Molecular Regulation of Muscle Protein Synthesis at Rest and Following Endurance Exercise

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Science
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Abstract

The purpose of this study was to investigate whether protein consumption after endurance exercise modulates postprandial fraction-specific muscle protein synthesis. In crossover trials, eight trained men received a primed continuous infusion of L-[ring-2H5]phenylalanine to measure changes in myofibrillar (MyoPS) and mitochondrial (MitoPS) protein synthesis in response to 20g of egg protein with 50 g carbohydrates at rest and after 1 h of treadmill running at 70%VO2peak. Muscle biopsies were obtained while fasted and at 0, 1 and 5h after beverage ingestion to determine Myo/MitoPS (LC/MS/MS) and intracellular signaling (Western blotting/immunofluorescence). We demonstrate that MitoPS is uninfluenced by feeding and exercise whereas MyoPS is primarily nutrient sensitive over a 5h postprandial period at rest and after endurance exercise, which may be underpinned in part by enhanced mRNA translational capacity mediated through an increase in 4E-BP1 phosphorylation and secondary to an increase in mTOR/Rheb colocalization and reciprocal decrease in TSC2/Rheb colocalization.
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List of Abbreviations

4E-BP1 - 4E-binding protein 1
ATP - Adenosine triphosphate
EAA - Essential amino acids
COX - cytochrome oxidase
eEF2 - eukaryotic elongation factor 2
eIF4E, eIF4F, eIF4G - eukaryotic translation initiation factor 4E, F and G
Erk1/2 - extracellular signal regulated kinases 1/2
GAP - GTPase activating protein
GDP – Guanosine diphosphate
GTP - Guanosine triphosphate
GTPases - Guanosine triphosphatases
IGF-1 - Insulin-like growth factor 1
MAPK - Mitogen activated protein kinase
MitoPS - Mitochondrial protein synthesis
MPB – Muscle protein breakdown
MPS - Muscle protein synthesis
mTOR - mechanistic target of rapamycin
mTORC1 and mTORC2 – mechanistic target of rapamycin complex 1 and 2
MyoPS - Myofibrillar protein synthesis
NB – Net protein balance
PGC-1α - Peroxisome proliferator-activated receptor-γ coactivator
Rheb - Ras homolog enriched in brain
rpS6 - Ribosomal protein S6
S6K1 - Ribosomal protein S6 kinase 1
TBST - Tris-buffered saline with 0.1% Tween 20
TSC1 and TSC2 - Tuberous sclerosis proteins 1 and 2
WGA - Wheat germ agglutinin
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Chapter 1
Review of Literature

1.1 Introduction

The following overview will discuss the current literature examining the nutrient and exercise regulation of skeletal muscle protein turnover, primarily through examining changes in muscle protein synthesis (MPS). To further the understanding of skeletal muscle plasticity, emphasis on the effects of feeding and exercise on fraction-specific muscle proteins, such as the force-generating myofibrillar and energy-producing mitochondrial proteins, will be discussed (Section 1.2). To provide mechanistic insight on skeletal muscle plasticity (i.e. the ability for muscle to change in muscle size and/or function), an explanation of the molecular mechanisms that regulate fraction-specific MPS will be reviewed (Section 1.3) and traditional vs. novel methods to assess their regulatory pathways (Section 1.4) will be discussed. To conclude, the proposed study will be briefly introduced (Section 1.5). Although some parallels may be drawn between other exercise modalities (e.g. resistance exercise), a focus on endurance exercise-induced regulation of MPS will be emphasized throughout the review.
1.2 Muscle Protein Turnover

1.2.1 Nutrient regulation of muscle protein synthesis

Skeletal muscle compromises 40% of human body weight and besides its obvious function towards the musculoskeletal system to generate movement and force, acts as a major site for macronutrient metabolism. Skeletal muscle is a highly plastic tissue as evidenced by the ability of contractile activity (i.e. exercise) to induce significant acute remodeling that can ultimately translate into chronic changes in muscle size, shape, and/or activity. This remodeling occurs through an increase in protein turnover, which is the simultaneous synthesis (i.e. making of new proteins with appropriate amino acid building blocks) and breakdown of old and/or damaged proteins into their constituent amino acids (Figure 1). Muscle mass is altered through the regulation of net protein balance (NB), which is the algebraic difference between MPS and muscle protein breakdown (MPB). As highlighted by several-fold increases in MPS with relatively little change in MPB, the feeding-induced stimulation of MPS is the highly regulated variable in the non-diseased state (Biolo et al., 1997, Rennie et al., 2004, Greenhaff et al., 2008). For this reason, most studies examining the acute effects of skeletal muscle anabolism (when MPS exceeds MPB) and/or remodeling (repairing old muscle proteins via MPS) measure MPS.

Feeding dietary protein and/or crystalline amino acids stimulates mixed MPS, an event that is driven by the essential amino acid (EAA) content of the nutrition (Tipton et al., 1999a, Tipton et al., 1999b, Volpi et al., 2003). It has been suggested that the EAA-induced stimulation of MPS is dependent on extracellular amino acid availability (Bohe et al., 2003) and is generally uninfluenced by systemic hyperinsulinemia provided there is a normal basal concentration of the latter (Greenhaff et al., 2008, Phillips, 2008, Staples et al., 2011, Trommelen et al., 2015). Throughout the day, muscle protein turnover is in a continuous state of metabolic flux, such that, in the fasted state, muscle NB is negative (Phillips et al., 1997) but becomes positive (primarily due to an amino acid-induced stimulation of MPS) with the ingestion of a protein-containing meal (Biolo et al., 1997). Skeletal muscle has a remarkable capacity to adapt to various external stimuli; however, skeletal muscle is not a homogeneous tissue. As such, the traditional measure of mixed MPS (i.e. the average response of all muscle proteins and specific fractions) may not adequately
capture the protein synthetic response of a specific muscle protein fraction to a given physiological stimulus (Moore et al., 2009, Kim et al., 2005). Therefore, understanding the effects of feeding on different muscle protein fractions, such as the force generating myofibrillar and energy producing mitochondrial proteins, is vital to advance our understanding of this tissue’s incredible plasticity.

**Figure 1.** Changes in muscle protein synthesis (MPS) and muscle protein breakdown (MPB) in response to dietary protein or amino acids. Figure adapted from (Burd et al., 2009)

1.2.1.1 Nutrient regulation of myofibrillar protein synthesis (MyoPS)

Constituting ~65% of the volume of the muscle fiber, the contractile myofibrillar proteins (e.g. actin, myosin, sarcomeric structural proteins, etc.) represent the major protein fraction in skeletal muscle. The continuous turnover of myofibrillar proteins ensures that old and/or damaged proteins are broken down primarily through the ubiquitin proteasome pathway into their constituent amino acids and made available for: i) the resynthesis of new myofibrillar and/or other muscle proteins; ii) the exportation from the muscle to provide substrates for the synthesis of other bodily proteins, and/or; iii) the deamination or transamination to provide substrates for oxidative metabolism. As skeletal muscle is the main “storage reservoir” for amino acids from dietary protein (Rennie et al., 2004), understanding how feeding regulates myofibrillar protein synthesis (MyoPS) is critical to maintain and/or enhance human muscle health and performance.

Numerous studies have examined the effect of dietary protein-induced stimulation of mixed MPS (Volpi et al., 2003, Moore et al., 2009, Koopman et al., 2006, Koopman et al., 2005, Tang et al., 2008). A limitation of quantifying direct rates of mixed MPS results in a mixed average of all muscle proteins and does not provide insight into the remodeling of fraction-specific muscle
proteins (i.e. myofibrillar, mitochondrial, sarcoplasmic, collagen, ribosomes etc). For example, the force generating myofibrillar, the structural collagen, and the remaining organelles (e.g. energy producing mitochondria, catabolic lysosomes, etc.) contained within the sarcoplasm all have different nutrient sensitivities, turnover rates and expression levels in skeletal muscle (Mittendorfer et al., 2005). In light of this, research has evolved to examine the differential regulation of fraction-specific MPS. In response to feeding 25g of dietary protein at rest, MyoPS and cytoplasmic (i.e. mitochondrial proteins, ribosomes, lysosomes, etc.) protein synthesis are elevated (Moore et al., 2009). Furthermore, ingested protein dose-response studies have demonstrated MyoPS plateaus after the ingestion of 20g (~0.25g/kg) of high quality (i.e. EAA-enriched) dietary protein at rest in young men (Cuthbertson et al., 2005, Moore et al., 2015). Therefore, to replenish fasted state losses of muscle NB at rest, ~0.25g/kg per bodyweight of dietary protein is required to enhance muscle protein remodeling and maximize MyoPS (Moore et al., 2015).

1.2.1.1 Nutrient regulation of mitochondrial protein synthesis (MitoPS)

Mitochondria, which represent ~10% of total skeletal muscle proteins, are commonly referred to as the energy organelles or the “powerhouse” of the cell as they represent the major site of adenosine triphosphate (ATP) production that cells require to function. When amino acids are intravenously infused at rest, there is an apparent stimulation of mitochondrial protein synthesis (MitoPS) (Bohe et al., 2003, Bohe et al., 2001), suggesting mitochondria may be responsive to nutrients. However, the physiological significance of these otherwise seminal studies is confounded by a limited number of observations (i.e. n=2) for MitoPS (Bohe et al., 2001), a group design (Bohe et al., 2003), and providing amino acids in a non-physiological primed, constant intravenous infusion (Bohe et al., 2001; Bohe et al., 2003); as such, based on our limited research into the nutrient sensitivity of this protein fraction, the robustness of the amino acid-induced stimulation of MitoPS is uncertain. Moreover, unlike that of MyoPS, we currently have relatively limited knowledge on the nutrient sensitivity of skeletal muscle MitoPS in response to bolus meal-protein intakes. Finally, the relative nutrient sensitivity of both MyoPS and MitoPS in response to physiologically relevant nutrient ingestion (i.e. bolus protein ingestion) is unknown. Although the plasticity towards MitoPS is understudied, advancements in techniques to optimize the measurement and reliability of MitoPS have been developed (Burd et al., 2015). As such, the direct
measurement of MitoPS, a kinetic measure of mitochondrial remodeling (Miller and Hamilton, 2012), following nutrient ingestion and contractile activity in healthy and/or diseased states may be used to provide insight into chronic changes towards skeletal muscle function and the mechanistic underpinnings of muscle plasticity.

1.2.2 Exercise regulation of MPS

Chronic resistance training results in increased muscle strength and muscle hypertrophy (e.g. greater muscle cross sectional area), the latter of which is an adaptive response that is ultimately driven by greater myofibrillar proteins (Phillips, 2014). On the contrary, chronic endurance training is characterized by adaptations that increase fatigue resistance, a result that would ultimately be underpinned in part by an enhanced oxidative capacity associated with mitochondrial biogenesis (Hawley, 2002). Regardless of the mode of exercise performed along the strength/endurance continuum, contractile activity induces a variety of metabolic and phenotypic responses in skeletal muscle that serve to minimize cellular disturbances during subsequent training sessions (Hawley et al., 2011); these coordinated responses are ultimately underpinned by changes in skeletal muscle protein turnover (Aguirre et al., 2013). For example, numerous studies have documented increases in both MPS and MPB in response to resistance (Phillips et al., 1997, Biolo et al., 1995, Phillips et al., 1999) and endurance exercise (Pikosky et al., 2006, Carraro et al., 1990, Rodriguez et al., 2007). The skeletal muscle adaptive response with respect to endurance exercise is associated with increases in the activities of key enzymes of the mitochondrial electron transport chain and a concomitant increases in mitochondrial biogenesis and/or protein concentration (Hawley, 2002, Hoppeler and Fluck, 2003). Although endurance exercise increases postprandial rates of MPS (Howarth et al., 2009, Bolster et al., 2005), this response may primarily be to increase muscle protein turnover rather than for the net expansion of myofibrillar protein pools; as such, this greater protein turnover may primarily function to repair and remodel skeletal muscle proteins, including those which may have been damaged during exercise (Moore and Stellingwerff, 2012). Hence, endurance exercise may not be the utmost strategy to induce muscle hypertrophy (Hawley et al., 2011) but can still be a potent stimulus to enhance skeletal muscle remodeling and protein synthesis.
The ingestion of 10g of dietary protein following 1h of cycling at 60% of VO2 max results in a 6- and 4-fold increase in indirect rates of MPS when compared to water and protein-free nutrition, respectively (Levenhagen et al., 2002). Notably, Levenhagen et al. (2002) measured MPS using arteriovenous balance, which generally shows greater changes than tracer incorporation techniques (Patterson et al., 1997). Nonetheless, the ingestion of as little as 10g of dietary protein following endurance exercise can shift muscle NB from negative to positive, which occurs primarily through the stimulation of MPS (Levenhagen et al., 2002). Furthermore, the timing of nutrient ingestion following endurance exercise may play a role in protein dynamics. For example, when compared to the 3h delay of nutrient ingestion (10g protein, 8g carbohydrate, 3g lipid), immediate nutrient ingestion following 1h of cycling at 60% of VO2 max results in a 3 fold increase in MPS, shifting leg NB from negative to positive (Levenhagen et al., 2001). Collectively, these data suggest that dietary protein or amino acid ingestion and timing play critical roles towards remodeling skeletal muscle immediately following endurance exercise (Moore et al., 2014). However, these collective studies measured mixed MPS; as such we currently have limited knowledge on the effect of endurance exercise on regulating rates of postprandial fraction-specific MPS (i.e. MyoPS, MitoPS). As the skeletal muscle adaptive response with exercise is likely to be attributed to the cumulative effect of repeated bouts of exercise (Mitchell et al., 2015), providing acute insight into the regulation of postprandial fraction-specific MPS will provide clues to such adaptations occurring following various training sessions. This information is critical towards designing optimal nutrition and/or exercise interventions aimed at optimizing muscle size, shape, and/or activity in a variety of populations (e.g. young/old) and applications (e.g. training adaptations or immobilization countermeasure).

1.2.2.1 Exercise regulation of MyoPS

When compared to rest, an acute bout of resistance exercise has been shown to sustain MyoPS for at least 5h after the ingestion of a bolus of dietary protein (Moore et al., 2009). In contrast, rates of postprandial sarcoplasmic PS are similar at rest and after resistance exercise in response to protein ingestion (Moore et al., 2009). Subsequently, this divergent response reinforces the importance of measuring fraction-specific MPS when investigating feeding and exercise interventions.
Similar to that of rested muscle, 20g or ~0.25g/kg (body weight) of high quality protein maximizes MyoPS following resistance exercise in young men (Witard et al., 2014). Although the ingested dose response of MyoPS following endurance exercise in young men has yet to be characterized, 20g or ~0.25g/kg of high quality protein is thought to maximize MyoPS (Breen et al., 2011) and potentially stimulate adaptations that promote an endurance phenotype (Moore et al., 2014). Moreover, nutrient ingestion has been shown to increase MyoPS following resistance exercise (West et al., 2011, Churchward-Venne et al., 2012) repeated sprints (Coffey et al., 2011), endurance exercise (Breen et al., 2011, Rowlands et al., 2015) and concurrent training (Camera et al., 2015). Therefore, given the generally consistent response in MyoPS with protein ingestion across exercise modalities, it is likely that the dose of protein to maximally stimulate MyoPS after endurance exercise would be within a 20-25g dose (Moore et al., 2014). Ultimately, with contractile activity being such a potent stimulator of MPS, it is not surprising that the largest constituent of skeletal muscle (i.e. myofibrillar) is highly responsive towards any cellular disturbance.

1.2.2.2 Exercise regulation of MitoPS

Skeletal muscle mitochondria are thought to exist in 2 distinct subpopulations, the subsarcolemmal and intermyofibrillar mitochondria (Krieger et al., 1980). These subpopulations have been shown to respond differently with respect to morphological changes and content in response to anabolic/catabolic stimuli (Chomentowski et al., 2011). In humans, the effect of endurance exercise on mitochondrial biogenesis has been suggested to be similar between the subsarcolemmal and intermyofibrillar mitochondria (Hoppeler et al., 1985). Moreover, methodological difficulties often preclude the ability to isolate sufficient amounts of subsarcolemmal and intermyofibrillar mitochondria for enrichment analysis in human muscle (Burd et al., 2015, Wilkinson et al., 2008); as such both skeletal muscle mitochondrial protein fractions are combined for enrichment analysis (Burd et al., 2015). Hence, the studies presented examining MitoPS are “mixed” measures combining both mitochondrial sub-fractions.

The muscular phenotypes induced by chronic resistance training (e.g. myofibrillar protein accretion) and endurance training (e.g. mitochondrial expansion) ultimately result from differential
stimulation of MyoPS and MitoPS (Phillips, 2014, Rodriguez et al., 2007). However, the exercise-induced stimulation of MitoPS is equivocal. For example, MitoPS has been reported to be minimally stimulated during “early” post-exercise recovery periods (0.5-4.5 h) in the fasted state whereas there is a robust increase 24-28h after an acute bout of high (~70%VO2max) but not low-intensity (~35%VO2max) endurance exercise (Di Donato et al., 2014). These findings are in apparent contrast to that of Wilkinson et al. (2008) who reported that endurance exercise stimulates MitoPS, but not MyoPS, in both the untrained and trained states during the early 4-h post-exercise recovery period (Wilkinson et al., 2008) (Figure 2). Notably, subjects in the Wilkinson et al. (2008) study were under conditions of sustained hyperinsulinemia and hyperaminoacidemia; thus, a fasted vs. fed response of MitoPS could not be obtained. In contrast, resistance exercise increased both MyoPS and MitoPS before training. However, chronic training results in a more orchestrated response, as indicated by increases in only MyoPS but not MitoPS (Wilkinson et al., 2008). Thus, chronic exercise training induces differential stimulation of fraction-specific MPS that serve to minimize cellular disturbances during subsequent training sessions.

Additional research examining the effects of feeding on skeletal muscle protein-subfractions following endurance exercise have been conducted. Recently, Breen et al. (2011) demonstrated that the ingestion of protein/carbohydrate supports greater rates of MyoPS but not MitoPS compared to a protein-free control following prolonged endurance (i.e. cycling) exercise (Breen et al., 2011). Similarly, the ingestion of protein/carbohydrate in close proximity to repeated bouts of high-intensity sprint exercise amplifies MyoPS but not MitoPS relative to a protein-free control (Coffey et al., 2011). As mitochondria compromise about 10% of the muscle protein pool, increases in protein synthesis from exogenous amino acids may not be required to facilitate the remodeling process (Coffey et al., 2011) and/or the mitochondrial remodeling may lie outside the 4 hour feeding period (Breen et al., 2011, Coffey et al., 2011). However, in the absence of basal rates of PS, previous study designs (Breen et al., 2011, Coffey et al., 2011) precluded the assessment of exercise-specific effects on MPS of various sub-fractions. Furthermore, none of these previously mentioned studies examined the effect of their nutrient supplements on MitoPS at rest. Given that MitoPS may be sensitive to amino acids at rest (Bohe et al., 2003, Bohe et al.,
2001), it is unclear if the exercise-induced stimulation of MitoPS (if present) would have been greater than (or potentially additive to) the fed-state response at rest.

In the presence of basal rates of MyoPS and MitoPS, only the former but not the latter is responsive to dietary protein ingestion following concurrent exercise (i.e. resistance exercise following by cycling) (Camera et al., 2015). Although the same training stimuli has been shown to stimulate MitoPS in untrained men (Di Donato et al., 2014), the subjects in the Camera et al. (2015) study were trained (> 1 yr of concurrent training); thus cycling for 30 min at 70% VO2 max may have been a suboptimal training stimuli to generate a robust increase in MitoPS (Camera et al., 2015). In light of the generally discrepant findings, it is likely that interpretation of the responsiveness of MitoPS to exercise and/or nutrition is influenced by the study’s design (i.e. fasted-measures, nutrition vs. no nutrition, timing of muscle biopsies) and/or the methodology of mitochondrial protein extraction and isolation wet muscle may (Burd et al., 2015). Previous research reports a ~100% increase in MitoPS with amino acid infusion at rest (Bohe et al., 2003, Bohe et al., 2001) and constant feeding following endurance exercise (Wilkinson et al., 2008). Hence, whether MitoPS is: i) increased by a physiological relevant protein bolus dose at rest, and; ii) is further enhanced by an endurance exercise stimulus is currently unknown.
Figure 2. Stimulation of myofibrillar and mitochondrial fractional synthetic rate (FSR) in response to feeding, training status and 10 weeks of periodized endurance exercise. Untrained: cycling for 30 min at 75% VO₂peak. Trained: cycling for 60 min at 75% VO₂peak. REST: ingestion a beverage (75% carbohydrate, 18% protein and 7% fat), every 30 min (22 times) for the duration of the metabolic study. EX: endurance exercise with the same feeding pattern as REST. *different from REST in same condition †different from untrained, P<0.05. Figure adapted from Wilkinson et al. 2008.
1.3 Molecular regulation of muscle protein synthesis

1.3.1 Mechanistic target of rapamycin complex 1 (mTORC1)

The mammalian/mechanistic target of rapamycin (mTOR) is an evolutionary conserved serine/threonine protein kinase that encompasses at least 2 distinct complexes, mTORC1 and mTORC2 (Laplante and Sabatini, 2009). Although there is some evidence of cross-talk between the 2 complexes (Xie and Proud, 2013), the mode of regulation, subunit composition, substrate specificity, and cellular functions are different between them (Laplante and Sabatini, 2009). Briefly, mTORC1 has been implicated in regulating cell proliferation, growth and autophagy (Ekim et al., 2011, Zhou et al., 2013), whereas to maintain cell integrity, mTORC2 modulates the actin cytoskeleton regulation (Kamada et al., 2005). Described as the “master regulator” of mRNA translation initiation and subsequently MPS, mTORC1 plays an essential role in the maintenance and/or growth of muscle mass (Phillips, 2009, Bentzinger et al., 2008). A distinguishing feature of the complexes is that while mTORC1 is sensitive to the immunosuppressant drug rapamycin, mTORC2 in general is not (Loewith et al., 2002). As such, to understand the effects of feeding and exercise, rapamycin is often administered to directly inhibit mTORC1 activity and subsequently blunt MPS (Dickinson et al., 2011, Drummond et al., 2009). With remarkable capabilities to respond to amino acid availability (Dickinson et al., 2011), contractile activity (Apro et al., 2013), growth factors (Efeyan and Sabatini, 2013) and energy status (Mounier et al., 2011), mTORC1 has taken centre stage in research aiming to understand the regulation of the anabolism and/or remodeling of skeletal muscle. To further appreciate the signaling cascade of mTORC1, a brief understanding of upstream effectors and downstream targets is warranted.

1.3.1.1 Regulators of mTORC1

The tuberous sclerosis proteins 1 and 2 (TSC1 and TSC2) form a heterodimeric complex which acts as negative regulator of mTORC1 signaling (Laplante and Sabatini, 2009). TSC1 and TSC2 inhibit mTORC1 activity through the GTPase activating protein (GAP) characteristics of TSC2, which serves to convert and hydrolyze the active GTP form of the Ras homolog enriched in brain (Rheb) complex into the inactive GDP Rheb complex. The significance of inactive Rheb is that it cannot directly bind and co-localize with mTORC1, which subsequently inhibits the activation of mTORC1 (Inoki et al., 2003). Therefore upon IGF-1 activation, Rheb directly binds
to and co-localizes with mTORC1 and activates its kinase activity in a growth factor-dependent manner (Garami et al., 2003).

**Figure 3.** A schematic of TSC1/TSC2 regulation in human embryonic kidney cells. Rheb functions downstream of TSC1/TSC2 and upstream of mTOR. TSC2 acts as a GTPase activating protein to inactivate Rheb by directly stimulating GTP hydrolysis, preventing Rheb from interacting with mTORC1. Rheb interacts and stimulates mTOR when amino acids turn the negative regulator TSC2 “off”, allowing for increased translation. Nutrient (amino acids) and cellular energy status (ATP) signals through TSC2. LAMP2 is a lysosome-associated membrane protein. TSC1 and TSC2: tuberous sclerosis proteins 1 and 2, a negative regulator of mTORC1. Rheb: Ras homolog enriched in brain, a positive regulator of mTORC1. Figure adapted from Inoki et al. 2003.

The extracellular signal regulated kinase1/2 (ERK1/2) has also been shown to regulate proteins involved in mRNA translation initiation and elongation (Wang et al., 2001, Roux et al., 2007) independent of amino-acid induced mTOR activity (Drummond et al., 2009, Williamson et al., 2003). As ERK1/2 is a mitogen activated protein kinase (MAPK), it is activated in response to a cellular disturbances (e.g. exercise), (Wang et al., 2001, Roux et al., 2007) and therefore may play a role in regulating post-exercise induced increases towards MyoPS.

### 1.3.1.2 Downstream targets of mTORC1

The most well characterized proteins downstream of mTORC1 are the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase 1 (S6K1), the former and latter being negative and positive regulators, respectively (Figure 3). By binding to eIF4E, 4E-BP1 prevents the formation of the eIF4F complex, which subsequently inhibits its ability to bind mRNA and initiate the rate-controlling step for MPS of mRNA.
translation (Kimball et al., 1999). Upon nutrient and/or growth factor (e.g. insulin or IGF-1) activation of mTORC1, 4E-BP1 becomes phosphorylated allowing eIF4E to dissociate from 4E-BP1, forming the eIF4F complex, upregulating mRNA translation initiation (Kimball et al., 1999, Proud and Denton, 1997). Furthermore, the activation of the mTORC1/S6K1 signaling cascade is required to promote mRNA translation and MPS in both amino acid and growth factor-dependent manners (Laplante and Sabatini, 2009).

1.3.2 Nutrient regulation of mTORC1

Much of our understanding of the molecular regulation of mTORC1 action comes from in vitro and animal studies, which collectively help inform suitable targets in skeletal muscle to help understand the molecular regulation of human MPS. For example, in vitro (Shen et al., 2005) and animal (Shen et al., 2002, Balage et al., 2001) studies support the requirement of insulin to regulate the assembly of eIF4F complex and, ultimately, protein synthesis. Insulin has also been shown to activate Rheb, subsequently allowing it to bind and activate mTORC1 (Garami et al., 2003). In rat skeletal muscle, insulin acts directly to enhance the association of eIF4E with eIF4G coincided with stimulation of protein synthesis (Kimball et al., 1997). Human studies also demonstrate a role for insulin in stimulating mTOR signalling within skeletal muscle in an apparent dose-dependent manner (Greenhaff et al., 2008). However, unlike animal and cell culture, the presence of amino acids is the prime variable regulating MPS with insulin playing a permissive role (Greenhaff et al., 2008, Phillips, 2008, Staples et al., 2011, Trommelen et al., 2015), suggesting an apparent dissociation between mTOR and mRNA translation in vivo. As such, in human skeletal muscle, amino acids are necessary for the insulin-induced activation of mTORC1 and PS (Barazzoni et al., 2012, Drummond et al., 2008).

Early investigations revealed that, in the fasted state, amino acids are required to stimulate protein synthesis in skeletal muscle of rodents (Garlick et al., 1973, Fulks et al., 1975) and humans (Rennie et al., 1982), a process now known to be under the control of mTORC1 (Laplante and Sabatini, 2009). Subsequent studies in cultured mammalian cells confirmed that a mixture of all 20 amino acids activated mTORC1(Hara et al., 1998, Wang et al., 1998) and that the combination of amino acid and/or growth factor signaling (i.e. IGF-1) (Balage et al., 2001) is necessary to
promote mTORC1 signal transduction. Although leucine (Dodd and Tee, 2012) and arginine (Hara et al., 1998) are critical for mTORC1 activation, the presence of the remaining 18 amino acids is required to ultimately support optimal rates of protein synthesis (Phillips, 2009). With respect to skeletal muscle, leucine appears to have unique capabilities to regulate mTORC1 activity and subsequently postprandial MPS in rodent (Norton et al., 2012) and humans (Churchward-Venne et al., 2012, Churchward-Venne et al., 2014). Moreover, a single oral dose of a potent mTORC1 inhibitor (rapamycin) prior to 10g of EAA ingestion blunts mixed MPS and attenuates the activation of mTORC1/S6K1 signaling proteins (Dickinson et al., 2011). As extracellular and intracellular amino acid concentrations are similar when rapamycin is and is not administered (Dickinson et al., 2011), this study demonstrates that mTORC1 activation is a key regulator of human MPS/MyoPS in response to increased EAA availability.

When cells are deprived of amino acids, in vitro research suggests mTORC1 is relatively dispersed throughout the cytoplasm (Bar-Peled and Sabatini, 2014). However, in response to amino acid exposure and mediated by raptor, mTORC1 rapidly translocates to the lysosomal surface where it interacts with the small GTPase Rheb in cell models (Sancak et al., 2010). The amino acid-dependent translocation of mTORC1 is activated by the Rag guanosine triphosphatases (GTPases), a family of four small GTPases (RagA/B-RagC/D GTPases) that reside in the lysosome (Sancak et al., 2008). Furthermore, the Ragulator interacts with the Rag GTPases and is essential for mTORC1 activation (Sancak et al., 2010). Thus, Rag-Ragulator-mediated translocation of mTORC1 to lysosomal membranes is an important event in amino acid signaling to mTORC1, as suggested by in vitro models (Figure 4). As these findings were made in vitro, the significance of these mechanisms and whether they operate in human skeletal muscle as a means to regulate protein metabolism requires further study.
Figure 4. A schematic of Rag-Ragulator mediated translocation of mTORC1 to lysosomal membranes in human embryonic kidney cells. In the presence of amino acids, the Rag GTPases, which are tethered to the lysosomal surface by the Ragulator, allow mTORC1 to be activated by Rheb at the lysosomal membrane. Whether exercise results in a similar signaling response remains under investigation. Ragulator: protein complex associated with Rag proteins (RagD and RagB) and is a positive regulator of mTORC1. GDP: Guanosine diphosphate, GTP: Guanosine triphosphate. Figure reproduced from Sancak et al. 2010.

1.3.2.1 mTORC1 and MyoPS

It has been well established that feeding stimulates MPS (see previous). As such, in young healthy muscle, the feeding of 10g of EAA (the equivalent of ~25g dietary protein) maximizes MyoPS and is underpinned by elevations in mTORC1/S6K1 phosphorylation (Cuthbertson et al., 2005). Some studies have suggested that transition from a fasted to fed state occurs relatively rapidly in human muscle as the ingestion of a bolus dose (48g) of whey protein has been reported to stimulate a ~3-fold increase in MyoPS that peaks ~1.5 h after feeding before returning to baseline by 2 h (Atherton and Smith, 2012). The return to basal rates of MyoPS occurred in parallel with markers of anabolic signalling and in spite of a sustained elevation of amino acids, which led the authors to suggest that this “refractory” period is consistent with a “muscle full effect” (Atherton et al., 2010). However, contrasting data demonstrate that ingestion of 25g of whey protein at rest enhances S6K1 activity at 1h (Moore et al., 2011), where MyoPS peaks at 1-3 hours (Moore et al., 2009, Churchward-Venne et al., 2012). The significance of this time-discordance is unclear but could suggest the ability of anabolic signalling proteins to “turn on” the protein synthetic machinery is more rapid than the removal of that signal to “turn it off”.

Aside from “snapshot” assessment of anabolic signalling through traditional Western blotting, we currently have limited knowledge on the effects of feeding on mTORC1 translocation and protein complex association in human skeletal muscle, which represent an important level of
biological regulation that has yet to be explored. Whether feeding and/or contractile activity would promote mTORC1 translocation towards the lysosome where it can interact with positive regulators (i.e. Rheb) and dissociate with negative regulators (i.e. TSC2) in humans requires further investigation. Unpublished work from human muscle utilizing immunofluorescence indeed suggests that mTOR/LAMP2 (i.e. marker of lysosome) are highly co-localized and that in response to resistance exercise this complex dissociates from TSC2, translocates to the cell periphery, and co-localizes with Rheb as early as 15min after exercise and up to 3h into recovery (Song et al., in review, personal communication); whether similar events occur in response to nutrient ingestion at rest and/or after endurance exercise remains to be determined.

1.3.2.2 mTORC1 and MitoPS

Although it has been well established that mTORC1 function is critical for muscle and metabolic health (Phillips, 2014, Bentzinger et al., 2008), the link between mTORC1 and MitoPS has received relatively little attention and subsequently represents a gap in our understanding of the potential plasticity of skeletal muscle. With respect to mitochondrial biogenesis, the transcriptional coactivator peroxisome proliferator-activated receptor-γ coactivator (PGC-1α) is a critical regulator of mitochondrial content and function in skeletal muscle (Uguccioni and Hood, 2011).

The chronic inhibition (12 weeks) of mTORC1 and subsequently protein synthesis through rapamycin has been shown to decrease mixed and sarcoplasmic MPS, but maintain MitoPS in rodents (Drake et al., 2013). Furthermore, recent data suggest that in sedentary mice, rapamycin does not blunt MitoPS (Philp et al., 2015) suggesting that the translation of mitochondrial genes in rodents is not dependent on mTORC1 and is perhaps alternatively regulated through a mitochondrial-specific ribosome pool (Pechmann et al., 2013). Presently, how MitoPS is regulated in human skeletal muscle is largely unknown (Hallberg and Larsson, 2014). Needless to say, limited attention linking mTORC1 towards MitoPS in response to feeding at rest and/or after endurance exercise have yet to be conducted in humans.
1.3.3 Exercise regulation of mTORC1

One of the hallmarks of an adaptation to endurance training is an increase in the pool of muscle mitochondria (Holloszy, 1967). The main mechanism by which mitochondrial biogenesis occurs is through the AMP-activated protein kinase (AMPK)/PGC-1α signaling pathway; as a comparison, hypertrophy induced by resistance exercise is generally mTORC1/S6K1-dependent (Atherton et al., 2005). Notably, one of the biological negative regulators of mTORC1 activity is energy status given that mRNA translation and protein synthesis is energetically expensive (Laplante and Sabatini, 2009). Hence, low cellular energy can induce a decrease in the ATP/AMP ratio that can increase AMPK phosphorylation and subsequently suppress mTORC1 signaling and protein synthesis (Kim et al., 2011). In turn, this suppression in protein synthesis may be attributed to Rheb binding to TSC2 rather than mTORC1 (Laplante and Sabatini, 2009, Efeyan and Sabatini, 2013), although this proposed mechanism has yet to be investigated following endurance exercise, which has been shown to increase AMPK phosphorylation (Mounier et al., 2011, Atherton et al., 2005). Additionally, elevated intracellular Ca²⁺ in response to exercise has been shown to blunt protein synthesis through the phosphorylation and subsequent activation of the negative regulator of translation elongation, eukaryotic elongation factor 2 (eEF2) phosphorylation (Rose and Richter, 2009). To minimize cellular disturbances during subsequent training sessions increased AMPK/PGC-1α activity induces mitochondrial biogenesis in response to endurance exercise. However, to remodel skeletal muscle proteins following endurance exercise, rates of MPS are elevated (Howarth et al., 2009, Mascher et al., 2011), which is associated with increased mTORC1/S6K1 (Mascher et al., 2011, Mascher et al., 2007). Therefore, there appears to be an intricate, and perhaps redundant, regulation of skeletal muscle to promote MPS and/or mitochondrial biogenesis in response to energy status.

When compared to resting levels, endurance exercise (i.e. cycling for 1h at 70% VO₂ peak) induces enhanced AktThr308/Ser473 and mTORSer2448 phosphorylation, which peaks 30-60 minutes post-exercise (Camera et al., 2010). Furthermore, the enhanced AktSer473 and mTORSer2448 phosphorylation remains elevated up to 2h post endurance exercise, returning to baseline at 3h (Mascher et al., 2007). Despite increased mTOR activity following endurance exercise, S6K1Thr389 phosphorylation is not elevated post exercise (Mascher et al., 2007, Coffey et al., 2006) as this kinase appears to be especially responsive to high force contraction (i.e. resistance exercise)
The lack of S6K1\textsuperscript{Thr389} phosphorylation despite an increase in mTOR phosphorylation suggests another role for mTOR\textsuperscript{Ser2448} following endurance exercise. Where the phosphorylation of mTOR \textsuperscript{Ser2448} is elevated up to 2 h following endurance exercise, eEF2\textsuperscript{Thr56} phosphorylation is reduced from 30 min to 3 h of recovery (Mascher \textit{et al.}, 2007), which may indicate that translation elongation is activated and protein synthesis rates are increased (Kimball and Jefferson, 2010). In the absence of direct measures of MPS via stable isotopes, the aforementioned studies make it difficult to ascertain whether endurance exercise \textit{per se} results in increases in MPS (Mascher \textit{et al.}, 2007, Camera \textit{et al.}, 2010, Coffey \textit{et al.}, 2006). However, when stable isotopes are employed and in the presence of resting levels, MPS is significantly elevated up to 3h with concomitant increases in mTOR/S6K1 activity following endurance exercise (Mascher \textit{et al.}, 2011). As previously mentioned, the measurement of mixed MPS is a weighted average of all muscle protein fractions and therefore does not provide insight into myofibrillar and/or mitochondrial protein remodeling, which ultimately limits our understanding of the fraction-specific regulation of skeletal muscle protein synthesis. Therefore, additional research that combines markers of mTOR activation (e.g. Western blotting, immunofluorescence) with parallel measures of fraction-specific MPS are warranted to further our understanding of the importance of the former in regulating the latter after endurance exercise in human muscle.

1.3.3.1 mTORC1 and MyoPS: impacts of exercise

The importance of mTORC1 in regulating MPS in humans has been advanced by the use of the macrolide drug rapamycin, which impairs mTORC1 signaling and can blunt the acute amino acid (Dickinson \textit{et al.}, 2011) and resistance exercise (Drummond \textit{et al.}, 2009, Gundermann \textit{et al.}, 2014) induced increases in MPS. Furthermore, in rodents, rapamycin-mediated inhibition of mTORC1 suppresses mechanical loading induced muscle hypertrophy (Bodine \textit{et al.}, 2001), a response dependent on mTORC1 activity and not to off-target effects of rapamycin (Goodman \textit{et al.}, 2011). Through transgenic rodent studies, the muscle-specific upregulation of mTOR is necessary to promote muscle hypertrophy (Goodman \textit{et al.}, 2010). Through the control of synthesizing new muscle proteins, these studies collectively suggest that mTORC1 is a critical regulator of skeletal muscle size in response to resistance exercise.
With respect to endurance exercise, studies examining the effects of endurance exercise alone or with feeding have answered numerous questions regarding skeletal myofibrillar remodeling following endurance exercise. For instance, an increase in the phosphorylation status of mTORSer2448 has been shown to increase at 0.5h following high-intensity endurance exercise, which is correlated with rates of MyoPS during early and late recovery (Di Donato et al., 2014). Furthermore, this increase in the phosphorylation of mTORSer2448 following cycling exercise is consistent with previous literature, which may suggest that mTORC1 is underpinning the increases towards MyoPS (Breen et al., 2011, Coffey et al., 2011, Mascher et al., 2007, Camera et al., 2010). Despite the observation that mTORSer2448 phosphorylation was elevated 0.5h after high-intensity endurance exercise, phosphorylation of S6K1Thr389 (downstream of mTORC1) was unaltered (Di Donato et al., 2014); this finding aligns with previous studies of aerobic exercise when biopsies were taken at similar time points (Coffey et al., 2011, Mascher et al., 2007, Camera et al., 2010, Coffey et al., 2006) as this kinase appears to be especially responsive to high force contraction (i.e. resistance exercise) (Atherton et al., 2005, Nader and Esser, 2001). With this in mind, the phosphorylation of Aktthr308/ser473, mTORser2448, S6K1thr389, and rps6ser235/23515 have been shown to increase as early as 15 minutes post exercise (Coffey et al., 2011) and S6K1thr389 phosphorylation has been shown to be sustained up to 4 hours post endurance exercise (Breen et al., 2011). In neither of these previous studies (Breen et al., 2011, Coffey et al., 2011) was a resting rate of MyoPS reported, and so it is not possible to determine the magnitude of MyoPS stimulation and/or mTOR signaling, regardless of nutrition provided. The apparent dissociation of signaling across studies could be attributed to the various timing of muscle samples, missing changes in phosphorylation status of the target phosphoproteins measured (e.g. mTOR/S6K1), and/or other phosphorylation sites that regulate mTOR activity, which could provide novel mechanisms that regulate MyoPS following endurance exercise.

It is not clear what role, if any, that the rapamycin-mediated inhibition of mTORC1 may play in mediating a divergent protein synthetic response of myofibrillar and mitochondrial muscle proteins in response to endurance exercise. Using an exercise only rodent model, Philp et al. (2015) recently demonstrated that when compared with an unexercised control the acute rapamycin-mediated inhibition of mTORC1 blunts S6K1Thr389 phosphorylation at 0.5h, and 3h post-endurance exercise and that this was coincident with ~10-fold decreases in S6Ser235/236 and S6Ser240/244.
phosphorylation that persisted throughout the 6h recovery period. Furthermore, rapamycin blunted 4E-BP1\textsuperscript{Thr37/46} phosphorylation ~2-3-fold throughout the entire post-exercise period (Philp \textit{et al.}, 2015). Area under the curve analysis of MyoPS revealed that when compared to control rodents, rapamycin blunted MyoPS throughout the 6h recovery period despite being transiently blunted at 0.5h and increasing normally (~2 fold) 3 and 6h post exercise (Philp \textit{et al.}, 2015). Collectively, these data suggest that the remodeling of MyoPS following endurance exercise is mTORC1 dependent. The question of whether feeding (i.e. dietary protein) could “rescue” the inhibition of mTORC1/MyoPS post endurance exercise remains to be determined; however, studies examining the effects of endurance exercise and feeding on mTORC1 and MyoPS have begun to receive more attention. Nonetheless, this body of research suggests that feeding before or after exercise stimulates MyoPS and is associated with changes in mTORC1 signaling; in contrast, the responsiveness of MitoPS following endurance exercise has arguably received considerably less attention.

1.3.3.2 mTORC1 and MitoPS: impacts of exercise

A well-orchestrated series of cellular and molecular signaling transduction is necessary to convert the mechanical stimuli of contractile activity into the production of new mitochondria. It has been well established that the “master regulator” of exercise-induced mitochondrial biogenesis is PGC-1\textalpha (Uguccioni and Hood, 2011). However, the relationship between mTORC1 signaling and mitochondrial biogenesis after contractile activity has not been well characterized. Despite only increases in MyoPS following endurance-types of exercise, some studies show increases in Akt\textsuperscript{ser473}, mTOR\textsuperscript{ser2448} S6K1\textsuperscript{thr389} phosphorylation 15 minutes and 1 hour post exercise (Coffey \textit{et al.}, 2011, Camera \textit{et al.}, 2015), while others do not (Wilkinson \textit{et al.}, 2008, Di Donato \textit{et al.}, 2014). These dissociations between the anabolic signaling of muscle and direct measures of MPS have been previously reported (Greenhaff \textit{et al.}, 2008), and will be discussed in depth in the following section. Through the rapamycin-mediated inhibition of mTORC1 (3h/day, over 4 days), Carter and Hood (2012) demonstrated that in murine skeletal muscle myotubes, contractile activity-induced mitochondrial biogenesis is not reliant on mTORC1 as indicated by 1.6-fold increase in cytochrome oxidase (COX) activity, 2.5-fold increase in protein expression of COX subunit IV and the 1.5-fold increase in protein expression of the mitochondrial transcription factor A (Tfam), markers of mitochondrial biogenesis. Notably, the study did not directly measure rates of MitoPS,
thus an appreciation of time dependent changes in the synthesis of these mitochondrial proteins is not clear. Consistent with these findings, Philp et al. (2015) demonstrated that when compared to unexercised control rodents, acute rapamycin-mediated inhibition of mTORC1 suppresses mTORC1 activity and MyoPS but does not affect MitoPS 6h post endurance exercise. Thus, the authors suggested endurance exercise stimulates MitoPS independently of mTORC1 (Philp et al., 2015).

Given that mTORC1 phosphorylation by Western blotting was unaltered irrespective of exercise intensity, the late divergent response of MitoPS between the exercise intensities could not ostensibly be explained by changes in mTORC1 activity (Di Donato et al., 2014); this could ultimately suggest an independent mechanism in promoting MitoPS. Furthermore, whether there is a necessity for dietary protein provision to robustly stimulate MitoPS, as there is with MyoPS (Moore et al., 2009, Moore and Stellingwerff, 2012) either at rest or after endurance exercise requires further investigation. Given that mTORC1 is an indispensable protein complex to regulate the plasticity of skeletal muscle and that mitochondria are critical to maintain muscle function, it would be intuitive to link the evolutionary conserved mTORC1 to mitochondrial protein turnover in humans. Thus, more human work is needed to determine a link, if any, towards mTORC1 and MitoPS.

1.4 Methods to measure mTORC1 Activity

1.4.1 Traditional methods to measure mTORC1 activity

Ever since researchers published the first detailed description of detecting and quantifying specific proteins in 1979 (Towbin et al., 1979), the Western blot has been a staple method in exercise and/or nutritional science labs. The Western blot allows for the identification of specific proteins from a complex mixture of proteins or homogenates extracted from cells (Kurien and Scofield, 2006). Briefly, the technique uses three elements to accomplish this task: (1) denaturation and separation of proteins by size (i.e. molecular weight) through gel electrophoresis, (2) transferring the proteins to a solid support, producing a band for each protein and (3) marking target protein using a proper primary and secondary antibody to visualize the protein of interest (Mahmood and Yang, 2012). As the ideal antibody only binds to the protein of interest, only one band should be visible, where the thickness and/or darkness (i.e. “intensity”) of the band
corresponds to the amount of antigen present (i.e. total protein or phosphorylated residue) (Mahmood and Yang, 2012). With respect to studies in the exercise and nutritional sciences, Western blotting is often used to measure mTORC1 activity through changes in protein phosphorylation (Drummond et al., 2009, Mascher et al., 2007, Koopman et al., 2007). In response to exercise and nutrition, S6K1\textsuperscript{Thr389} appears to be an appropriate surrogate to measure mTORC1 activity (Koopman et al., 2007, McGlory et al., 2014, Koopman et al., 2006, Apro et al., 2015). However, additional methodologies are necessary in complementing the Western blot in explaining the molecular “signature” that may translate into altered rates of MPS, as there are some reports of a dissociation between these acute “snapshot” markers of mTORC1 kinase activity and the direct measures of subsequent muscle protein remodeling (Greenhaff et al., 2008, Wilkinson et al., 2008, Areta et al., 2013). Moreover, proteins within the mTORC1 cellular milieu are ultimately regulated by protein-protein interactions and specific cellular compartmentalization (Laplante and Sabatini, 2009). The Western blot does not provide insight into whether proteins are in a position in the cell that would allow them to be active, such as bound to positive or negative regulators and/or within regions with the greatest ribosomal density. Given that Western blotting does not provide information about protein-protein interactions and protein colocalization, using additional methods (i.e. immunofluorescence) in conjunction with Western blotting can undoubtedly provide higher resolution “snapshot” markers into the regulation of mTORC1 in response to feeding and/or exercise.

### 1.4.2 Immunofluorescence to visualize mTORC1 activity

Immunofluorescence is a powerful technique that utilizes fluorescent-labeled antibodies to detect specific target antigens that, when coupled with cross sections of skeletal muscle, permits the visualization of native proteins and their associated distribution within the cell (Odell and Cook, 2013). Studies in vitro and in non-muscle cells have indicated that the cellular localization and protein-protein interaction of mTORC1 activity in response to physiological stimuli (e.g. nutrients, growth factors) may be fundamentally important in the regulation of the mTORC1 signaling cascade (Laplante and Sabatini, 2009; Betz and Hall, 2013); this information is unfortunately lost during the homogenization and protein denaturing steps of Western blotting. Despite mTORC1 translocation to the lysosome being a critical component in kinase activity (Sancak et al., 2010) that would not be detected by traditional Western blotting approaches, studies
examining the visual translocation and colocalization of mTORC1 to the lysosomal surface in human skeletal muscle in response to feeding and endurance exercise have yet to be conducted. To this end, immunofluorescence can be utilized to determine protein colocalization (Odell and Cook, 2013) by demonstrating whether TSC2 dissociates from Rheb, which would be required for enhanced mTOR kinase activity (Inoki et al., 2003) and subsequent stimulation of MPS (Phillips, 2009). In response to feeding and/or contractile activity, immunofluorescence techniques could be utilized to visualize the cellular translocation of mTORC1 (i.e. in the cytoplasm or lysosome) in human skeletal muscle. This information will help further our knowledge of the regulatory events associated with enhance mTOR activity and may ultimately shed light on the potential dissociation between anabolic signalling and MPS previously reported in human research (Greenhaff et al., 2008, Moore et al., 2009, Wilkinson et al., 2008, Areta et al., 2013). Furthermore, the site of MPS in skeletal muscle is largely unknown. Notably, rRNA is generally located around the peripheries of muscle cells (Horne and Hesketh, 1990) where new proteins appear to be synthesized around the plasma membrane in vivo (Goodman et al., 2012). Thus, immunofluorescence can be utilized to determine if mTOR translocates towards the periphery of muscle cells, the potential cite of protein synthesis, and from the standpoint of its amino acid-induced regulation (Bar-Peled and Sabatini, 2014), in close proximity to the capillaries that deliver anabolic nutrients.

Unpublished preliminary results in humans utilizing immunofluorescence in response to feeding (20g protein/40g carbohydrate/1g fat) or energy-free control demonstrate that following resistance exercise mTOR translocates towards the plasma membrane, associating with positive regulators (i.e. Rheb and eIF3F), consistent with an enhanced mRNA translational capacity (Song et al., in review, personal communication). Therefore, in addition to Western blotting approaches, the use of immunofluorescence to quantify protein-protein interactions (i.e. Rheb/mTOR) and cellular translocation (i.e. mTORC1 translocation to the plasma membrane) has great potential to enhance our understanding of the mechanisms involved in the regulation of mTORC1 and fraction-specific protein synthesis with nutrients at rest and following endurance exercise.
1.5 Conclusions, gaps in the literature

1.5.1 Proposed study

Using stable isotopes to directly measure both MyoPS and MitoPS, the proposed study will examine the effect of dietary protein as a strategy to provide amino acid building blocks to remodel fraction-specific skeletal muscle proteins (i.e. myofibrillar and mitochondrial proteins) at rest and following endurance exercise. Moreover, to provide insight into the molecular regulation of fraction-specific MPS, immunofluorescence (for colocalization and cellular compartmentalization) in conjunction with Western blotting (for mTORC1 phosphorylation) will be utilized to quantify mTORC1 activity in human skeletal muscle in response to nutrient ingestion at rest and following endurance exercise.

1.5.2 Rationale

The role of post exercise dietary protein to remodel damaged skeletal muscle proteins following endurance exercise has received little attention. Moreover, how MitoPS is regulated at rest and following endurance exercise is largely unknown. Therefore the purpose of this study is 3 fold; i) to determine if MitoPS is responsive to a physiologically relevant bolus feeding (20g intrinsically-labeled egg protein and 50g carbohydrates) at rest and, if so, whether its relative nutrient sensitivity is similar to MyoPS; ii) to determine if endurance exercise and feeding further enhance MyoPS and MitoPS when compared to rest, and; iii) for the first time in humans, to utilize immunofluorescence (to measure protein colocalization) in conjunction with Western blotting to explain the molecular mechanisms that regulate MyoPS and MitoPS in response to dietary protein/carbohydrate ingestion at rest and following endurance exercise.

1.5.3 Hypotheses

It is hypothesized that: i) the feeding of dietary protein/carbohydrates will similarly enhance rates of MyoPS and MitoPS at rest, demonstrating the latter is responsive to nutrient ingestion; ii) when compared to rest, the ingestion of dietary protein/carbohydrates following an acute bout of prolonged endurance exercise, will yield higher rates of both MyoPS and MitoPS, and; iii) dietary protein ingestion at rest and following endurance exercise will be associated with
greater mTORC1 signaling via immunofluorescence and Western blotting. Further to this final point, we hypothesize that immunofluorescence will reveal greater mTOR colocalization with its positive regulators (e.g. Rheb) rather than negative regulators (e.g. TSC2) and that Western blotting will demonstrate changes in phosphorylation of proteins downstream of mTORC1 that would be consistent with their greater activity and, hence, muscle protein remodeling.
Chapter 2
Research Proposal

2.1 Introduction

Based on the previously described knowledge base, the proposed research was divided into a total of 2 research phases. The first phase consisted of a screening phase, and the second phase consisted of two separate experimented test days. Therefore, subjects were required to attend to 3 laboratory visits. The research proposal section will serve as a brief introduction of the purpose and objectives associated with each research phase. Lastly, a timeline of the study data collection will be presented and the potential methods to translate knowledge from the obtained data upon the study’s completion will be explained. A more comprehensive explanation of the materials and methodology will be provided in Chapter 3.
2.2 Phases of research

A total of 8 active, healthy male participants were recruited for the present study. Previous studies demonstrate that 8 subjects are required to determine changes in MyoPS/MitoPS and mTORC1 activity from fasted to fed states (Camera et al., 2015, Camera et al., 2010, Burd et al., 2012). As chronic training increases exercise-mode and fraction-specific protein synthetic responses (Wilkinson et al., 2008), trained subjects were recruited. Each participant engaged in a total of 3 laboratory visits (initial screening day + 2 experimental trials) encompassing the 2 phases of the research study. The three phases of the proposed study are described below.

Phase I – Screening Day (1 x 2h):

The initial screening phase consisted of a single laboratory visit lasting approximately 2 hours, and had two primary objectives. In order to ensure that they were properly informed before being asked to provide consent, the first objective served to provide the participants with a comprehensive oral introduction to the study protocol. Once participants have been provided a detailed overview of the study protocol, they were given the opportunity to ask questions, and signed the required consent document if they wished to participate in the study. Furthermore, information regarding the participants’ general health and habitual physical activity levels using the Physical Activity Readiness Questionnaire (PAR-Q) and a Medical History Form was utilized to determine if subjects qualified for the study. Secondly, after initial recruitment and informed consent, participants underwent a body composition measurement (DEXA) and VO₂ max testing.

Phase 2 – Experimental Test Days (2 x 8.5h):

Each participant participated in 2 trials (lasting ~8.5 h), each separated by ≥ 7 days. The first trial was a rested control trial. The subsequent trial was randomized and counterbalanced. These trials differed in the inclusion of exercise and/or nutrient intake. Contemporary research demonstrates that to maximize and elicit robust increases in MPS/MyoPS 20g bolus dose of high quality dietary protein (i.e. egg protein) is required at rest and following resistance exercise (Moore et al., 2009, Witard et al., 2014). Arguably, this recommended dose is transferable towards endurance exercise modalities as well (Moore et al., 2014). Furthermore, 50g of carbohydrates has been shown to play a pivotal role in recovery following endurance exercise (Burke et al., 2011), and this dose was used to aid in post-exercise glycogen replenishment.
2.3 Knowledge translation

The aim of the present study is to improve our scientific understanding on how dietary protein ingestion impacts skeletal muscle protein metabolism at rest and following an acute bout of endurance exercise. The results obtained from this study may aid in the designing of optimal nutrition and/or exercise interventions aimed at managing muscle size, shape, and quality and/or preventing muscle loss in humans. Furthermore, the knowledge obtained from the molecular mechanisms of muscle remodeling has potential impact in the development of neutraceutical and/or pharmacological targets aimed at optimizing muscle mass in humans. Upon study completion, data analysis and interpretation, the objective will be to communicate these results to the scientific community through non-peer-reviewed publications, peer-reviewed journal article(s), and through conference presentations.

2.4 Project Timeline

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<th>Stage</th>
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<th>Details</th>
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<td>June 2015</td>
<td>July 2015</td>
<td>In collaboration with the University of Illinois at Urbana Champaign (UIUC). Ethic approved from UIUC and UofT.</td>
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<td><em>Proposal defense</em></td>
<td>October 2015</td>
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<td><em>Optimizing methods for immunofluorescence and Western blotting</em></td>
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<td><em>Data analysis</em></td>
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<td><em>Writing and defence</em></td>
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Chapter 3
Methodology

3.1 Introduction

This section will provide a comprehensive description of the screening day, and the two experimental trial days. The study design, materials and methods relevant to each phase of the study will be elucidated. Additionally, a schematic of the proposed study will be shown. Lastly, statistical analyses utilized to analyze the data will be discussed.
3.2 Participants & study design

In total, 8 healthy (BMI <25 kg∙m$^2$) active young male subjects (18-35 y) were recruited for this study. Previous research by Moore et al. (2009), utilized the most similar methodological and intervention standpoint to the present study and was used to power the present study to detect a difference in the primary outcome (i.e. fraction-specific MPS). In the study by Moore et al. (2009), MyoPS increased from 0.025%/h ± 0.06 (fasted) to 0.049 ± 0.2 and 0.07%/h ± 0.2 following feeding (i.e. 25g whey protein) at rest and after resistance exercise, respectively. Therefore, with an $\alpha = 0.05$, $\beta = 0.8$, and utilizing a within participant comparison, $n=7$ participants were determined to be sufficient to detect a significant difference between the protein ingestion at rest and following endurance exercise, when compared to a rested fasted control. To account for a potential ~10% drop out rate, we recruited $n=8$ participants for the crossover design. Participants were recruited from various sports teams from UIUC. Inclusion and exclusion criteria were as follows:

Table 1. Inclusion/exclusion criteria

<table>
<thead>
<tr>
<th>Inclusion</th>
<th>Exclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Male</td>
<td>• Sedentary</td>
</tr>
<tr>
<td>• Aged 18-35 y</td>
<td>• Smoking</td>
</tr>
<tr>
<td>• Active (aerobic activity 3-6 times per week)</td>
<td>• Known allergies to eggs</td>
</tr>
<tr>
<td>• Healthy (BMI &lt; 25 kg∙m$^2$)</td>
<td>• Vegans</td>
</tr>
<tr>
<td>• $\text{VO}_{2}\text{peak} &gt; 50$ ml/kg/min</td>
<td>• Diagnosed GI tract diseases</td>
</tr>
<tr>
<td></td>
<td>• Arthritic conditions</td>
</tr>
<tr>
<td></td>
<td>• History of neuromuscular problems</td>
</tr>
<tr>
<td></td>
<td>• Previous participation in amino acid tracer studies</td>
</tr>
<tr>
<td></td>
<td>• Predisposition to hypertrophic scarring or keloid formation</td>
</tr>
<tr>
<td></td>
<td>• Medications known to affect protein metabolism (i.e. corticosteroids, NSAIDs, prescription-strength acne medications)</td>
</tr>
<tr>
<td></td>
<td>• High blood pressure (systolic &gt; 140 mmHG; diastolic &gt;90 mmHG)</td>
</tr>
</tbody>
</table>
Furthermore, subjects were asked to complete a Medical History Form to aid in the evaluation of the exclusion criteria. In order to determine if it is safe for the subjects to engage in exercise, participants were asked to complete a PAR-Q (Physical Activity Readiness Questionnaire). The total participant commitment was spanned over 3 laboratory visits, and had an approximate time commitment of 19 hours (1 x 2h, 2 x 8.5h).

To determine exercise effects of endurance exercise, subjects served as their own control. Therefore each subject was required to engage in two separate trials (REST and EX), each separated by ≥ 7 days. The trials were randomized and counterbalanced.

3.3 Subject introductory session and familiarization to study

After initial recruitment, all individuals who qualified for the study and agreed to participate underwent a body composition assessment (DEXA). Subjects were instructed to lie down on a table and stay motionless for approximately 3 minutes during which the body scan takes place. In addition, the subjects’ body height, body weight, and blood pressure were assessed. Also, subjects’ peak oxygen uptake (VO\(_2\)peak; marker of aerobic fitness) was determined via a graded treadmill exercise test and a gas + flow analyzer. After a 5 min warm-up at 1.7 mph at a 5 % grade, a modified Bruce testing protocol was employed to determine subjects’ VO\(_2\)peak as previously described (Wilkinson et al., 2008).

The test ended when subjects were unable to continue, despite verbal encouragement from the researchers. It was expected that most subjects reached VO\(_2\)peak within 8-12 min (Stage 4-6). Before leaving the laboratory, subjects were instructed to refrain from alcohol and any sort of heavy physical exercise for 2 days before each trial day. The subjects were instructed to consume their regular diet for 2 days prior to the start of the first trial day (trial 1). Subjects were also asked to record the food that they eat during those days in a 2-day food dairy. For the subsequent trial days, subjects were asked to consume a similar diet as was consumed before the first trial day, using the 2-day food diary as their guidance. This screening visit lasted ~2 h in duration.
3.4 Stable isotopes

The determination of MPS rates requires an intravenous infusion of a stable isotope amino acid tracer coupled with the collection of small pieces of thigh muscle from the research subject. A stable isotope tracer is an amino acid that is a slightly heavier version of the abundant form of the amino acid (tracee) in the body. The tracer is chemically and functionally identical to the tracee amino acid, but the tracers’ distinct mass enables its precise detection in the muscle, whole body proteins, and breath. To reliably assess the stimulation of protein synthesis, it is important that the stable isotope amino acid is continuously and steadily infused over the measurement period. Furthermore, a certain fraction of all of the carbon and hydrogen within the body is already in the same form as that of the stable isotope. Hence, the infusion of the stable isotope-labeled amino acid will simply result in a slight increase in the amount of stable isotope within the participants’ body; termed as "enriching" the amount of stable isotope. Thus, the infusion of the stable isotope will be a tracer dose that will increase the naturally occurring levels by 1-5%.

The objective of the proposed research was to examine protein co-ingestion with carbohydrates to stimulate fraction-specific MPS rates following an acute bout of treadmill running in healthy, young adults. To achieve this objective, intrinsically labeled L-[5,5,5-2H3]leucine eggs, in combination with the primed continuous intravenous infusion of L-[ring-2H5]phenylalanine was utilized. With the use of these stable isotopes, the dietary protein digestion and absorption kinetics, leucine oxidation rates and the post-meal (postprandial) stimulation of whole body and MPS rates after an acute bout of endurance exercise can be quantified.

3.5 Experimental trials

The study employed a randomized crossover design with all subjects performing 2 trials, each lasting ~ 7-8.5 h. During the first trial, the subjects remained sedentary and protein synthesis was determined during a rested control state (REST) (Figure 5). During the exercise trial, subjects ran on a treadmill for 1 h at 70 % VO2peak (EX). During REST and EX subjects ingested 20g of intrinsically labeled egg protein and 50g of carbohydrates (50 % dextrose and 50 % maltodextrin) (FED) at 0H (Figure 5). Blood and muscle biopsy samples were collected during the experimental trials.
Protein drinks: Intrinsically labeled egg protein powder (20g of protein) was utilized to maximize MyoPS (Moore et al., 2009, Witard et al., 2014) and carbohydrates (50% dextrose and 50% maltodextrin; 50g) served to aid in glycogen replenishment (Burke et al., 2011). Drinks were dissolved in 350 ml water and were uniformly flavoured with 2 mL of vanilla. The test beverages remained refrigerated at ~2°C prior to administration to the subjects.

3.5.1 Rest trial

Subjects were asked to report to the laboratory fasted, to ensure MPS rates are in a basal state prior to feeding and/or exercise. At 08.00 h, topical anaesthesia (EMLA cream) was used prior to the placement of a Teflon catheter (flexible plastic tube) into the antecubital vein. Subsequently, the participants received a priming dose of L-[ring-\(^2\)H\(_5\)]phenylalanine (2.0 \(\mu\)mol·kg\(^{-1}\)), prior to initiating a continuous infusion of L-[ring-\(^2\)H\(_5\)]phenylalanine (0.05 \(\mu\)mol·kg·min\(^{-1}\)) and filtered through a sterile filter (0.2 \(\mu\)m), using a calibrated infusion pump (PHD 2000, Harvard apparatus, Natick MA). To determine fasted rates of MPS, the change in protein-bound \(^2\)H\(_5\)phenylalanine enrichment was determined by GC-MS between -2 and 0h, according to established methods (Patterson et al., 1997). Upon feeding of the protein beverage immediately following the 0h muscle biopsy, postprandial MPS was determined between 0h and 5h to capture the entire postprandial period (Moore et al., 2009). Alongside with the biopsies used to determine rates of MPS, the muscle biopsy obtained at 1h was utilized for mTORC1 signaling via immunofluorescence and Western blotting. The extra muscle biopsy was taken at 1h, as this time point is thought to be reflective of greatest mTORC1 signaling in response to feeding and/or exercise (West et al., 2011, Moore et al., 2011). Blood samples were collected throughout various time points during the test day to determine plasma EAA and insulin concentrations (Figure 5). Following the end of the trial, subjects were provided with a “Biopsy Care Kit” to help aid in the proper care for their wounds after leaving the laboratory. Within 5 days of completion of a trial, the subjects were asked if the incisions had healed properly.
3.5.2 Exercise trial

During the exercise trial, participants reported to the lab after an overnight fast to receive a primed constant infusion of L-ring-[\(^{2}\)H\(_5\)]phenylalanine. Following 1.5h of infusion, participants performed 1h of treadmill running at 70% of their previously established VO\(_2\)peak (i.e. -1 to 0h). Since our subjects were healthy, young and trained (exercising 3 – 6 times/wk); running for 1h at 60-70 % VO\(_2\)peak was attainable for our subjects. This notion is supported by various previous studies that had participants of similar activity level exercising at such intensities for durations of 60 min or more (Bolster et al., 2005, Wilkinson et al., 2008, Bowtell et al., 2000). Immediately after exercise and the first muscle biopsy (0h), subjects consumed 20g of egg protein and 50g of carbohydrates (FED). Subjects consumed the protein/carbohydrate beverage immediately after exercise as previous research has demonstrated that delaying nutrient ingestion up to 3h following endurance exercise impairs MPS (Levenhagen et al., 2001). As the rest trial served to obtain fasted rates of MPS, only postprandrial rates of MPS were obtained from the biopsies obtained at 0h and 5h during the exercise trial. Similar to the muscle biopsies obtained at 0 and 5h, the muscle biopsy at 1h was used for mTORC1 signaling via immunofluorescence and Western blotting. Blood was collected throughout various time points during the test day (Figure 5).
3.6 Study schematic

**Figure 5:** During the rested (REST) and exercise (EX) trials, subjects consumed 20g of egg protein + 50g of carbohydrates (Drink) at 0h to induce a REST or EX postprandial response (FED). Throughout the duration of the trial, subjects received a primed continuous infusion of L-[ring-\(^{2}\)H\(_5\)] phenylalanine. To determine fasted MPS, muscle biopsies were taken at -2, 0H. Another biopsy was obtained at 5H to obtain Fed MPS. In conjunction with the biopsies used to determine rates of MPS, the muscle biopsy obtained at 1h was utilized for mTORC1 signaling via immunofluorescence/Western blotting. Trials were randomized and counterbalanced. EX trial involved 1h of treadmill running at 70% VO\(_2\)-peak from -1 to 0h.

3.7 Blood and muscle analysis

All blood samples were collected in heparinized evacuated containers and kept on ice until they were centrifuged to obtain plasma, which was subsequently aliquoted, frozen, and stored at -20°C until further analysis. Plasma insulin was performed as described in Appendix 1. Plasma L-[ring-\(^{2}\)H\(_5\)]phenylalanine enrichments were determined as previously described (Moore *et al.*, 2009) (see Appendix 2). Purified free amino acids were then converted to their heptafluorobutyrate (HFB) derivatives and analyzed for L-[ring-\(^{2}\)H\(_5\)]phenylalanine enrichment by GC-MS (Agilent 7890A GC/5975C; MSD) as previously described (Moore *et al.*, 2009, Burd *et al.*, 2015).

A 100-mg piece of wet muscle was homogenized using a glass homogenizer in ice-cold homogenization buffer (10ul/mg; 0.01 M sucrose, 0.01 M Tris·HCl, 0.05 M KCl, 0.001 M EDTA) with protease and phosphatase inhibitor cocktail tablets (Complete Mini, PhosSTOP; Roche Applied Science, Mannheim, Germany). The homogenate was transferred to an Eppendorf tube.
and centrifuged at 700 g for 10 min at 4°C to pellet myofibrillar proteins. The supernatant was transferred to another Eppendorf tube and centrifuged at 12 000 g for 20 min at 4°C to pellet mitochondrial proteins. The supernatant was transferred in a separate Eppendorf tube and stored at -80°C for Western blot analysis (to be described later).

Amino acids were obtained from the mitochondrial pellet as described previously (Burd et al., 2015, Burd et al., 2012). Briefly, the pellet was washed once with ice-cold homogenization buffer and centrifuged at 12 000g for 15 min at 4°C. Following the removal of the supernatant, the mitochondrial pellet was washed once with ethanol, and then dried under vacuum. Proteins were hydrolyzed by adding 6 M HCl and heating at 110°C for 18 h. The free amino acids from the mitochondrial and myofibrillar enriched fractions were purified using cation exchange chromatography and converted to their N-acetyl-n-propyl ester derivatives for 5500 QTRAP LC–tandem MS (Sciex) analysis. For full muscle analysis, see Appendix 3.

3.7.1 Myo/MitoPS calculations

Plasma L-[ring-2H5]-phenylalanine was determined using gas chromatography-mass spectrometry (GC-MS) analysis as previously described (Moore et al., 2009) (Appendix 2). The fractional synthetic rates (FSR) of MyoPS and MitoPS were calculated using the standard precursor–product method as described (Moore et al., 2009, Burd et al., 2012):

\[
FSR \text{ (% h}^{-1}\text{)} = \frac{\Delta E_p}{E_m} \times \frac{1}{t} \times 100
\]

Where \(\Delta E_p\) is the change in bound protein enrichment between two biopsy samples, \(E_m\) is the average enrichment of plasma phenylalanine across the two biopsy samples, and \(t\) is the time between biopsies. Specifically, fasted FSR was calculated from the change in bound protein enrichment between the baseline biopsy (-2h) and the biopsy taken at 3 h after the start of the infusion using the intracellular phenylalanine enrichment at 0 h. FSR at 0 h was calculated from the change in bound protein enrichment from the fasted biopsy to the 0 h biopsy after feeding in both the REST or EX conditions, respectively. The change in bound protein enrichment between subsequent biopsies within each condition was used to calculate FSR in REST and EX at 0–5 h (i.e. FSR expressed over 5 h intervals).
3.7.2 Western blot methodology to measure mTORC1 activity

To provide mechanistic insight towards feeding and exercise protein synthetic responses we measured the phosphorylation of the 2 well characterized proteins downstream of mTORC1, S6K1\textsuperscript{Thr389} and 4E-BP1\textsuperscript{Thr37/46}, regulators of mRNA translation initiation. As a surrogate measure for mRNA translation elongation and contractile-induced activation of mTORC1, we measured phosphorylation of eEF2\textsuperscript{Thr56} and Erk1/2\textsuperscript{Thr202/Tyr204}, respectively. Lastly, as a surrogate marker for MitoPS, we measured p38\textsuperscript{Thr180/Tyr180} phosphorylation, an upstream regulator of PGC-1α, the master regulator of mitochondrial biogenesis.

The protein concentrations of the extracts were determined using the BCA assay (Thermo Fisher Scientific, Rockford, IL). Samples were prepared to the same concentration by dilution with the homogenization buffer used to extract the myofibrillar and mitochondrial proteins, denatured with Laemmlli sample buffer and heated to 95°C for 5 min. Samples (10μg) were loaded on 4–20% gradient polyacrylamide gels and were separated by electrophoresis (200 V for 45 min), and subsequently transferred to nitrocellulose membrane (wet transfer, 100 V for 1 h). Membranes were blocked at room temperature for 1 h using 5% wt/vol milk in Tris-buffered saline with 0.1% Tween 20 (TBST) before overnight incubation in primary antibody at 4°C. Primary antibodies, diluted 1:1000 in TBST and 3% milk, were from: Cell Signaling Technologies (Danvers, MA, USA) --, phospho-S6K1\textsuperscript{Thr389} (cat. 9205), phospho-eEF2\textsuperscript{Thr56} (cat. 2331), phospho-4E-BP1\textsuperscript{Thr37/46} (cat. 9459), phospho-mTOR\textsuperscript{Ser2448} (cat. 2971), phospho-p38 MAPK\textsuperscript{Thr180/Tyr182} and phospho-ERK\textsuperscript{Thr202/Tyr204} (cat. 4377). Following overnight incubation, membranes were washed in TBST (3 x 5 min) and incubated in secondary antibody (1:20,000 with 3% milk) for 1 h at room temperature before washing in TBST (3 x 5 min) and detection by chemiluminescence (Millipore; cat. WBKLS0500). Membranes were imaged using Fluorochem E Imaging system (Protein Simple; Alpha Innotech, Santa Clara, CA). Bands were quantified using Protein Simple AlphaView SA software and normalized to Ponceau S staining as Ponceau S has been validated as an alternative loading control (i.e. actin) (Rivero-Gutierrez et al., 2014, Romero-Calvo et al., 2010).
3.7.3 Immunofluorescence methodology to measure mTORC1 localization

Muscle biopsy samples (~25mg) were freed from any visible fat and connective tissue prior to being mounted in Optimal Cutting Temperature Compound (Tissue-Tek®, VWR) and frozen in isopentane cooled by liquid nitrogen prior to storage at -80°C for subsequent immunofluorescence analysis. Embedded muscle samples were fixed on the position in front of the blade of a microtome and serial sections (7μm) were collected onto room temperature uncoated glass slides. To remove excess crystallized water inside sections under storage, sections were left to air dry at room temperature for ~10min.

Sections (7 μm) were fixed in acetone and ethanol (3:1) solution for 5 min and then washed for 3 x 5min in phosphate buffered saline (PBS) to remove fixation reagent. Subsequently, sections were incubated in primary antibody solution for 2h (Table 1) diluted 1:200 (depending on antibody) with 5% normal goat serum. Following incubation, sections were washed for 3 x 5min in PBS and incubated in the appropriate secondary antibody for 30min at room temperature. Sections were finally incubated with Wheat Germ Agglutinin (WGA) (Invitrogen cat. W11263) for 20min at room temperature to mark the sarcolemmal membrane. The slides were left to air dry until the visual water stains evaporated for 1-2 min at room temperature. Sections were then mounted with 20μL of fluorescence mounting media (Dako, Denmark) and sealed by glass cover slips to protect the muscle sections and to preserve fluorescence signals. Slides were then left overnight before observation under an EVOS FL Auto Cell imaging microscope (Thermo Fisher, CA) at 20x0.45 magnification. For full mTOR/Rheb and TSC2/Rheb colocalization protocol see Appendix 4.

For images capture, DAPI UV (340–380nm) filter was used to view WGA-350 (blue) signals and mTOR stains tagged with Alexa 594 fluorophore (red) was visualized under the Texas red (540–580 nm) excitation filter. GFP (465–495nm) excitation filter was left to capture signals of mTOR-associated proteins, which were conjugated with Alexa Fluor 488 fluorophore (Table 1). All image capturing was kept constant between images, including exposure time, gain, image frame and light intensity. For colocalization analysis, 3-5 areas per section (6-7 fibers) were randomly selected, imaged under the same capture settings and averaged (r-value). All image processing and quantitation was carried out in ImagePro Premier v9.2 (Media Cybernetics, MD,
USA) where Pearson’s correlation coefficient was used to quantify correlations between mTOR/TSC2 with Rheb.

**Table 2:** Antibody information for mTOR colocalization using immunofluorescence

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Source</th>
<th>Dilution</th>
<th>Secondary antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoclonal anti-mTOR antibody weight mouse antigen, isotype IgG y1 kappa</td>
<td>Millipore, 05-1592</td>
<td>1:200</td>
<td>Goat anti-mouse IgGy1 Alexa594</td>
<td>1:200</td>
</tr>
<tr>
<td>Monoclonal anti-Rheb antibody with rabbit antigen, isotype IgG</td>
<td>Abcam, Ab92313</td>
<td>1:50</td>
<td>Goat anti-rabbit Alexa488</td>
<td>1:200</td>
</tr>
<tr>
<td>Monoclonal anti-TSC2 antibody with mouse antigen, isotype IgGy1</td>
<td>Abgent, AM1919b</td>
<td>1:200</td>
<td>Goat anti-mouse IgGy1 Alexa594</td>
<td>1:200</td>
</tr>
<tr>
<td>Wheat Germ Agglutinin</td>
<td>ThermoFisher, W11263</td>
<td>1:20</td>
<td>Alexa350 conjugated</td>
<td></td>
</tr>
</tbody>
</table>

### 3.8 Results, statistical analysis

Protein synthesis data were analyzed using a one-way ANOVA; blood and cell signaling data were analyzed by two-way ANOVA (two factor, time x condition) with repeated measures with Student–Newman–Keuls post hoc analysis when P<0.05. Plasma [³H]-phenylalanine enrichment was analyzed using linear regression. GraphPad version 6.00 for Windows (GraphPad Software, San Diego California USA) was subsequently used for all statistical analyses.
Chapter 4
Results

4.1 Subject characteristics

Eight trained men (Age = 25±6 y; Height = 177.3±6.1 cm; Body Mass = 72.4±7.6 kg; Fat Mass = 9.4±2.2 kg; Lean Mass = 60.4±5.7 kg; BMI = 23±2 kg/m²; VO₂peak = 62±7 ml/kg/min; means±SD) were recruited for the present study. All participants were active and currently trained, most having competed for a varsity cross country team. All participants met the inclusion criteria pertaining to health and fitness having obtained a minimum VO₂peak of 50mL/kg/min and having engaged in moderate-vigorous activity for at least 3d/week.

4.2 Plasma $[^2]H_5$-phenylalanine enrichment

Plasma $[^2]H_5$-phenylalanine enrichment was steady across time and not different (P=0.54; Figure 6) between REST (5.4±0.4 MPE) and EX (5.1±0.4 MPE) over the infusion period.

![Figure 6: Plasma $[^2]H_5$-phenylalanine enrichment (mole percent excess; MPE) before (-120-0min) and after (0-300min) physiological protein ingestion at rest (REST) and after endurance exercise (EX) over a 5h postprandial period. Dotted lines indicate endurance exercise (EX) from -60 to 0 min at 70% of VO₂ peak.](image-url)
4.3 Plasma insulin

Plasma insulin concentrations (Figure 7) were similarly increased after feeding in REST and EX, with values peaking at 30 min (~20 fold; P<0.0001) and returning to baseline at 120 min (P=0.86). There were no significant differences between REST and EX during the 300-min postprandial period (P=0.52).

Figure 7: Plasma insulin concentration (mU/L) in REST and EX. * denotes difference from -180 min in REST and EX (P<0.0001).
4.4 MyoPS & MitoPS

MyoPS was increased (P<0.01; Fig. 8A) above fasted at rest (FED; ~2.1-fold) and following 1h of treadmill running 70%VO$_{2peak}$ (EX FED; ~2-fold) during the 5h postprandial period. MyoPS was not different (p=0.67) between FED and EX FED. There was no effect (P=0.135; Fig. 8B) of feeding or exercise on MitoPS.

![Myofibrillar FSR](image1)

![Mitochondrial FSR](image2)

**Figure 8:** Myofibrillar (A) and mitochondrial (B) fractional synthetic rate (FSR) in the fasted state (FASTED) and after physiological protein ingestion at rest (REST) and after endurance exercise (EX) over a 5h postprandial period. *Different from FASTED, P < 0.05.
4.5 mTORC1 signaling: Western blot

4E-BP1\textsuperscript{Thr37/46} phosphorylation was increased above FASTED (P<0.01; Fig. 9A.) in REST (~2-fold) and EX (~1.8-fold) 1h after beverage ingestion. There was no effect of exercise or nutrition on other candidate intracellular signaling molecules (i.e. mTORC1\textsuperscript{ser2448}, S6K1\textsuperscript{Thr389}, eEF2\textsuperscript{Thr56}; Fig. 9B-D). A ~1.8-fold increase (P=0.056; Fig. 9E) in ERK1/2\textsuperscript{Thr202/Tyr204} phosphorylation above REST late in recovery (5h) during EX and a ~1.5-fold greater p38 MAPK\textsuperscript{Thr180/Tyr180} phosphorylation for EX than REST (main effect; P<0.01; Fig 9F) occurred after nutrient ingestion. For representative Ponceau S stain as a loading control see Appendix 5.
Figure 9: Change in (a) mTOR$^{\text{Ser2448}}$ (b) S6K1$^{\text{Thr389}}$ (c) 4E-BP1$^{\text{Thr37/46}}$ (d) eEF2$^{\text{Thr56}}$ (e) Erk1/2$^{\text{Thr202/Tyr204}}$ (f) p38$^{\text{Thr180/Tyr180}}$ phosphorylation before and after physiological protein ingestion at rest (REST) or after endurance exercise (EX). *Different from FASTED, $P < 0.05$. †Different from condition at same time point, $P = 0.056$. 

Exercise effect, $P < 0.01$.
4.6 mTORC1 colocalization: Immunofluorescence

There was a main effect for condition (EX>REST; P<0.001) for the association of mTOR with Rheb. Furthermore, in response to feeding, mTOR colocalization increased above FASTED, during REST and EX at 1 and 5H, by ~18% and 26%, respectively (P<0.01) (Figure 10). mTOR colocalization with WGA similarly increased ~27-28% (P<0.01) above FASTED during REST and EX at 1h but was not different (P>0.44) from baseline at 5H FED in REST and EX (Figure 11).

Furthermore, immediately following EX, TSC2 colocalization with Rheb increased ~20%, when compared to REST FASTED (P<0.001). Following beverage ingestion, TSC2/Rheb colocalization in FED and EX FED similarly decreased (P<0.01) at 1H and 5H (~13-20%), when compared to REST FASTED (Figure 12). TSC2 colocalization with WGA similarly decreased ~22-32% below FASTED during REST and EX (P<0.01) but was not different (P>0.15) from baseline at 5H FED in REST and EX (Figure 13).
Figure 10: Immunofluorescence quantification of mTOR (Red) and Rheb (Green) interaction, displayed as a composite image (Merge) and WGA (Blue). Orange regions represent mTOR and Rheb interaction. (a) Each panel represents one subject from REST and EX across the experimental time course. (b) Group data are quantified and reported; circles represent REST, squares represent EX. All data presented relative to REST FASTED. Scale bar represents 200μm area. Data presented as mean ± SD. *Different from FASTED in same condition (P<0.01). **main group effect for EX (P<0.001).
Figure 11: Immunofluorescence quantification of mTOR (Red) and WGA (Blue) interaction, displayed as individual images. (a) Each panel represents one subject from REST and EX across the experimental time course. (b) Group data are quantified and reported; circles represent REST, squares represent EX. All data presented relative to REST FASTED. Scale bar represents 200μm area. Data presented as mean ± SD. *Different from FASTED in same condition (P<0.01).
Figure 12: Immunofluorescence quantification of TSC2 (Red) and Rheb (Green) interaction, displayed as a composite image (Merge) and WGA (Blue). Orange regions represent mTOR and Rheb interaction. (a) Each panel represents one subject from REST and EX across the experimental time course. (b) Group data are quantified and reported; circles represent REST, squares represent EX. All data presented relative to REST FASTED. Scale bar represents 200 μm area. Data presented as mean ± SD. *Different from FASTED in same condition (P<0.01). †Different from REST at same time point (P<0.001)
Figure 13: Immunofluorescence quantification of TSC2 (Red) and WGA (Blue) interaction, displayed as individual images. (a) Each panel represents one subject from REST and EX across the experimental time course. (b) Group data are quantified and reported; circles represent REST, squares represent EX. All data presented relative to REST FASTED. Scale bar represents 200μm area. Data presented as mean ± SD. *Different from FASTED in same condition (P<0.01).
Chapter 5
Discussion

5.1 Introduction

Dietary protein increases the synthesis of skeletal muscle proteins when consumed at rest and after resistance exercise as a means to remodel and repair this vital tissue (Phillips, 2009). Currently, our understanding of the role that dietary protein plays in stimulating the synthesis of fraction-specific muscle proteins at rest and following endurance exercise and the underlying mechanisms that orchestrate the remodeling process in vivo in humans is at a relative infancy. Therefore, using stable isotopes to directly measure fraction-specific MPS, our primary outcome was to examine the effects dietary protein ingestion towards MPS at rest and following a single bout of endurance exercise in trained young men. Furthermore, to provide mechanistic insight into nutrient and exercise regulation of MPS, we employed Western blotting and immunofluorescence methodologies, where the latter was used to characterize the association of positive and negative regulators of the primary growth regulatory mTORC1 signaling pathway. We hypothesized that feeding (20g PRO + 50g CHO) would enhance both Myo/MitoPS at rest and that 1h of endurance exercise would enhance the postprandial response as a means to remodel these skeletal muscle proteins.

In order for a robust MPS to be accurately measured by a primed constant tracer infusion the enrichment in the precursor pool for protein synthesis (i.e. tRNA) or a proxy surrogate (e.g. plasma) of the labelled isotope must be in isotopic steady state (Patterson et al., 1997). In the present study, plasma \( \[^2\text{H}_5\] \)-phenylalanine enrichment was steady across time and similar across conditions (REST and EX). Therefore, our modeling for MPS was satisfactory as we met the requirements of isotopic tracers in metabolic research (i.e. constant steady state of labelled tracer in response to a physiological stimulus) (Patterson et al., 1997). To this point, we demonstrate that: i) MyoPS is primarily nutrient sensitive over a 5h postprandial period at rest and after endurance exercise in trained young men; ii) MitoPS is uninfluenced by acute exercise or nutritional interactions; iii) mTOR/MAPK (i.e. 4E-BP1\(^{Thr37/46}\), ERK1/2\(^{Thr202/Tyr204}\), p38 MAPK\(^{Thr180/Tyr180}\)) signaling was elevated with feeding and exercise via Western blotting methodology, and; iv) the association of positive and negative regulators of mTORC1 using
immunofluorescence revealed that mTOR/Rheb colocalization increased with feeding/exercise with a reciprocal decrease in disassociation with TSC2/Rheb following feeding/exercise, which would be consistent with an increased activation and attenuated suppression, respectively, of the mTOR signalling pathway. The following sections will contextualize our data with previous literature examining the effects of nutrient and/or exercise-induced regulation of MPS, where most parallels will be drawn from endurance exercise-based studies. Lastly, in light of the current study, strength and limitations will be reviewed and suggestions for future research to fill in potential gaps in the area will be discussed.

5.2 MyoPS

In the fasted state, rates of MyoPS are generally ~0.03–0.05% h⁻¹ (Moore et al., 2009, Witard et al., 2014, Camera et al., 2015), due to a lack of amino acids as substrates to stimulate MyoPS. Fasted rates of MyoPS in the present study were 0.013 ± 0.01% h⁻¹, which are generally lower than what has been previously described utilizing a stable isotope of phenylalanine (Moore et al., 2009, Witard et al., 2014, West et al., 2011, Camera et al., 2015). This apparently lower fast MyoPS could be attributed in part to using plasma tracer (i.e. [²H₅]-phenylalanine) enrichment, rather than intracellular tracer enrichment, as done in the previous studies (Moore et al., 2009, Witard et al., 2014, Camera et al., 2015). For example, the enrichment of a labelled tracer (i.e. [²H₅]-phenylalanine) would be higher in the plasma, rather than in the muscle intracellular pool, which would result in a lower FSR (Witard et al., 2014, Smith et al., 2011). Therefore, when we calculate FSR (section 4.7.1.) by dividing ΔEₚ, the change in bound protein enrichment ([²H₅]-phenylalanine) between two biopsy samples, by Eₘᵢ, the average enrichment of plasma (higher [²H₅]-phenylalanine enrichment) or intracellular (lower [²H₅]-phenylalanine enrichment) phenylalanine across the two biopsy samples, would result in a lower FSR using the plasma as a surrogate marker to measure FSR. Regardless of the method used, the comparison of relative rates of MyoPS can be made across studies (Smith et al., 2011). Furthermore, the design of a study allows the relative differences of the FSR between conditions (i.e. fasted vs. fed) to be determined. To this point, in the present study, we demonstrated that MyoPS was elevated above the fasted state at rest (~2.1-fold) during the 5h postprandial period, which is agreement with previous findings that protein ingestion enhances MyoPS (Moore et al., 2009, Witard et al., 2014, Robinson
et al., 2013). Within these studies, protein was fed in isolation (i.e. not co-ingested with CHO), and enhanced MyoPS above fasted ~1.5 fold (Witard et al., 2014) and ~2.6 fold (Moore et al., 2009), respectively. In the present study we fed both PRO + CHO, and the doubling of MyoPS observed at rest was likely primarily driven by amino acids as substrates, and not insulin per se, to remodel myofibrillar proteins (Phillips, 2008, Staples et al., 2011, Atherton and Smith, 2012). Consistent with our findings, the ingestion of a mixed macronutrient meal (~25g PRO, 23g CHO, 6g fat), resulted in a ~3.6 fold increase in MyoPS above fasted (Churchward-Venne et al., 2014). Notably, Churchward-Venne et al. (2014) measured MyoPS using intracellular tracer enrichment, resulting in a slightly higher rates when compared to other studies that utilized plasma tracer (i.e. $[^2\text{H}_5]$-phenylalanine) enrichment (Witard et al., 2014, Smith et al., 2011). However, as expected, we observed a robust feeding response towards MyoPS at rest, replicating previous data (Moore et al., 2009, Moore et al., 2015, Witard et al., 2014, Churchward-Venne et al., 2014) suggesting that the myofibrillar protein fraction is sensitive to dietary protein ingestion at rest.

In response to resistance exercise, feeding has been shown to synergistically enhance MyoPS above that during the rested fed state (Moore et al., 2009, Witard et al., 2014, Robinson et al., 2013). Based on the importance of remodeling skeletal muscle during recovery from exercise, we speculated that this additive effect on MyoPS of feeding and resistance exercise would also extend to our endurance exercise stimulus in order to promote recovery through an increase in protein turnover (Moore et al., 2014) However, in disagreement with our initial hypothesis, our data demonstrated that endurance exercise did not further enhance postprandial MyoPS.

The apparent lack of effect of endurance exercise towards enhancing postprandial MyoPS may be related to the training status of our participants. For example, we speculate that the exercise intensity and/or duration (i.e. 70% VO$_2$peak for 1h) was insufficient to further amplify postprandial MyoPS as our subjects were trained (i.e. VO$_{2\text{peak}}$=62±7 ml/kg/min). To this end, in untrained participants, both low (60 min at 35% VO$_2$max) and high (30min at 70% VO$_2$max) intensity exercise enhances fasted state MyoPS during 4.5h of recovery (Di Donato et al., 2014), suggesting a sufficient exercise stimuli to induce myofibrillar protein remodeling. In agreement with our findings, Wilkinson et al. (2008) also demonstrated that in trained participants, endurance exercise (~75% VO$_2$max for 1h) did not enhance postprandial MyoPS, suggesting the insufficient exercise
stimuli to increase myofibrillar protein turnover. Therefore, the exercise intensity in the present study may have been insufficient to have an additive effect of MyoPS in response to feeding.

In addition to a suboptimal exercise intensity, the modality (i.e. running) may have dampened any additive effect feeding had following endurance exercise. To elaborate, the endurance exercise modality in the present study (i.e. treadmill running) may have been suboptimal in activating the vastus lateralis (biopsy cite) (Harber et al., 2009, Bijker et al., 2002), in turn, failing to further amplify MyoPS. For example, cycling involves only concentric contractions, while in contrast, running involves both concentric and eccentric contraction of the legs (Bijker et al., 2002). Furthermore, eccentric muscle actions (i.e. running) require less metabolic energy (i.e. storing elastic recoil energy during normal locomotion and/or running) than concentric contractions (i.e. cycling), hence the greater electromyography activity observed in the vastus lateralis from cycling vs. running exercise (Bijker et al., 2002). However, in the present study, we chose running as our exercise stimuli as we believe it has greater ecological validity when compared to cycling (i.e. humans are bipedal). Furthermore, running is an understudied exercise modality as the majority of endurance-based studies use cycling as their exercise modality to stimulate MyoPS (Di Donato et al., 2014) Therefore, in the present study, running may have been a suboptimal exercise modality to engage the vastus lateralis, and as a result, may have contributed towards not further stimulating postprandial MyoPS.

The postprandial response to dietary protein ingestion at rest typically occurs over a 4-6h period after nutrient ingestion (Moore et al., 2009, Witard et al., 2014, Atherton et al., 2010). In the present study, we measured MyoPS over 5h as we wanted to examine if endurance exercise enhances the postprandial response of MyoPS when protein is ingested at rest. However, previous literature has demonstrated that MyoPS is enhanced for at least 24h following endurance-based exercise (Di Donato et al., 2014, Burd et al., 2011, Cuthbertson et al., 2006, Doering et al., 2016). For example, it has been previously reported that MyoPS in the fasted state following endurance exercise (Di Donato et al., 2014) and in the fed state following low-load (30% 1-RM) resistance exercise to failure is elevated 24h post exercise (Burd et al., 2011). Furthermore, MyoPS has also been shown to be elevated 24h following 12 minutes of dynamic shortening or lengthening exercise in response to bolus feeding (45g EAA + 135g CHO) (Cuthbertson et al., 2006). Additionally, MyoPS has been shown to be elevated 72h following 1 h of one-legged kicking.
exercise at 67% (Wmax) (Miller et al., 2005). Collectively, these data suggest that endurance-based exercise has a sensitizing effect towards enhancing MyoPS that lasts day(s), which would be consistent with enhanced muscle protein remodeling following exercise. In the present study, we measured MyoPS over a 5h postprandial period, and as such, we acknowledge that in the fed state, MyoPS may be elevated 24h post endurance exercise (Doering et al., 2016). In turn, this elevation of MyoPS up to 24h post-exercise would assist in muscle protein remodeling and renewal (Doering et al., 2016). Nonetheless, our data suggest that endurance exercise did not enhance postprandial MyoPS in trained young men, when measured over 5h.

5.3 MitoPS

In the present study, we also hypothesized that feeding would enhance MitoPS at rest, which would serve to enhance the remodelling of this vital organelle. To this point, when amino acids are intravenously infused, there is an apparent stimulation of MitoPS at rest (Bohe et al., 2003, Bohe et al., 2001), suggesting that mitochondria may be responsive to amino acids. However, the physiological significance of these studies may be confounded by a limited number of observations (i.e. n=2) for MitoPS (Bohe et al., 2001) or group comparisons between constant infusion rates (Bohe et al., 2003). To clarify, the advantage of using a within-subject design, whereby each individual serves as their own fasted/rested control, is that acute changes in FSR can be attributed primarily to the nutritional and/or exercise stimulus rather than to inter-subject variability. Furthermore, in both studies (Bohe et al., 2003, Bohe et al., 2001), participants received a continuous infusion of amino acids, which resulted in a ~2-3 fold increase in plasma amino acid concentrations that was sustained over 4h. This is in contrast to the transient increases in amino acid concentrations in response to physiologically-relevant bolus feeding (Moore et al., 2009, Witard et al., 2014, West et al., 2011). Therefore, to overcome these limitations in the present study, we utilized a repeated measures (within-subject) design and provided a physiologically discrete dose of protein that has been shown to maximize myofibrillar and sarcoplasmic protein synthesis (Cuthbertson et al., 2005, Witard et al., 2014). Contrary to our initial hypotheses, our data suggest that MitoPS is uninfluenced by protein and carbohydrate ingestion in trained young men over a 5h postprandial period. Drawing parallels between Bohe et al. (2001) and Bohe et al. (2003), whose data suggests a sustained aminoacidemia can promote MitoPS, the moderate 20g dose of protein may have been insufficient to increase the peak and/or
duration of aminoacidemia that would be necessary to stimulate the synthesis of this protein fraction. It is noteworthy that the relative size of the skeletal muscle mitochondrial protein pool (~10%) is substantially smaller than the major myofibrillar protein pool (60–70%), the latter of which is clearly nutrient responsive (Moore et al., 2009, Cuthbertson et al., 2005, Moore et al., 2015, Witard et al., 2014, Churchward-Venne et al., 2014). It is possible the moderate increases in amino acids after discrete protein ingestion may not be required for the stimulation of MitoPS in the rested state due to a compartmentalization of amino acids towards and/or within within mitochondria (Hallberg and Larsson, 2014). For example, it has been suggested that due to the presence of their own DNA, the synthesis of new mitochondria (through enhanced ribosomal biogenesis, translation etc.) may be intrinsically (i.e. within the mitochondria) regulated (Hallberg and Larsson, 2014), which could generally dissociate its acute regulation from external regulatory factors such as moderate dietary protein ingestion. As such, our data suggest that the mitochondria are not sensitive to a discrete intake of a moderate dose of exogenous amino acids. Therefore, unlike myofibrillar proteins, the availability of intracellular amino acids may be prioritized towards maintaining MitoPS at rest; this could ultimately serve to optimize ATP production and/or cell viability, independent of nutrient ingestion.

In addition to examining the nutrient sensitivity of the mitochondria at rest, we also sought to examine whether endurance exercise enhances postprandial MitoPS as a means to support the traditional phenotypic adaptations associated with chronic endurance exercise (e.g. mitochondrial biogenesis). Previous findings suggest that post-endurance exercise protein/amino acid ingestion does not enhance MitoPS (Breen et al., 2011, Coffey et al., 2011). The lack of basal rates of protein synthesis in these studies (Breen et al., 2011, Coffey et al., 2011), however, precluded the ability to determine if there were exercise-specific effects on MitoPS during a postprandial period. In the present study, our design allowed us to assess the exercise effects of MitoPS at rest and following endurance exercise. Contrary to our hypothesis, we demonstrated that endurance exercise did not alter MitoPS.

Similar to that of MyoPS, previous research has suggested that training status and/or exercise intensity can influence MitoPS. For example, in response to moderate intensity cycling (55% VO2peak for 40 min), 20g of protein enhances MitoPS above fasted, in untrained, middle
aged men (Donges et al., 2012). In contrast, Wilkinson et al. (2008) demonstrated that MitoPS is increased after endurance exercise in the fed state before (30min at 70% VO₂ max) but not after (60min at 70% VO₂ max) 8 weeks of endurance training. In contrast to Donges et al. (2012) but similar to Wilkinson et al. (2008), our participants were previously trained, which could suggest that there was an attenuated MitoPS response to our exercise stimulus. It is possible that a greater exercise intensity (i.e. high intensity interval training) and/or duration (i.e. 1.5h) would have been required to stimulate MitoPS in our trained population. Therefore, within the present study, endurance exercise did not enhance postprandial MitoPS, suggesting chronic training may dampen the response towards mitochondrial remodeling.

Similar to that of the myofibrillar protein fraction, we speculate that the timing of our measurement may have not captured the enhancement of MitoPS. MitoPS is not increased early (0.5-4.5h) in recovery after an acute bout of endurance exercise (~70% VO₂ max for 30min) in the fasted state (Di Donato et al., 2014). However, MitoPS is elevated 24-28h following this endurance exercise stimulus (Di Donato et al., 2014), indicating remodeling of this organelle occurs beyond the early acute 5h recovery period. In the present study, we measured MitoPS over 5h as we sought to determine if endurance exercise enhances the postprandial response of protein ingestion, which would be a sufficient measurement period to capture the complete feeding response to protein ingestion (Moore et al., 2009). Therefore, while we did not observe an increase towards postprandial MitoPS immediately after endurance exercise, we cannot rule out that we may have missed any remodeling of the mitochondria that would have occurred late (i.e. 24h later) in recovery.

Finally, it has been previously demonstrated that higher protein intakes (~64 g) following endurance exercise enhance mitochondrial energy pathway signaling (i.e. peroxisome proliferator-activated receptor gamma family expression) up to 48h post-endurance exercise (Rowlands et al., 2011), which may suggest mitochondrial protein remodeling at this time point. This enhancement in mitochondrial transcriptome that occurred late (48h) in recovery (but not at 3h) (Rowlands et al., 2011) could be related to the relatively large post-exercise bolus protein dose, which would result in an abundance of amino acids to repair all protein fractions (i.e. both mitochondrial and myofibrillar pools) (Moore and Stellingwerff, 2012). Nevertheless, the ingestion of 24g of protein,
with 4.8g leucine and 50g of carbohydrates before exercise did not enhance MitoPS after repeated high-intensity sprints (Coffey et al., 2011). Moreover, the ingestion of 20g of protein with 50g of carbohydrates following endurance exercise (90min at ~77% VO₂max) did not augment MitoPS (Breen et al., 2011). Therefore, in both studies (Breen et al., 2011, Coffey et al., 2011), the dose required to provide ample amino acids to remodel the mitochondria may have been suboptimal. In the present study, 20g of protein with 50g of carbohydrates within the present study did not enhance postprandial MitoPS, which leads us to believe that a greater dose may be required to enhance MitoPS following endurance exercise. To this end, dose response studies following resistance exercises suggest that 20g of high quality protein maximizes MyoPS (Moore et al., 2009, Witard et al., 2014). However, the amount of protein required (through a dose response study) to maximize MitoPS and subsequently optimize recovery from endurance exercise, has yet to be determined. Regardless, our data suggest that postprandial MitoPS is uninfluenced by acute endurance exercise in trained young men after the ingestion of 20g of protein with 50g of carbohydrates.

5.4 Western blot signaling

To provide mechanistic insight towards MyoPS and MitoPS, we examined the phosphorylation of mTORC1 (i.e. p-mTOR_{Ser2448}), the master regulator of MPS (by regulating mRNA translation initiation) (Dickinson et al., 2011, Drummond et al., 2009), and various protein candidates within this anabolic signaling cascade. In the present study, there was no change in mTOR_{Ser2448} phosphorylation in response to feeding, which is consistent with previous findings that fed 25g of whey protein at rest (Moore et al., 2011). Furthermore, high insulin doses of 72 and 167 mU/l does not alter mTOR_{Ser2448} phosphorylation at rest (Greenhaff et al., 2008). As such, we speculate that feeding may have phosphorylated downstream activators of mTORC1, which would be more reflective of mTORC1 activity (Greenhaff et al., 2008, Ekim et al., 2011, Atherton et al., 2010). Therefore, at rest, mTOR_{Ser2448} phosphorylation may not be regulating changes towards MyoPS and/or MitoPS.

Similar to resistance exercise (Moore et al., 2011), mTOR_{Ser2448} phosphorylation following endurance exercise and feeding was unaltered over 5h within the present study. These findings are
in disagreement with pervious literature demonstrating that \( \text{mTOR}^{\text{Ser2448}} \) phosphorylation is enhanced in the fasted state at 0.5h following 0.5-1h of cycling at \( \sim 75\% \) \( \text{VO}_2\text{max} \) (Di Donato et al., 2014, Mascher et al., 2007). However, as mentioned previously, participants in Di Donato et al. (2014) were untrained; hence this increase towards \( \text{mTOR}^{\text{Ser2448}} \) phosphorylation may have contributed to increased myofibrillar protein remodeling. Furthermore, prior training has been shown to attenuate the exercise-specific signaling responses (e.g. dampened AMPK and \( \text{mTOR} \) phosphorylation following endurance and resistance exercise, respectively) involved with exercise (Coffey et al., 2006), which may explain the lack of \( \text{mTOR}^{\text{Ser2448}} \) phosphorylation following endurance exercise within the present study. Therefore, a greater exercise stimulus may have been required to promote \( \text{mTOR}^{\text{Ser2448}} \) phosphorylation, potentially stimulating MyoPS. Nonetheless, we demonstrate that \( \text{mTOR}^{\text{Ser2448}} \) phosphorylation is unaltered with feeding at rest and following endurance exercise.

The most well-characterized proteins downstream of mTORC1 that respond to feeding are arguably S6K1 and 4E-BP1, which directed our decision to measure S6K1\(^{\text{Thr389}}\) and 4E-BP1\(^{\text{Thr37/46}}\) phosphorylation as a proxy of mTOR activity (Moore et al., 2011, Atherton et al., 2005, Koopman et al., 2007, Koopman et al., 2006). When 4E-BP1 is phosphorylated, it disassociates from eIF4E, allowing the formation of the eIF4F complex, which subsequently allows 4E-BP1 to bind mRNA and initiate the rate-controlling step for MPS of mRNA translation (Kimball et al., 1999). Furthermore, the phosphorylation of S6K1 subsequently phosphorylates its translational downstream targets (i.e. eIF4B, rpS6) which then recruits S6K1 into the eIF3 preinitiation complex, promoting ribosomal assembly and subsequently enhancing mRNA translation (Laplante and Sabatini, 2009). Consistent with a lack of effect on \( \text{mTOR}^{\text{Ser2448}} \) phosphorylation, we demonstrate that at rest the ingestion of 20g of protein and 50g of carbohydrates does not enhance S6K1\(^{\text{Thr389}}\) phosphorylation. This finding is in disagreement with Moore et al. (2011) who demonstrated that S6K1\(^{\text{Thr389}}\) phosphorylation is elevated 1h following 25g of whey protein ingestion at rest but returned back to baseline by 5h. Although the discrepancy between studies is unclear, it has been recently demonstrated that S6K1\(^{\text{Thr389}}\) phosphorylation may not adequately represent the true kinase activity in response to protein ingestion (McGlory et al., 2014), suggesting a disassociation between phosphorylation and kinase activity. On the contrary, 1h following feeding 4E-BP1\(^{\text{Thr37/46}}\) phosphorylation was elevated at rest (\( \sim 2\)-fold) which may have
mediated the ~2-fold increase we observed with MyoPS at rest. These findings are in agreement with previous studies, demonstrating that protein ingestion enhances 4E-BP1<sup>Thr37/46</sup> phosphorylation at rest (Atherton et al., 2010, Moore et al., 2011, Fujita et al., 2007), suggesting that 4E-BP1 is nutrient sensitive. Notably, 4E-BP1<sup>Thr37/46</sup> phosphorylation did not mirror the lack of change in MitoPS, suggesting a mechanism that promotes MitoPS independent from mTORC1 (Philp et al., 2015). However, our data suggest that the transient increase towards 4E-BP1<sup>Thr37/46</sup> phosphorylation at 1h following feeding (and presumably increased mRNA translation initiation) may contribute, in part, to the stimulation of MyoPS at rest.

In addition to examining the phosphorylation of S6K1 and 4E-BP1 at rest, we also sought to determine how endurance exercise and feeding influence these downstream targets of mTORC1. Previous research has demonstrated that S6K1<sup>Thr389</sup> phosphorylation is elevated following resistance exercise and protein ingestion (Moore et al., 2009, West et al., 2011, Drummond et al., 2009, Moore et al., 2011). In the present study, we demonstrated that endurance exercise and feeding did not further enhance S6K1<sup>Thr389</sup> phosphorylation. This finding is in agreement with previous endurance exercise studies that took muscle biopsies at similar time points (Coffey et al., 2011, Di Donato et al., 2014, Camera et al., 2010, Coffey et al., 2006). Collectively, these data suggest that the type of contractile activity (i.e. resistance vs endurance) appears to influence the S6K1<sup>Thr389</sup> phosphorylation. To elaborate, resistance exercise is characterized by high frequency/force contractions whereas endurance exercise is characterized by low frequency/force contractions. As a result, S6K1<sup>Thr389</sup> phosphorylation appears to be responsive towards high force contractile activity (i.e. resistance exercise) (Atherton et al., 2005, Koopman et al., 2006) and not endurance exercise, per se. Consistent with this notion, we demonstrate that S6K1<sup>Thr389</sup> phosphorylation was not enhanced with feeding and endurance exercise. In the present study, we observed that MyoPS was elevated above fasted ~2-fold following endurance exercise and feeding, which, similar to that at rest, may have been mediated in part through a similar increase in 4E-BP1<sup>Thr37/46</sup> phosphorylation 1h after feeding. Our findings are in agreement with previous literature demonstrating that 4E-BP1<sup>Thr37/46</sup> phosphorylation is elevated following endurance exercise in the fed state (Coffey et al., 2011, Morrison et al., 2008). Therefore, we demonstrate that anabolic signalling that would be consistent with an enhanced mRNA translation initiation (i.e. increased...
4E-BP1\textsuperscript{Thr37/46} phosphorylation) 1h after feeding and endurance exercise may contribute to the increase in MyoPS.

In addition to understanding the regulation of mRNA translation initiation (i.e. S6K1, 4E-BP1) in response to feeding and endurance exercise, we also assessed how mRNA translation elongation is influenced by feeding at rest and following endurance exercise. We measured eEF2\textsuperscript{Thr56} phosphorylation, a negative regulator of mRNA translation elongation, to provide further insight towards MyoPS. At rest, we demonstrate that eEF2\textsuperscript{Thr56} phosphorylation is unaltered with feeding, which is consistent with previous findings (Moore et al., 2011); this suggests that, in our hands, mRNA translation elongation may not contribute to the postprandial stimulation of MyoPS at rest. Furthermore, previous data suggest MPS is suppressed immediately after exercise, which may be attributed to enhanced eEF2\textsuperscript{Thr56} phosphorylation that functions to suppress mRNA translation elongation (Rose and Richter, 2009, Rose et al., 2005). In fact, in the absence of protein ingestion following endurance exercise, eEF2\textsuperscript{Thr56} phosphorylation is elevated, which may serve to constrain the exercise-induced stimulation of MyoPS (Breen et al., 2011). However, in the present study, protein and carbohydrate ingestion following endurance exercise did not alter eEF2\textsuperscript{Thr56} phosphorylation, which is consistent with Breen et al. (2011) who demonstrated that eEF2\textsuperscript{Thr56} phosphorylation was unaltered at 0 and 4h following endurance exercise in response to protein and carbohydrate ingestion. Therefore, these data suggest that mRNA translation initiation, rather than elongation, may be the predominant mechanism mediating the increases in MyoPS in response to feeding at rest and following endurance exercise.

We also measured ERK1/2\textsuperscript{Thr202/Tyr204} phosphorylation as it has been shown to regulate proteins (i.e. p90RSK, eEF2) involved in mRNA translation initiation and elongation (Wang et al., 2001, Roux et al., 2007), independent of mTOR activity (Drummond et al., 2009, Williamson et al., 2003). As such ERK1/2 may play a role in regulating post-exercise induced increases in MyoPS. Consistent with our findings, ERK1/2\textsuperscript{Thr202/Tyr204} phosphorylation has previously been shown to be unresponsive towards nutrition at rest (Moore et al., 2011). In contrast, resistance exercise enhances ERK1/2\textsuperscript{Thr202/Tyr204} phosphorylation following 1h of bolus protein ingestion (Moore et al., 2011), suggesting this kinase is responsive to muscle contraction. Consistent with this notion, it should be noted that ERK1/2\textsuperscript{Thr202/Tyr204} phosphorylation is elevated 24h following...
resistance exercise in the fasted state (Deldicque et al., 2008), which may in part contribute towards the previously demonstrated enhancement of MyoPS 24h after exercise endurance exercise (Di Donato et al., 2014). In the present study, we observed an increase in ERK1/2\textsuperscript{Thr202/Tyr204} phosphorylation above rest late in recovery (5h) following endurance exercise and feeding, which may suggest delayed myofibrillar remodelling. Therefore, although endurance exercise did not enhance postprandial MyoPS over 5h within the present study, the elevated the ERK1/2\textsuperscript{Thr202/Tyr204} phosphorylation observed above rest at 5h, may suggest that MyoPS is elevated outside of our 5h measurement period.

As mentioned previously, mRNA translation initiation (i.e. mTOR, S6K1, 4E-BP1) did not provide insight towards MitoPS as exercise and/or feeding did not enhance the synthesis of this muscle protein sub-fraction. Furthermore, recent evidence suggests that the stimulation of MitoPS following endurance exercise can occur independently of mTORC1 activity (Philp et al., 2015). Therefore, to provide mechanistic insight towards mitochondrial-specific remodeling, we measured the phosphorylation of the MAPK p38\textsuperscript{Thr180/Tyr180}, which is an upstream kinase of the transcription factor generally regarded as the master regulator of mitochondrial biogenesis PGC-1\textalpha (Uguccioni and Hood, 2011, Akimoto et al., 2005). We demonstrated that p38\textsuperscript{Thr180/Tyr180} phosphorylation was not enhanced with feeding at rest, which is consistent with the lack of change in MitoPS. With respect to contractile activity, previous reports demonstrate that p38\textsuperscript{Thr180/Tyr180} phosphorylation is elevated 4.5h following high and low intensity endurance exercise (Di Donato et al., 2014), which may contribute towards enhanced MitoPS 24h following endurance exercise. Although in the present study MitoPS was uninfluenced by nutrition and/or exercise, the increase of p38\textsuperscript{Thr180/Tyr180} phosphorylation with endurance exercise may suggest that mitochondrial remodeling and/or MitoPS may have been enhanced beyond the 5h recovery period we measured in this study. Mechanistically speaking, transcriptional activity (through p38\textsuperscript{Thr180/Tyr180} phosphorylation) may have been elevated during 5h; however, protein translation and ultimately MitoPS, may have occurred outside our 5h window.

In summary, the data obtained from the Western blot has demonstrated that mRNA translation initiation was elevated in response to feeding and exercise (i.e. 4E-BP1\textsuperscript{Thr37/46}), potentially underpinning the increases towards MyoPS. Furthermore, endurance exercise
enhanced the phosphorylation of MAPK, ERK1/2$^{\text{Thr202/Tyr204}}$, corroborating an increase towards MyoPS, and p38 MAPK$^{\text{Thr180/Tyr180}}$, demonstrating a dissociation between signaling (i.e. protein content) and MitoPS measured over a 5h period. Therefore, the analysis of different targets and/or use of different methods (i.e. immunofluorescence) will serve to further understand the molecular mechanisms regulating skeletal muscle plasticity.

5.5 Immunofluorescence signaling

Numerous studies in humans have demonstrated that mTORC1 activity (measured by phosphorylation through Western blotting) is enhanced following exercise with and without protein ingestion (Coffey et al., 2011, Di Donato et al., 2014, Burd et al., 2010). Although Western blotting techniques have provided tremendous insight towards linking mTORC1 and skeletal muscle plasticity, additional methodologies are required to increase our understanding of how mTORC1 is activated in human skeletal muscle at rest, in response to contractile activity (i.e. endurance exercise), and under different nutrient conditions. This in turn could help elucidate how mTORC1 activates pathways regulating mRNA translation and, potentially, MyoPS. To this end, we have incorporated, for the first time, the measurement of MPS in conjunction with Western blotting and immunofluorescence approaches to characterize the molecular mechanisms that underpin skeletal muscle remodeling at rest and following endurance exercise.

*In vitro* studies have revealed that TSC1/2 forms a heterodimeric complex which acts as negative regulator of mTORC1 signaling (Laplante and Sabatini, 2009). TSC1 and TSC2 inhibit mTORC1 activity through the GTPase activating protein characteristics of TSC2, which converts and hydrolyzes the active GTP form Rheb complex into the inactive GDP Rheb complex. In turn, the inactive Rheb cannot directly bind and co-localize with mTORC1, which subsequently inhibits the activation of mTORC1 (Inoki et al., 2003). Therefore, when mTOR is activated, it then co-localizes with Rheb (Garami et al., 2003), which would support an increase in protein synthesis. Since Rheb is the direct activator of mTOR, the disassociation of the negative regulator TSC2 away from Rheb (turning the inhibitor “off”) may be an important regulatory step in promoting protein synthesis and/or cell remodeling.
Within the present study, TSC2/Rheb colocalization decreased below fasted at 1 and 5 hours following feeding. This could suggest that the disassociation between the negative regulator TSC2, away from the positive regulator Rheb, may be an important regulatory mechanism to increase MyoPS at rest (Betz and Hall, 2013, Marcotte et al., 2015). Furthermore, as expected, TSC2/Rheb colocalization increased by ~22% immediately after endurance exercise above fasted, which would be consistent with a decreased protein synthetic response immediately following endurance exercise (Rose et al., 2005, Norton and Layman, 2006). This attenuated protein synthetic response during exercise could occur through an increase AMPK, which can activate TSC2 to promote its interaction with Rheb, blunting mTOR activity (Mounier et al., 2011, Bar-Peled and Sabatini, 2014, Kimball and Jefferson, 2010). However, similar to that of rest, we observed a ~13-16% decrease (relative to fasted) in TSC2/Rheb colocalization following endurance exercise and feeding, which would support the notion that TSC2 must be “turned off” in human muscle in order to “turn on” protein synthesis (Inoki et al., 2003, Atherton et al., 2005).

Importantly, we demonstrated that the decreased TSC2/Rheb colocalization after the ingestion of 20g of egg protein and 50g of carbohydrate was associated with a concomitant increase in mTOR/Rheb colocalization, which would be consistent with a feeding-induced stimulation of MyoPS. Interestingly, endurance exercise with feeding further increased the colocalization (~10%), when compared to rest (main effect for exercise) following feeding, providing the necessary conditions (i.e. mTOR in a position to be active) for potentially greater mRNA translation initiation following endurance exercise. However, this ~10% increase towards mTOR/Rheb colocalization was not consistent with a greater postprandial MyoPS response following endurance exercise, which may be partly explained by: i) the magnitude of change (~10%) not being robust enough to further stimulate MyoPS, and/or; ii) other signaling candidates may be dampening MyoPS (i.e. endurance exercise did not further dissociate TSC2 from Rheb when compared to rest). Nevertheless, our results are generally consistent with preliminary unpublished data in human muscle (Song et al., in review) demonstrating that mTOR/Rheb are colocalized following post-resistance exercise with protein ingestion as early as 15min after exercise and up to 3h into recovery. Collectively, our data demonstrating the translocation and association of mTOR with the plasma membrane and Rheb, would be consistent with enhanced mRNA translational activity following feeding at rest and after endurance exercise.
The site of protein synthesis in vivo in human skeletal muscle is largely unknown. In rodent skeletal muscle, ribosomes (i.e. rRNA) are generally located around the periphery of muscle cells (Horne and Hesketh, 1990). The peripheral ribosomal clustering would be consistent with localization of new proteins in vivo, as determined by immunofluorescence visualization of puromycin (Goodman et al., 2012). Furthermore, the presence of ribosomes in close proximity to the capillaries that deliver anabolic nutrients would ideally position these regulatory proteins to respond to amino acid-induced to enhancement of mRNA translation (Bar-Peled and Sabatini, 2014). Thus, immunofluorescence can be utilized to determine if mTOR translocates towards the periphery of muscle cells, the potential site of protein synthesis. As a result, immunofluorescence analysis has recently revealed that eccentric contractions in rat skeletal muscle result in a dissociation of TSC2 from the lysosome as a means to promote mTOR/Rheb interaction (Jacobs et al., 2013), which suggests these events are fundamental to regulate mTOR signaling. In our hands, we observed TSC2 to be located at the plasma membrane and colocalized with Rheb in the rested fasted state, which would be consistent with low rates of MyoPS in the absence of an anabolic stimulus (e.g. amino acids and/or exercise). In response to feeding at rest and following endurance exercise, we observed the translocation of TSC2 away from the plasma membrane at 1h. In contrast, we observed mTOR translocation towards the plasma membrane had increased 1h following feeding at rest and after endurance exercise. Collectively, these data may suggest that the location of TSC2 at the plasma membrane in conjunction with enhanced TSC2/Rheb colocalization may be an important step in inhibiting mTOR signaling. Conversely, when TSC2 is inactivated and mTOR is activated by feeding and/or endurance exercise, TSC2 disassociates away from the plasma membrane (and Rheb), facilitating the translocation of mTOR to the plasma membrane (with Rheb). As such, we demonstrate that mTOR translocates towards the plasma membrane in response to feeding at rest and following endurance exercise, which may be a potential site of protein synthesis. We speculate the translocation of mTOR towards the plasma membrane is physiologically relevant, as in theory, this would put mTOR in close proximity to receive amino acid substrates, which would contribute to enhanced muscle protein remodeling. Therefore, in corroborating the previous data demonstrating that mTOR/TSC2 colocalization with Rheb may be an important regulatory step in promoting protein synthesis, we also demonstrate that the location of mTOR/TSC2 with the plasma membrane may also influence the regulation of MyoPS at rest and following endurance exercise.
In the present study, we observed that feeding and exercise resulted in an increase in mTOR/Rheb and a reciprocal decrease in TSC2/Rheb colocalization, which collectively would be consistent with an increase in mRNA translational activity and protein synthesis. As expected, TSC2/Rheb colocalization increased immediately following endurance exercise, which would be consistent with a suppressed protein synthetic response during exercise (Rose et al., 2005, Norton and Layman, 2006). However, mTOR/Rheb colocalization also increased above fasted (main effect) immediately following exercise, which appears at odds with the greater TSC2/Rheb colocalization that would be suggestive of a protein synthetic response. It is possible that these divergent responses may be related in part to the colocalization of mTOR with other intracellular components such as the lysosome and/or plasma membrane. For example, it is possible that an increase in autophagy during endurance exercise (Sanchez et al., 2014) could sequester mTOR away from the lysosome, in turn inhibiting autophagy (Zhou et al., 2013). As such, mTOR colocalization with the lysosome (i.e. LAMP2) would provide insight into endurance exercise-induced autophagy, which would support muscle protein remodeling (Sanchez et al., 2014). Alternatively, the discrepancy between mTOR/TSC2 colocalization with Rheb immediately after endurance exercise could be explained by the location of mTOR/TSC2 with the plasma membrane. In this regard, although mTOR/TSC2 colocalization with Rheb had both increased following endurance exercise, we speculate that mTOR was not at a site to be active. To this end, immediately following endurance exercise, the translocation of mTOR toward and TSC2 away from the plasma membrane, did not occur, suggesting that protein synthesis was suppressed. Therefore, we speculate a coordinated (i.e. TSC2 and mTOR colocalization with Rheb must decrease and increase, respectively) and integrated (i.e. translocation of TSC2 away and mTOR towards the plasma membrane) series of processes must concomitantly occur to increase the efficiency of protein synthesis.

In summary, we demonstrate that when dietary protein and carbohydrates are consumed at rest, the association of mTOR with the positive regulator Rheb is increased and that endurance exercise further enhances the association between mTOR and Rheb. In addition, mTOR colocalization with the plasma membrane is increased with feeding at rest and following endurance exercise, suggesting a potential site for protein synthesis. Furthermore, immediately after
endurance exercise, we demonstrate that the association of the negative regulator TSC2 is enhanced with Rheb, where feeding then decreased the colocalization between TSC2/Rheb (at rest and following endurance exercise). Herein, TSC2 colocalization with the plasma membrane decreased, attenuating the inhibitory effect TSC2 has towards protein synthesis. Collectively, our data suggests the potential importance of understanding mTOR colocalization with positive and negative regulators and illustrate another level of complexity of the molecular regulation of protein synthesis at rest and following endurance exercise.

5.6 Strengths, limitations, & future research

Within the present study, there exist numerous strengths inherent towards the study design. First, previous studies examining the effects of feeding at rest (Tipton et al., 1999a, Tang et al., 2009) and following endurance exercise measured mixed MPS (Howarth et al., 2009, Mascher et al., 2011). Therefore, in the present study, we measured the nutrient and exercise sensitivity of fraction-specific Myo/MitoPS, as mixed MPS is not representative of fraction-specific MPS, following resistance exercise (Moore et al., 2009), and presumably following endurance exercise. Furthermore, by using a repeated measured design, with a rested fasted condition, we are the first to demonstrate that MitoPS is not responsive to a moderate dose of dietary protein at rest and that endurance exercise does not augment the postprandial response. Arguably, the most important strength of the present study is that to our knowledge, we are the first to provide mechanistic insight towards fraction-specific MPS using both Western blotting, and for the first time in human skeletal muscle, immunofluorescence. Studies in vitro and in rat skeletal muscle have indicated that the cellular localization and protein association of mTORC1 with positive and negative regulators in response to physiological stimuli (e.g. amino acids, growth factors) may be fundamentally important in the regulation of the mTORC1 signaling cascade (Laplante and Sabatini, 2009, Betz and Hall, 2013). However this information is unfortunately lost during the homogenization and protein denaturing steps of Western blotting. As such, in the present study, we examined both the protein phosphorylation through Western blot as well as mTOR localization via immunofluorescence in an effort to further our understanding of the molecular mechanisms regulating MyoPS (and perhaps MitoPS). Therefore, both techniques complemented each other by providing insight as to whether mTORC1 is active based on its phosphorylation and/or whether
mTOR is associated with a negative/positive regulator, the former and latter were determined by Western blot and immunofluorescence, respectively.

Although the use of immunofluorescence in the present study provided incredibly novel mechanistic insight towards mTORC1 regulation in human skeletal muscle, there do exist limitations inherent to its practicability in future studies. First, we measured fraction-specific MPS over a 5h postprandial period using stable isotope amino acids. As such, our narrow window may have missed any Myo/Mito remodeling that would have occurred outside this time frame; although we measured MPS over 5h as we were interested if endurance exercise augmented the postprandial response, as shown previously using resistance exercise models (Moore et al., 2009, Witard et al., 2014). However, we chose to measure MPS over a 5h time period as delaying protein ingestion 3h following endurance exercise suppresses MPS (Levenhagen et al., 2001). As such, had we measured Myo/MitoPS outside this period, we may have observed elevated rates of protein synthesis in both sub-fractions after endurance exercise, as observed previously, (Di Donato et al., 2014, Burd et al., 2011, Cuthbertson et al., 2006, Miller et al., 2005). Furthermore, we speculate that these elevated rates of protein synthesis may have been mediated with sustained mTOR/TSC2 association/disassociation with Rheb, respectively. Nevertheless, in the present study, our 5h window was sufficient to understand how endurance exercise modulates postprandial fraction-specific MPS when protein is ingested at rest.

To determine if endurance exercise enhances postprandial Myo/MitoPS, we assessed the effects of feeding at rest compared to feeding following endurance exercise. As such, our study did not allow for the assessment of endurance exercise alone (i.e. exercise fasted). Hence, we could not determine if endurance exercise in the fasted state suppressed MyoPS and/or mTORC1 signaling. This would allow us to determine whether protein ingestion following endurance exercise is beneficial (through comparing it to a rest fed, and exercise fasted conditions). Mechanistically speaking, the colocalization of mTOR/TSC2 with Rheb endurance exercise with no nutrient provision would have provided more information to how mTOR colocalization regulates MyoPS. To this end, we would speculate that in the fasted state, TSC2/Rheb colocalization would be sustained following endurance exercise with a reciprocal decrease in mTOR/Rheb colocalization, collectively suggesting suppression in MyoPS in the fasted state.
To this end, as we demonstrated in the present study, feeding following endurance exercise would induce changes in protein colocalization that would be conducive to “turning on” protein synthesis, such as increased mTOR/Rheb colocalization with a reciprocal decrease in TSC2/Rheb colocalization. While we cannot determine if exercise specifically stimulated MyoPS, our immunofluorescence results would suggest that post-exercise skeletal muscle remodeling would at the very least not be constrained with protein/carbohydrate coingestion relative to what might be expected in the fasted state.

Within the present study, the Western blot allowed us to assess mTORC1 activation through protein phosphorylation of various targets within this signaling cascade. Herein, we normalized the phosphorylation of proteins within the mTORC1 pathway to Ponceau S, as Ponceau S has been validated as an alternative loading control (i.e. actin) to ensure equal amount of protein was loaded per well (Rivero-Gutierrez et al., 2014, Romero-Calvo et al., 2010). However, we do recognize that quantifying the phosphorylation of proteins within the mTORC1 pathway over the total amount of protein present (i.e. phosphorylation of mTORC1 over the total amount of mTORC1) within skeletal muscle may be an alternative way to effectively determine the phosphorylation status of a protein (Philp et al., 2015, McGlory et al., 2014). However, previous research has demonstrated that the total amount of protein within the mTORC1 pathway does not change in response to acute nutrition and/or exercise (Fujita et al., 2007, Dreyer et al., 2008), suggesting that within the present study, the use of Ponceau S as a loading control was effective to assess the phosphorylation of proteins within the mTORC1 pathway.

We also assessed colocalization and cellular compartmentalization of mTOR using immunofluorescence. As mTOR encompasses two complexes (i.e. mTORC1/mTORC2), which differ in subunit composition and cellular function (Ekim et al., 2011, Bar-Peled and Sabatini, 2014), the antibody used against mTOR for the immunofluorescence analysis was not specific towards mTORC1 and could have interacted with mTORC2. Therefore, to overcome this limitation, the colocalization of mTOR with Raptor would confirm that it is indeed mTORC1 that is being activated in response to feeding at rest and/or after endurance exercise. However, our data demonstrating that the association of mTOR and related proteins localize and translocate in response to feeding and/or endurance exercise, would be consistent with our current understanding of the mTORC1 regulatory pathway. Therefore, we believe that the specificity of our antibody is
more reflective of mTORC1, rather than mTORC2, as the former is more responsive to feeding and/or contractile activity (Ekim et al., 2011, Bar-Peled and Sabatini, 2014). Although our immunofluorescence analysis provided novel insight towards the complexity of mTOR regulation in response to feeding at rest and following endurance exercise, our colocalization analysis primarily generally aligned more with MyoPS (postprandial increase) compared to MitoPS (unaltered). We demonstrated that mTOR colocalization with the plasma membrane is enhanced in response to feeding at rest and following endurance exercise, where the majority of ribosomes appear to be located in rodent skeletal muscle (Horne and Hesketh, 1990, Goodman et al., 2012). However, as mitochondria are dispersed throughout skeletal muscle (Hoppeler and Fluck, 2003), the colocalization of mTOR towards the plasma membrane suggests that the plasma membrane may be the main site of protein synthesis. As such, proteins within the mitochondria may be remodeled through an intrinsic mechanism (i.e. intracellular trafficking proteins) (Hallberg and Larsson, 2014), independent of mTOR signaling. As such, an mTOR/COX IV stain may provide some insight towards this possibility as it might demonstrate that mTOR not only moves towards the plasma membrane (potential site of protein synthesis) but also to the mitochondria specifically. As such, future avenues examining the colocalization of mitochondrial proteins (i.e. COX IV) with positive and negative regulators in response to a physiological stimuli will further the molecular understanding of this essential organelle.

Lastly future research utilizing the use of rapamycin, to inhibit mTORC1 at rest and following endurance exercise, will elucidate which muscle protein fraction is dependent on this critical evolutionary kinase, which has great therapeutic potential towards human health. Similar to that of rapamycin blunting protein phosphorylation of mTOR and downstream targets via Western blotting (Dickinson et al., 2011, Drummond et al., 2009), we would expect that rapamycin would attenuate mTOR/Rheb colocalization and increase TSC2/Rheb colocalization, in turn suppressing protein synthesis. Furthermore, the translocation of mTOR to the plasma membrane, the potential site of protein synthesis, would be suppressed, whereas TSC2 colocalization to the plasma membrane would be elevated; collectively suggesting a suppression in protein synthesis. On the contrary, we would expect that blunted mTOR/Rheb colocalization and an increase TSC2/Rheb colocalization in response to rapamycin would not influence MitoPS, as recent evidence suggests that MitoPS can occur independent of mTORC1 activity following endurance
exercise (Philp et al., 2015). Therefore, this avenue of research would have tremendous human application towards developing nutraceutical, therapeutic and/or pharmacological agents aiming to maximize muscle mass.

5.7 Conclusion

The purpose of the present study was to determine the effect of bolus protein ingestion on fraction-specific MPS at rest and following a single bout of endurance exercise in trained young men. We demonstrated that: i) MyoPS is nutrient sensitive over a 5h postprandial period at rest and is not enhanced by endurance exercise; ii) MitoPS is uninfluenced by acute exercise or nutritional interactions; iii) enhanced mTOR (i.e. 4E-BP1\textsuperscript{Thr37/46}) and MAPK (ERK1/2\textsuperscript{Thr202/Tyr204}, p38\textsuperscript{Thr180/Tyr180}) signaling via Western blotting and; iv) enhanced mTOR signaling via immunofluorescence (i.e. mTOR/Rheb colocalization and reciprocal decrease in TSC2/Rheb colocalization), which would be consistent with enhanced mRNA translational capacity, and potential protein synthesis in response to feeding at rest and following endurance exercise. As such, future avenues of research should continue to further the advancement and understanding of skeletal muscle plasticity in response to feeding and exercise, using concurrent measures of muscle protein synthesis and Western blotting/immunofluorescence approaches, which has great relevance towards exercise performance and/or optimizing human health.
References


Betz C & Hall MN (2013). Where is mTOR and what is it doing there?. *J Cell Biol*203, 563-574, DOI: 10.1083/jcb.201306041 [doi].


Appendix 1

Insulin ELISA Assay

All reagents and samples must be brought to room temperature before use.

1. Prepare enzyme conjugate 1X solution and wash buffer 1X solution.

2. Prepare sufficient microplate wells to accommodate Calibrators, controls and samples in duplicate.

3. Pipette 25 µL each of Calibrators, controls and samples into appropriate wells.

4. Add 100 µL of enzyme conjugate 1X solution to each well.

5. Incubate on a plate shaker (700-900 rpm) for 1 hour at room temperature (18-25°C).

6. Wash 6 times with 700 µL wash buffer 1X solution per well using an automatic plate washer with overflow-wash function, after final wash, invert and tap the plate firmly against absorbent paper. Do not include soak step in washing procedure.

   Or manually,

   Discard the reaction volume by inverting the microplate over a sink. Add 350 µL wash buffer 1X solution to each well. Discard the wash solution, tap firmly several times against absorbent paper to remove excess liquid. Repeat 5 times. Avoid prolonged soaking during washing.

7. Add 200 µL Substrate TMB into each well.

8. Incubate on the bench for 15 minutes at room temperature (18-25°C).

9. Add 50 µL Stop Solution to each well. Place plate on a shaker for approximately 5 seconds to ensure mixing.

10. Read optical density at 450 nm and calculate results. Read within 30 minutes.
Appendix 2

Plasma amino acid (i.e. L-[ring-2H₅]phenylalanine) extraction

**Supplies needed:**
1. 1.5 ml Eppendorf Tubes

**Reagents needed:**
1. MIX A (3:3:2: Acetonitrile:Isopropanol:ddH₂O)
2. Internal standard (DL-p-chlorophenylalanine)
3. 0.1 M HCL

**Protocol**
2. Put 0.2 mL of Plasma in the Eppendorf with MIX A and vortex.
3. Place the mixed sample in freezer (-20°C) for 30 min.
4. Spin at 14000 RPM for 10 min (4°C).
5. Transfer 1.0 mL of supernatant to new Eppendorf.
6. Make fresh internal standard (DL-p-chlorophenylalanine); 1 mg/mL in 0.1 HCL.
7. Add internal standard (10 µl per sample).
8. Ready for derivitization and amino acid analysis on GC-MS.

The L-[²H₅]leucine enrichments of the myofibrillar and plasma protein bound samples were determined by multiple reaction monitoring (MRM) at m/z 166.0 à 103.0 and m/z 171.0 à 106.0 for unlabeled and labeled L-[ring-²H₅]phenylalanine
Appendix 3

Isolation of mitochondria for enzyme and FSR measurements

*other extraction procedures, which use different buffer components/ionic strength and centrifugation speeds, are available.

**Buffer 1**

<table>
<thead>
<tr>
<th></th>
<th>M.W. (g/mol)</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>sucrose</td>
<td>342.3</td>
<td>10 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>372.2</td>
<td>1 mM</td>
</tr>
<tr>
<td>Tris/HCL</td>
<td>121.14</td>
<td>10 mM</td>
</tr>
<tr>
<td>KCL</td>
<td>74.55</td>
<td>50 mM</td>
</tr>
</tbody>
</table>

- Adjust the pH to 7.4, supplement with protease inhibitors (e.g., Roche Applied Science) if required for Western blot analysis

**Buffer 2**

<table>
<thead>
<tr>
<th></th>
<th>M.W. (g/mol)</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>sucrose</td>
<td>342.3</td>
<td>10 mM</td>
</tr>
<tr>
<td>EGTA/Tris</td>
<td>380.4</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>Tris/HCL</td>
<td>121.14</td>
<td>10 mM</td>
</tr>
</tbody>
</table>

- Adjust the pH to 7.4, supplement with protease inhibitors (e.g., Roche Applied Science)

**Note:** All buffers must be ice cold and the samples kept on ice

1. Place Potter-Elvehjem glass tube on ice to cool.
2. Weigh ~100 mg of wet muscle tissue in glass tube (mince tissue with small scissors)
3. Add 10 µl per mg of wet muscle of Buffer 1 to muscle in glass tube and homogenize on ice with the Potter-Elvehjem glass homogenizer.
4. Centrifuge the resultant homogenate at 700× g for 10 min at 4 °C.
5. Transfer the supernatant into a eppendorf tube
6. Repeat the centrifuge at 700× g for 10 min at 4 °C
7. Transfer the supernatant into new eppendorf tubes
**Mitochondrial Isolation**

8. Centrifuge the muscle mitochondrial protein supernatants at 12,000x g for 20 min at 4 °C.
9. Remove the supernatant
10. Wash the mitochondrial protein pellet with Buffer 1 and centrifuge at 12,000x g for 15 min at 4 °C.
11. Gently re-suspend the mitochondrial enriched pellet in a small volume (200-500 µL) of ice-cold Buffer 2 and freeze for further analysis

   OR

12. Centrifuge at 12,000x g for 15 min at 4 °C.
13. Remove supernatant
14. Add 500 µl 95% EtOH to the mitochondrial pellet
15. Centrifuge at 6000x g for 10 min at 4 °C.
16. Remove and discard EtOH
17. Lyophilize
18. Transfer mitochondrial protein pellet to 4 mL screw top tube
19. Add 2.0 ml of 6 M HCL and hydrolyze overnight at 110°C
20. Purify amino acids over Dowex cation exchange column and convert to specific derivative for analysis by GC-MS, GC-C-IRMS, or Tandem MS (e.g., LC/MS/MS)
Appendix 4

mTOR staining with Rheb

**Chemicals used**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>mTOR</td>
<td>Millipore #05-1592</td>
</tr>
<tr>
<td>TSC2</td>
<td>Abgent #AM1919b</td>
</tr>
<tr>
<td>Rheb</td>
<td>Abcam #Ab92313</td>
</tr>
<tr>
<td>10 % Normal goat serum (NGS)</td>
<td>Invitrogen #50-062Z</td>
</tr>
<tr>
<td>Wheat germ agglutinin (WGA)</td>
<td>Invitrogen #W11263</td>
</tr>
<tr>
<td>Goat anti-mouse IgGγ1 Alexa@594 secondary</td>
<td>abcam #ab150116</td>
</tr>
<tr>
<td>Goat anti-rabbit Alexa®488 secondary</td>
<td>abcam #ab150077</td>
</tr>
<tr>
<td>Mounting medium</td>
<td>Dako #S302380-2</td>
</tr>
</tbody>
</table>

**Fixation**

Muscle samples were cut into 7µm thickness sections. An acetone/ethanol solution mixture (3:1) was prepared and sections were immersed into the fixation solution for 5 min. Then sections were rinsed by 3 x 5 min in PBS solution under room temperature. For fixation and all the rinse steps in this protocol, sections were put in a “bath” to make sections fixed/washed evenly.

**Primary antibody staining**

mTOR (1 to 200 dilution) and Rheb (1 to 50 dilution) primary antibodies were mixed in 5 % NGS (diluted in PBS solution) and ~15µicroL was added to each section. Slides were then incubated for 2 hours at room temperature and then rinsed in PBS (3x5min).

**Secondary antibody staining**

Goat anti-mouse IgGγ1 Alexa®594 secondary antibody targeting mTOR (1 to 200 dilution) was mixed with Goat anti-rabbit Alexa®488 secondary antibody targeting Rheb (1 to 200 dilution) in PBS solution and ~15microl was added to each section. Slides were then incubated for 30 min at room temperature (dark).

**Cell membrane staining**

Following another 3 x 5 min wash in PBS solution, sections were incubated with WGA (1 to 20 dilution) for 30min at room temperature (dark).
Mounting

~15microL Dako mounting medium was added to each section and a cover slip was placed on the slide. Slides were left in the dark to dry overnight at room temperature.

Image capturing

Sections were imaged using the triple (DAPI-TxRed-GFP) fluorescence filters controlled by EVOS FL Auto. Rheb was observed under the GFP filter and mTOR signals were visualized under TxRed filter. WGA was observed under DAPI filter. All image capturing was kept constant between images, including exposure time, gain, and image frame and light intensity.

TSC2 staining with Rheb

Fixation

Muscle samples were cut into 7µm thickness sections. An acetone/ethanol solution mixture (3:1) was prepared and sections were immersed into the fixation solution for 5 min. Then sections were rinsed by 3 x 5 min in PBS solution under room temperature. For fixation and all the rinse steps in this protocol, sections were put in a “bath” to make sections fixed/washed evenly.

Primary antibody staining

TSC2 (1 to 50 dilution) and Rheb (1 to 50 dilution) primary antibodies were mixed in 5 % NGS (diluted in PBS solution) and ~15microL was added to each section. Slides were then incubated for 2 hours at room temperature then rinsed in PBS (3x5min).

Secondary antibody staining

Goat anti-mouse IgGγ1 Alexa®594 secondary antibody targeting TSC2 (1 to 200 dilution) was mixed with Goat anti-rabbit Alexa®488 secondary antibody targeting Rheb (1 to 200 dilution) in PBS solution and ~15microL was added to each section. Slides were then incubated for 30 min at room temperature (in the dark).

Cell membrane staining

Following another rinse by 3 x 5 min in PBS solution, sections were incubated with WGA (1 to 20 dilution) for 30min at room temperature (dark). Then slides were washed in PBS for 5min.
Mounting

~15 microL Dako mounting medium was added to each section and a cover slip was placed on the slide. Slides were left in the dark to dry overnight at room temperature.

Image capturing

Sections were imaged using the triple (DAPI-TxRed-GFP) fluorescence filters controlled by EVOS FL Auto. Rheb was observed under the GFP filter and TSC2 signals were visualized under TxRed filter. WGA was observed under DAPI filter. All image capturing was kept constant between images, including exposure time, gain, and image frame and light intensity.
Appendix 5: Representative Ponceau S stain of 10µg loaded per lane before and after physiological protein ingestion at rest (REST) or after endurance exercise (EX). Ponceau S staining was used to check equal loading of gels under different conditions by quantifying the whole lane.