treatment control; NTC) or LY294002 [10 μM (stimulated + LY; S+LY)] for 1 h ($n = 3$ in duplicate), then they were stimulated for 30 min in 20% fetal bovine serume (FBS) (S, stimulated). *Significantly different from NTC and S+LY. *D*: p70S6K1 activation by serum + amino acid stimulation and inhibition by rapamycin (100 nM). C_2C_{12} myotubes were serum and amino acid-starved for 3 h in PBS + 5 mM glucose and preincubated with either vehicle (NTC) or rapamycin [100 nM (stimulated + rapamycin; S+R) for 1 h ($n = 2$ in triplicate)], then they were stimulated for 30 min in 20% FBS + DMEM (stimulated; S). *E*: p70S6K1 activity in response to overnight starvation (WT-S) and 4 h of refeeding (WT-R), and 4 h of refeeding in p70S6K1$^{-/-}$ mice. *F*: antibody saturation curve for p70S6K1 antibody in 500 μg of pooled human lysate. *Insets* are representative Western blots. All data expressed as means ± SD. ND, nondetectable.

antibody to immunoprecipitate p70S6K1 from 500 μg of protein lysate extracted from pooled human muscle biopsy material from at least three volunteers. We found that despite increasing amounts of IgG-heavy chain (from the p70S6K1 antibody), the amount of p70S6K1 that was immunoprecipitated from 500 μg of protein lysate was saturated by 2 μg of antibody. We therefore used 4 μg of p70S6K1 antibody for every 500 μg of protein lysate to ensure that our antibody was always in excess.

Time-Dependent Saturation Curves

To select the most appropriate duration for each assay in human biopsy samples we carried out a time-dependant saturation curve for each assay from a pool of human muscle biopsies (Fig. 2). We carried out the AMPK assays for 7.5, 15, and 30 min, whereas PKB and p70S6K1 assays were carried out for 15, 30, and 60 min. These assays revealed linearity across the time course for each assay, indicating that assays carried out for anywhere between 7.5 and 30 min for panAMPK, and 15–60 min for panPKB and p70S6K1, would be within the linear range for time.

Validation of the Serial IP

To economize on tissue with human muscle samples, panPKB and p70S6K1 activity assays were carried out via serial IP with p70S6K1 immunoprecipitated first. To confirm that this serial IP process did not affect PKB activity we performed a validation of this procedure in response to maximal insulin

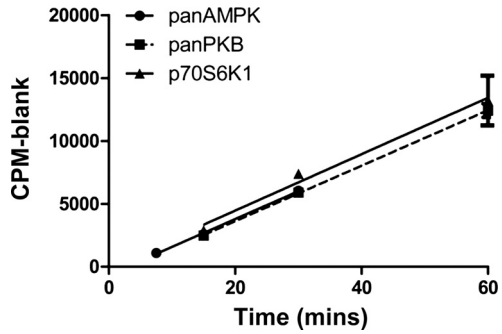

Fig. 2. Saturation time course of activity assays carried out from pooled human skeletal muscle protein lysate. R^2 values are as follows: AMPK = 0.969; panPKB = 0.982; and p70S6K1 = 0.856. All data are expressed as means ± SD.

stimulation (Fig. 3). Serially immunoprecipitating panPKB after p70S6K1 had no significant effect upon panPKB activity compared with a standard IP (Fig. 3).

Application of the [γ-^{32}P] ATP KA in a Physiological Context in Human Skeletal Muscle

We next determined whether we could measure the activity of panAMPK, panPKB, and p70S6K1 from the same human skeletal muscle sample following a well-defined anabolic stimulus in humans (23). In our study we identified a significant increase in p70S6K1 activity from pre-RE at 1 and 3 h post-RE ($P < 0.05$; Fig. 4*C*). However, there was no significant change in panPKB activity at any time point (Fig. 4*B*). Finally, panAMPK activity was significantly repressed ($P < 0.05$; Fig. 4*A*) at 3 h post-RE compared with pre-RE. To confirm that we were able to detect physiologically relevant changes in panPKB activity, we assessed the activation of panPKB in response to a physiologically relevant (0.36 nM) insulin stimulus in ex vivo mouse skeletal muscle (Fig. 4*B*, *inset*). Indeed, we detected a significant increase in panPKB activity in response to 50 min of insulin stimulation, thus confirming that this assay is capable of detecting changes in panPKB activity in a physiological context.

Western Blotting

Following the assessment of kinase activity as markers of anabolic responses in humans we next measured the phosphorylation of proteins that are typically used as surrogate readouts of anabolic signaling activity. The responses of kinases as determined by WB are shown in Fig. 5 (AMPK readouts), Fig. 6 (PKB readouts), and Fig. 7 (mTORC1 readouts). In response to RE and nutrition, there were no significant changes in phosphorylated mTOR^{ser2448} (Fig. 7*A*), ACC^{ser79} (Fig. 5*A*), Raptor^{ser792} (Fig. 5*B*), p70S6K1^{thr389} (Fig. 7*B*), PKB^{thr308} (Fig. 6*A*), and PRAS40^{thr246} (Fig. 6*B*). However, phosphorylated 4EBP1$^{thr37/46}$ was significantly elevated at 3 h post-RE com-

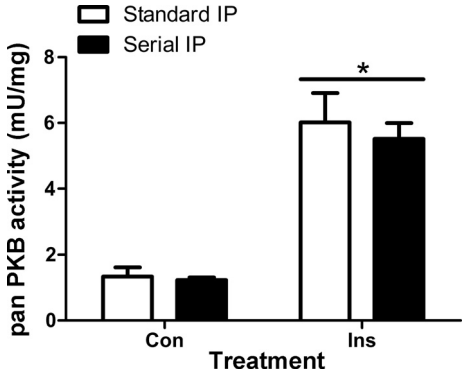

Fig. 3. Serial immunoprecipitation (IP) validation. IPs were set up to immunoprecipitate panPKB alone or p70S6K1 immunoprecipitated prior to immunoprecipitating with panPKB. *Significantly different from both control (Con) conditions. All data are expressed as means ± SE.

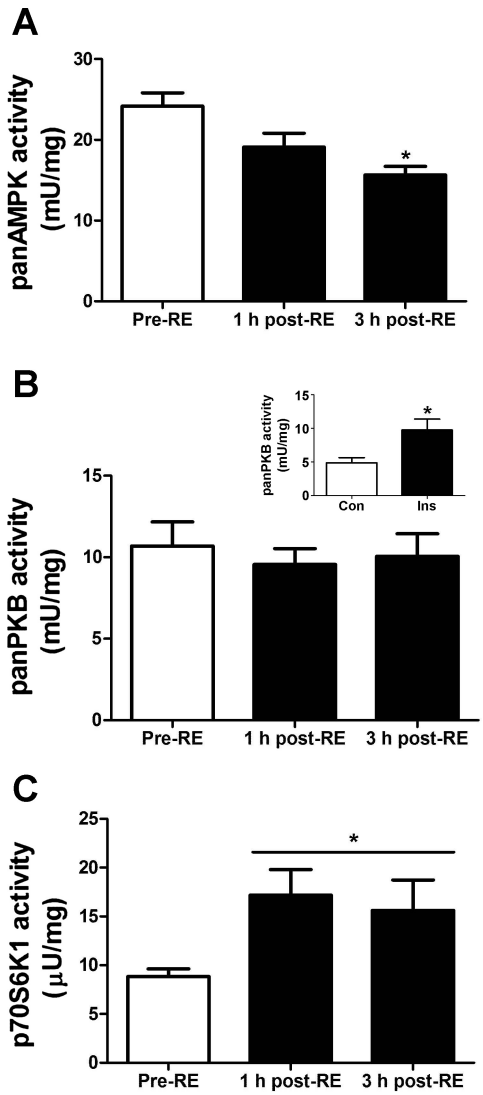

Fig. 4. Application of three kinase assays in human skeletal muscle in response to a physiological anabolic stimulus of resistance exercise combined with feeding 20 g of protein ($n = 6$). *A*: panAMPK activity was determined from 50 μg of lysate in a 20-min reaction against the synthetic substrate AMARA. *B*: panPKB activity serially immunoprecipitated after p70S6K1 IP. *Inset*: panPKB activity response to a physiological insulin stimulation of 0.36 nM for 50 min in ex vivo mouse skeletal muscle ($n = 6$). panPKB activity was determined from 200 μg of lysate in a 30-min reaction against the synthetic peptide substrate Crosstide. *C*: p70S6K1 activity was determined from 500 μg of lysate in a 45-min reaction against the synthetic peptide substrate S6K1tide. Pre-RE indicates biopsy taken prior to resistance exercise and feeding, 1 h post-RE indicates the biopsy taken 1 h following combined resistance exercise and feeding, 3 h post-RE indicates biopsy taken 3 h following combined resistance exercise and feeding. *Significantly different from Con or Pre-RE ($P < 0.05$). All data are expressed as means ± SE.

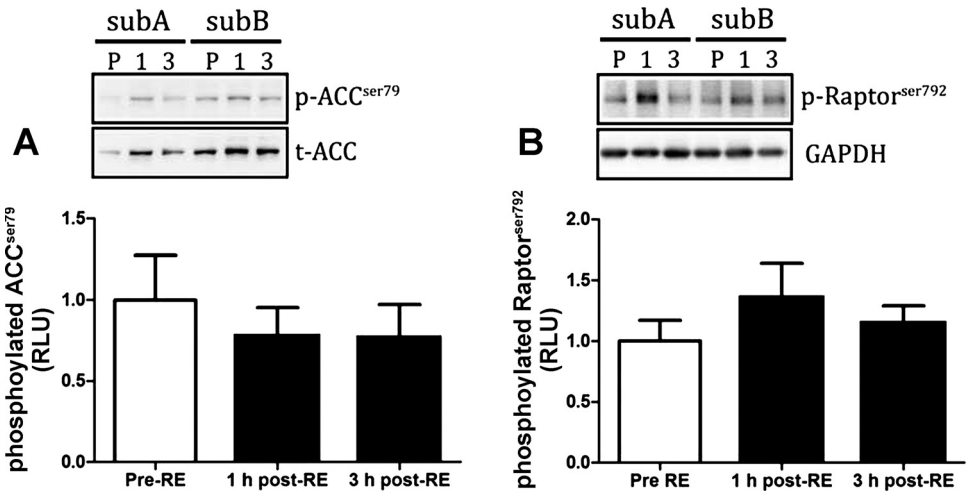

Fig. 5. Markers of AMPK activity in response to a physiological anabolic stimulus of resistance exercise combined with feeding 20 g of protein. Protein phosphorylation of ACC^{ser79} (*A*) and Raptor^{ser792} (*B*) obtained at pre-RE, and 1 and 3 h post-RE. All data are expressed as means ± SE.

pared with pre-RE ($P < 0.05$; Fig. 7*C*). Representative WB images appear as *insets* above each graph.

DISCUSSION

The main aim of the present methodological study was to develop and validate a quantitative p70S6K1 KA for use in human skeletal muscle biopsy samples. Second, we aimed to examine the physiological context of alterations in p70S6K1 activity by examining parallel alterations in PKB and AMPK activity in response to acute RE and protein feeding (23). For the first time we demonstrated that combined RE and protein feeding significantly increases p70S6K1 activity by approximately twofold, as determined by the KA with a similar, approximate twofold but nonsignificant change in p70S6K1^{thr389} phosphorylation. In addition, we observed a suppression of AMPK activity that was not apparent when assessing ACC^{ser79} phosphorylation, a known readout of AMPK activity (36). Furthermore, we demonstrate the capacity to achieve a dual measure of panPKB and p70S6K1 activity from the same sample via a serial IP protocol. This study therefore highlights the potential application of the KA described in this investigation to study the molecular signaling responses of skeletal muscle to RE and nutrition.

Although we observed a significant increase in p70S6K1 activity to RE and protein feeding, we detected no significant changes in the phosphorylation of p70S6K1^{thr389}. This finding is unexpected, given previous reports of significant, approximate twofold (5) and 12-fold (2) increases in phosphorylated p706K61^{thr389} to an acute bout of RE and protein feeding. Although the lack of detectable change in phosphorylated p70S6K1^{thr389} in our investigation appears to be related to low statistical power. Indeed, a post hoc sample size calculation from the present study determined that a participant sample of 12 would have been necessary to detect a statistically significant difference in phosphorylated p70S6K1^{thr389} between pre-RE and 1 h post-RE and protein ingestion. However, by utilizing the KA, we were able to detect a modest increase in p70S6K1 activity from pre-RE at 1 and 3 h post-RE and feeding. Thus these data highlight not only the precision but also the utility of this p70S6K1 KA to assess p70S6K1 activity to anabolic stimulation.

Due to issues associated with ethical practice and participant compliance in human research, muscle tissue availability is often a limiting factor. In this investigation we provided a validated, serial IP protocol for the dual assessment of p70S6K1 and panPKB activity from a single muscle homogenate. We showed that this serial IP protocol has no effect on panPKB activity, hence economizing on muscle tissue requirements. When applying this protocol to study panPKB responses of human skeletal muscle to RE and feeding, we

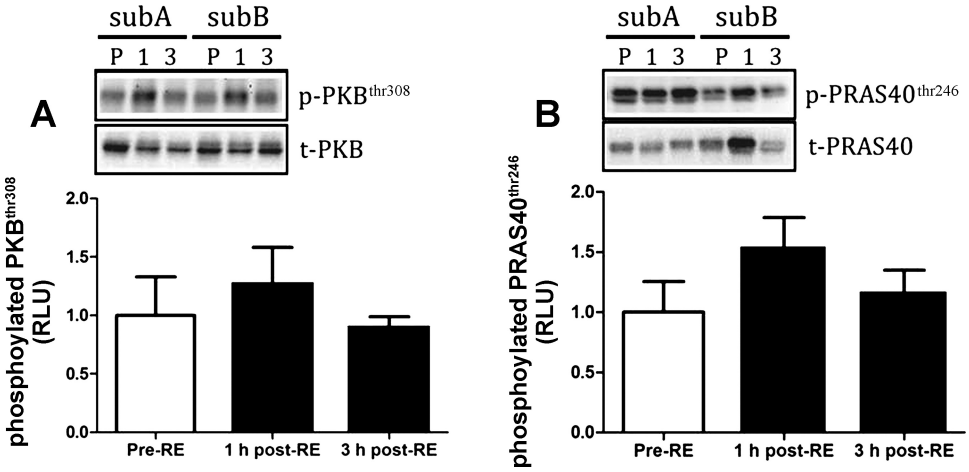

Fig. 6. Markers of panPKB activity in response to a physiological anabolic stimulus of resistance exercise combined with feeding 20 g of protein. Protein phosphorylation of PKB^{thr308} (*A*) and PRAS40^{thr246} (*B*). All data are expressed as means ± SE.

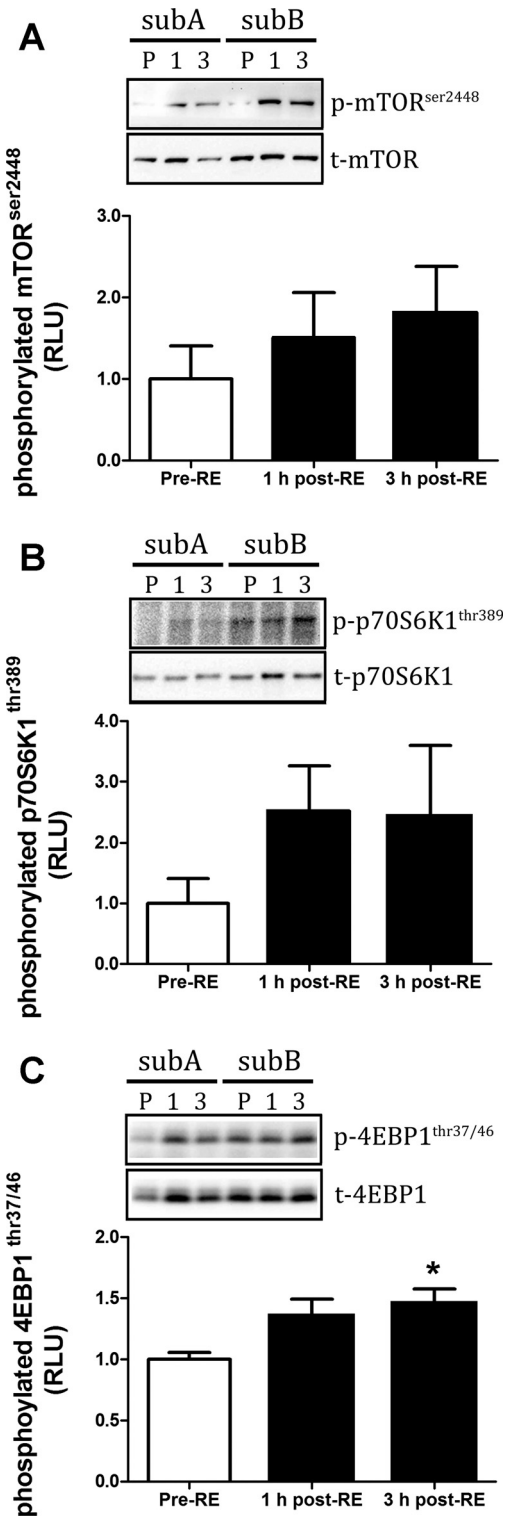

Fig. 7. Markers of mTORC1 activation in response to a physiological anabolic stimulus of resistance exercise combined with feeding 20 g of protein. mTOR^{ser2448} (*A*), p70S6K1^{thr389} (*B*), and 4EBP1$^{thr37/46}$ (*C*). *Significantly different from pre-RE ($P < 0.05$). All data are expressed as means ± SE.

showed no change in panPKB activity at any time point, a finding that corroborates previous reports (25, 28, 32). However, it is important to note that the panPKB KA described in this methodological investigation failed to provide information regarding PKB isoform-specific effects that could be useful in understanding cell growth and metabolism (30). The development of such a methodology is therefore a topic for future work.

The increase in p70S6K1 activity in our investigation was associated with a decrease in AMPK activity. These data are similar to findings showing that RE (1) or feeding (13) also repress AMPK^{thr172} phosphorylation, but these findings are incongruent with previous work that demonstrated RE increases AMPK α2 activity 1 h post-RE (10). However, in that study, RE was not followed by feeding, and one possibility is that the protein feeding in our study may have overridden RE-induced increases in AMPK activity, perhaps via restoration of the AMP:ATP ratio (15). Alternatively, it is known that p70S6K1 can inhibit AMPK via phosphorylation at Ser491 in mouse hypothalamic cells (8), although this latter hypothesis has yet to be observed in human skeletal muscle. A reduction in AMPK activity also is known to relieve inhibition on mTOR-p70S6K1 signaling (17), which could partially explain the sustained increase in p70S6K1 activation at 3 h post-RE and feeding in our investigation. Interestingly, the significant reduction in AMPK activity in our study was not mirrored by a reduction in ACC^{ser79} phosphorylation ($P = 0.70$). We chose to assess the phosphorylation of ACC^{ser79} as a readout of AMPK activity because phosphorylated AMPK^{thr172} possesses a low dynamic range that renders phosphorylated AMPK^{thr172} on this residue a poor surrogate of true AMPK activity (18). Therefore, the decrease in AMPK activity paralleled with a nonsignificant change in ACC^{ser79} phosphorylation further emphasizes the potential application of the KA to assess RE and nutrition-induced changes in signaling.

Both RE and protein ingestion are known to increase MPS via mTOR-p70S6K1 signaling (9, 11, 20). However, in response to RE and protein ingestion, we detected no significant change in the phosphorylation status of Raptor^{ser792}, PRAS40^{thr246}, or mTOR^{ser2448}. This finding was surprising because there was a significant increase in the phosphorylation of the mTOR substrate 4EBP1$^{thr37/46}$ at 3 h post-RE and protein feeding. Others have also shown no change in mTOR^{ser2448} phosphorylation to a 48-g whey bolus at both 1 and 3 h postfeeding despite increases in phosphorylated p70S6K1^{thr389} and 4EBP1$^{thr37/46}$ (3). Furthermore, it is also known that mutation of the ser2448 residue on mTOR fails to significantly affect p70S6K1 activity in cell-based systems (31). It therefore appears that mTOR^{ser2448} phosphorylation does not offer the most accurate readout of mTORC1 activity. Hence, studies that aim to infer changes in mTORC1 activity to anabolic stimulation using the WB technique may be better served by assessing changes in the phosphorylation of the mTOR substrates 4EBP1 and p70S6K1 rather than mTOR^{ser2448} phosphorylation itself.

In summary, this study provides a novel, fully quantitative methodology to assess p70S6K1-specific activity in human skeletal muscle. In addition, we provide a validated serial IP protocol that enables the dual assessment of PKB and p70S6K1 activity from a single skeletal muscle biopsy sample. Given that the number and yield of human muscle biopsies present

major limitations to routine analytical procedures, being able to assess the activity of three key kinases from the same muscle sample represents an attractive measurement strategy. However, it is important to acknowledge that this KA provides no information pertaining to the posttranslational modification of a protein such as phosphorylation. Indeed, it is important to recognize that phosphorylation is a critical regulatory step in protein function (19). Finally, it is important to acknowledge that the suitability of the KA is to provide a quantitative measurement of endogenous kinase activity, which would complement WB approaches to study protein PTM. In this manner, the KA would then allow a researcher to assess the physiological relevance of multisite PTM. Given the critical role of protein kinases in the regulation of MPS, the next logical step is therefore to combine the KA, WB, and direct measures of MPS to provide a more in-depth insight into changes in skeletal muscle signaling in response to perturbations such as age, exercise, and disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: C.M., B.D., G.L.C., D.P.M.M., I.T.C., A.P., J.P.M., and D.L.H. conception and design of research; C.M., A.T.W., C.T., I.T.C., S.S., J.P.M., and D.L.H. performed experiments; C.M., A.T.W., B.D., G.L.C., D.P.M.M., A.P., S.S., J.P.M., and D.L.H. analyzed data; C.M., A.T.W., A.P., J.P.M., and D.L.H. interpreted results of experiments; C.M. and D.L.H. prepared figures; C.M. and D.L.H. drafted manuscript; C.M., A.T.W., C.T., B.D., G.L.C., D.P.M.M., A.P., S.S., J.P.M., and D.L.H. edited and revised manuscript; C.M., A.T.W., C.T., B.D., G.L.C., D.P.M.M., I.T.C., A.P., S.S., J.P.M., and D.L.H. approved final version of manuscript.

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The Journal of Physiology

JOURNAL CLUB

Pattern of protein ingestion to maximise muscle protein synthesis after resistance exercise

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The maintenance of skeletal muscle mass is dependent upon the temporal and coordinated interaction between muscle/myofibrillar protein synthesis (MPS) and muscle protein breakdown (MPB). Resistance exercise (RE) alone elevates MPS and, to a lesser extent, MPB such that net muscle protein balance (NPB) remains negative. However, when RE is coupled with protein ingestion there is an accumulative effect on MPS resulting in a positive NPB (Phillips *et al.* 2005). Thus, repeated bouts of RE coupled with protein feeding is a viable strategy to maximise skeletal muscle hypertrophy and strength.

The impact of protein feeding on RE-induced increases in MPS has received much attention. One study has demonstrated that in young healthy males ∼20 g of high-quality protein is sufficient to maximise RE-induced rates of MPS over 4 h post-exercise (Moore *et al.* 2009). However, the interplay between the timing and quantity of protein consumed and subsequent anabolic responses throughout the course of a whole day is still poorly understood. In particular, there is a lack of data examining how the pattern of post-RE protein ingestion influences MPS later in the recovery phase (i.e. 4–12 h). A recent article published in *The Journal of Physiology* attempts to address this knowledge gap and in doing so provides valuable insights into how post-RE protein feeding strategies might be manipulated to optimise muscle anabolism. In an elegantly designed study, Areta *et al.* (2013) examined three groups of eight healthy, trained males. Participants performed a bout of bilateral leg extension RE followed by the consumption of 80 g of whey protein over 12 h of recovery ingested as either 8 × 10 g every 1.5 h, 4 × 20 g every 3 h or 2 × 40 g every 6 h. A stable isotope infusion was coupled with frequent skeletal muscle biopsy sampling to determine rates of MPS for 12 h post-RE. The data demonstrate that although all feeding strategies elevated MPS during the 12 h recovery period, consuming 20 g of whey protein every 3 h was the superior strategy for stimulating MPS rates. The authors concluded that these findings have the potential to maximise outcomes of resistance training designed to elicit a maximal hypertrophic response.

The data of Areta *et al.* show that manipulating the pattern of protein ingestion following RE can have a significant impact on the subsequent muscle anabolic response. The divergent feeding strategies of Areta *et al.* were used to mimic possible patterns of protein intake commonly observed in resistance-trained athletes. That is, 8 × 10 g every 1.5 h represents a 'grazing' approach, whereas 2 × 40 g every 6 h relates to the 'three square meals per day' approach. Yet, both of these strategies were inferior for stimulating MPS over 12 h of post-RE recovery compared with 4 × 20 g ingested every 3 h. However, it is important to note that this response was characterised when protein was ingested alone, and as the authors acknowledge, this finding cannot be evaluated in the context of a mixed meal. Indeed, it is commonplace to consume protein in the form of a mixed-macronutrient meal. Therefore, it is reasonable to postulate that macronutrient co-ingestion could alter intestinal transit, thus influencing amino acid absorption kinetics (Deutz *et al.* 1995) and perhaps MPS. Moreover, this study used high-quality whey protein and it remains to be seen if a similar pattern of MPS post-RE would be observed using the same feeding strategies with a slow-release protein such as casein. Such information may be valuable to individuals who choose not to (or are unable to) ingest high-quality protein in supplemental form following exercise, but instead consume whole-food protein sources.

Areta *et al.* should be highly commended for underlining the importance of not only the quantity, but particularly the pattern of post-RE protein ingestion to maximise the rate of MPS over 12 h. However, as a note of caution, their findings are limited to a healthy young male population. In this regard, recent evidence demonstrates that the elderly require more protein (40 to > 20 g) to elicit optimal increases in RE-induced rates of MPS than the young (Yang *et al.* 2012). It is therefore reasonable to consider whether the temporal influence of post-RE protein feeding on elderly muscle could be different compared to that of young. In this regard, the next logical step is to apply the model of Areta *et al.* in elderly and other populations, in whom maintenance of muscle mass is a critical determinant of longevity and quality of life. Yet, it should be acknowledged that Areta *et al.* afford data pertaining to only 12 h of recovery from RE. Hence, whether the acute responses of MPS to RE and protein feeding translate into a long-term functional response remains unknown.

The findings of Areta *et al.* will no doubt also grasp the attention of coaches and athletes alike. As such, some may cite the use of a bilateral exercise stimulus and absence of participants with large amounts of lean mass (>75 kg) as issues that preclude full applicability in a 'real-world' setting. To date, it is unclear whether exercising a greater volume of muscle mass is limiting for MPS in response to a given protein dose. Therefore, individuals with greater muscle mass or those engaged in whole-body RE training sessions may require ingestion of a greater protein dose to stimulate MPS maximally. With regard to the notion of applicability to the 'real-world' setting, it also may be significant that the participants entered the experimental trial in the fasted state. As a result the authors are unable to identify whether a pre-exercise meal would influence the MPS response to RE and various feeding strategies. This point becomes more relevant when considering the impact of insulin on MPB with regard to the true *growth* response and therefore the long-term applicability of the findings. Future studies assessing MPS and MPB in both the clinical and the athletic setting following RE and feeding are now required.

The study by Areta *et al.* also reveals novel nutrient–exercise interactions in cellular signalling. Phosphorylated mTOR^{Ser2448} was ∼2- to ∼6-fold above resting values throughout the 12 h recovery period independent of protein feeding strategy. Phosphorylation of p70S6K^{Thr389} was also increased above baseline, again in all feeding

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strategies. However, there was discordance between the degree of p70S6K^{Thr389} phosphorylation and the MPS response. In fact, the magnitude of phosphorylated p70S6K^{Thr389} displayed a 2×40 g to $> 4 \times 20$ g to $> 8 \times 10$ g pattern at 1 and 7 h post-RE. This finding is surprising given that phosphorylated p70S6K^{Thr389} is a key player in protein synthesis yet it was the 4×20 g strategy that induced the most favourable influence on MPS but median impact on phosphorylated p70S6K^{Thr389}. However, it is important to recognise that the timing of the biopsies at 1 and 7 h coincided with a greater volume of protein consumed prior to those biopsies for the 2×40 g condition, which may explain the discordance between p70S6K^{Thr389} signalling and MPS.

The common method employed to assay protein phosphorylation, a proxy of activity, in an exercise science setting, and in the present investigation, is Western blotting (WB). In contrast to the quantitative and reproducible techniques used to measure MPS, WB is a semi-quantitative method. Additionally, phosphorylated p70S6K^{Thr389} is recognised as a key controller of ribosomal biogenesis. So although the phosphorylation of p70S6K^{Thr389} post-RE does not correspond to the greatest acute MPS response it may in fact be leading to greater levels of ribosomal transcription. Interestingly, phosphorylation of p70S6K following RE often occurs in the nucleus, where ribosomal biogenesis commences. A caveat of the field is that no study has employed cellular fractionation techniques to reveal whether different RE and feedings strategies alter the ratio of nuclear to cytoplasmic phosphorylated p70S6K in human skeletal muscle. Hence, the lack of concordance between the MPS and signalling response in this and numerous other works emphasises the need for the development of new measures regarding readouts of ribosomal biogenesis in addition to fully quantitative methods to ascertain signalling activity following RE and nutrition.

To conclude, the study by Areta *et al.* contributes novel data to the body of literature highlighting the importance of the timing and quantity of protein consumed post-RE for muscle anabolism. By mimicking the habitual feeding strategies of many athletes engaged in resistance training, the authors move closer to bridging the gap between science and the applied setting. Future work that identifies the impact of different macronutrients consumed in combination, i.e. fat, carbohydrate, protein and fibre, on MPS in both elderly and young is warranted. Furthermore, there is growing interest in whether having greater amounts of muscle mass, or indeed exercising muscle mass involved in trainin impact RE-induced rates of MPS. Thus, future studies that examine the MPS response in individuals with large muscle mass, performing real-world RE, may provide informative data for clinical and athletic practice.

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