AEROBIC EXERCISE AND SKELETAL MUSCLE MYOFIBRILLAR SYNTHESIS

AEROBIC EXERCISE INTENSITY AFFECTS SKELETAL MUSCLE MYOFIBRILLAR PROTEIN SYNTHESIS AND ANABOLIC SIGNALING IN YOUNG MEN

By

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ABSTRACT

Aerobic exercise can stimulate mixed muscle protein synthesis (MPS) acutely post-exercise; however, the types of proteins synthesized as a result of aerobic exercise are not known by studying changes in mixed MPS. We aimed to study the effect of aerobic exercise intensity on the 4 and 24 h post-exercise fractional synthesis rate (FSR) of myofibrillar proteins. Using a within-subject design, eight males (21 ± 1 years, $VO_{2\ peak}$: 46.7 ± 2.0 mL kg^{-1} min^{-1}) underwent 2 trials with a primed constant infusion of L-[*ring*-$^{13}C_6$]phenylalanine in the fasted state for each work-matched exercise intensity (LOW: cycling for 60 min at 30% W_{max} and HIGH: 30 min at 60% W_{max}). Muscle biopsies were obtained to determine resting, 4 and 24 h post-exercise myofibrillar FSR. We also studied the phosphorylation of signaling proteins involved in protein synthesis at each time point using immunoblotting methods. Phospho-p38$^{Thr180/Tyr182}$ was greater at 4.5 h after exercise compared to 0.5, 24 and 28 h post-exercise ($p < 0.05$). Additionally, a strong trend was present for phospho-mTOR^{Ser2448} ($p = 0.056$) with 0.5 h post-exercise phosphorylation significantly higher after HIGH than after LOW exercise ($p < 0.05$). Myofibrillar protein synthesis was stimulated 1.5–fold 0.5 – 4 h post-exercise ($p < 0.05$), returning to rest in the LOW condition 24 h post-exercise, while 6 out of 8 subjects maintained increased myofibrillar FSR 24 h post HIGH exercise ($p < 0.05$). The increase in myofibrillar FSR 0.5 – 4 h post-exercise was correlated with phospho-mTOR^{Ser2448} 0.5 h post-exercise ($r = 0.698$, $p < 0.01$), indicating the role of this signaling pathway in myofibrillar protein synthesis. It is concluded that aerobic exercise has an effect on myofibrillar protein synthesis and intensity may play a role in the duration of this response.

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4EBP-1	eukaryotic initiation factor 4E binding protein 1
ACC	acetyl CoA carboxylase
ADP	adenosine diphosphate
AE	aerobic exercise
AMPK	AMP-activated kinase
ANOVA	analysis of variance
ATF-2	activating transcription factor-2
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BSA	bovine serum albumin
CaMK	calmodulin-activated protein kinase
CHO	carbohydrate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
eEF	eukaryotic elongation factor
eIF	eukaryotic initiation factor
ERK	extracellular signal regulated kinase
FSR	fractional synthesis rate
GβL	G-protein β-subunit-like protein
GC-C-IRMS	gas chromatography combustion isotope-ratio mass spectrometer
GMP	guanine monophosphate
HDAC	histone deacetylase
HFB	heptafluorobutyrate
HIGH	high intensity
HIIT	high intensity interval training
HRP	horseradish peroxidase
HR	heart rate
IC	intracellular
JNK	c-Jun NH_2-terminal kinases
LOW	low intensity
MAPK	mitogen activated protein kinase
MEF2	myocyte-enhancing factor 2
MEK	MAP kinase kinase
MHC	myosin heavy chain
MPB	muscle protein breakdown
MPS	muscle protein synthesis
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
NRF	nuclear respiratory factor

p70S6K1	p70 S6 kinase 1
p90RSK	p90 ribosomal S6 kinase
PCA	perchloric acid
PGC-1α	peroxisome proliferator-activated receptor 1 coactivator α
PI3K	phosphatidylinositol 3-kinase
PPAR	peroxisome proliferator-activated receptor
PRO	protein
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
RT	room temperature
SIRT1	sirtuin 1
SDS	sodium dodecyl sulfate
TBST	tris buffered saline with tween20
Tfam	mitochondrial transcription factor A
TSC	tuberous sclerosis complex
$VO_{2\ peak}$	maximal aerobic capacity
Vps34	vacuole protein sorting 34
W_{max}	maximum workload

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CHAPTER I

SKELETAL MUSCLE PROTEIN TURNOVER WITH DYNAMIC EXERCISE

I Dynamic Exercise

Dynamic exercise is characterized by continuous contraction utilizing multiple muscles or muscle groups. This type of exercise consists of an increase in activation frequency of motor units with relatively low force output and is fuelled by biochemical processes that are aerobic in nature (i.e., that involve oxidative metabolism of pyruvate) and includes activities such as running, swimming, or cycling (Brooks *et al.*, 2004). Dynamic exercise is predominately associated with type I and IIa fibre recruitment, which are more oxidative in nature and not as easily fatigueable as type IIx fibres. Adaptations to dynamic exercise are proposed to be confined to metabolic adaptations and result in changes in the content of enzymes involved in glycolysis, fat oxidation, the TCA cycle, and oxidative phosphorylation (Gollnick & Saltin, 1982; Holloszy & Coyle, 1984). These adaptations are a result of an increase in mitochondrial content. With continued practice of aerobic exercise (i.e., training), the muscle is better equipped to oxidize pyruvate generated from glycolysis by oxidative metabolism and to oxidize fat as a fuel (Gollnick & Saltin, 1982).

In activities such as cycling, the work performed by the muscle within a fixed time can be measured in Watts (W), and the maximum workload (W_{max}) can be determined during a maximum aerobic capacity ($VO_{2\ peak}$) test. Cycling at a percentage of W_{max} ($\%W_{max}$) is a relative intensity that can be standardized between persons; cycling at a higher $\%W_{max}$ requires more force to complete petal rotation than at a lower $\%W_{max}$. The size principle of motor recruitment states that as the force increases (i.e., a greater $\%W_{max}$), more motor units are recruited to generate the force necessary to complete the work without fatigue occurring, meaning that at a higher intensity cycling exercise more motor units are being utilized at a given time (Sale, 1987; Brooks *et al.*, 2004). It is thought that

adaptations can only occur in fibres that are activated during contraction and therefore more fibre recruitment could result in greater adaptations within a given fibre (Hood, 2001). As the $\%W_{max}$ increases the amount of oxygen required by the muscle also increases, resulting in a increased O_2 consumption and $\%VO_{2\,peak}$ for the exercise increases (Vanhatalo *et al.*, 2011).

Modes of dynamic exercise which may not be classified as traditional endurance training, such as low-volume high-intensity interval training (HIIT), are also potent stimuli of aerobic adaptations but may also lead to adaptations associated with resistance exercise, such as hypertrophy, in some cases (Ross & Leveritt, 2001). This may be due to the higher forces exerted during the exercise bout. High forces recruit more muscle fibres or more type IIa/IIx fibres than with a lower intensity longer duration exercise (Sale, 1987; Hood, 2001; Farina, 2004). Thus, even within the same mode of exercise, the intensity of exercise can have a profound impact on the adaptations that are observed with training.

II Protein Turnover in Skeletal Muscle with Exercise

Proteins in all tissues turn over, that is, they are broken down into their constituent amino acids, many of which are often reutilized to yield new proteins. This process of protein turnover consists of the balance between two processes, which in skeletal muscle are termed muscle protein synthesis (MPS) and muscle protein breakdown (MPB). The simultaneous and ongoing turnover of proteins provides a remodeling mechanism for cellular protein pools and ensures that damaged proteins can be removed and replaced. Damage to skeletal muscle proteins comes about through a variety of mechanisms, such as oxidation, nitrosylation, or mechanical disruption. The relative rates of MPS and MPB determine whether proteins accrue or decline. Altered functional demands of skeletal

muscle also leads to phenotypic adaptations, with increased loading (i.e., resistance exercise) leading to hypertrophy (MPS > MPB) (McCall *et al.*, 1996) and unloading leading to atrophy (MPB > MPS) (de Boer *et al.*, 2007).

Expansion of protein pools such as the mitochondria occur when skeletal muscle is repeatedly placed under high energy demand induced by repetitive low load contractions for prolonged periods (i.e., aerobic exercise) (Gollnick & Saltin, 1982; Holloszy & Coyle, 1984) or repetitive slightly higher load contractions for shorter but repeated bouts (i.e., sprinting) (Henriksson & Reitman, 1976). Thus, the altered functional demands in skeletal muscle lead to phenotypic changes, ultimately, through alterations in MPS and MPB of specific protein pools.

II.I Role of Protein Turnover in Adaptation

Skeletal muscle responds to exercise by adapting to the stimulus in a mode-specific manner. With repeated bouts of resistance exercise hypertrophy of muscle fibres occurs, and with repeated bouts of aerobic exercise the result is increased aerobic capacity. Although these modes of exercise are often believed to be antagonistic with distinct adaptations occurring with each mode that 'oppose' the other, this is not always the case. Increased muscle oxidative capacity has been observed with resistance exercise training (Ross & Leveritt, 2001; Tang *et al.*, 2006; Vanhatalo *et al.*, 2011), while hypertrophy can occur with aerobic exercise training in some populations (Harber *et al.*, 2009*b*). Modes of exercise such as HIIT, and low-load high volume resistance exercise to failure would be modes of exercise that highlight the overlap between resistive and aerobic exercise (Ross & Leveritt, 2001).

Changing the relative rates of protein turnover is a key response to exercise which leads to adaptation. Protein synthesis and breakdown are modulated by exercise as well as nutrition. Ultimately, the net balance between synthesis and breakdown determines the accretion or decrease in a pool of proteins in response to stimuli. Skeletal muscle is made up of a number of proteins that can be broadly classified into sarcoplasmic, myofibrillar, collagen, and mitochondrial proteins, which contribute to the metabolic and structural phenotype of the tissue. The turnover of each of these subcellular protein pools responds to exercise differently depending on the exercise type, duration, load, and intensity. The small transient changes in protein turnover after an acute bout of exercise accumulate over repeated bouts to make significant changes to the skeletal muscle that are specific to the exercise stimuli, leading to stimuli specific adaptation. This occurs in parallel with changes in gene expression both acutely (Mahoney *et al.*, 2005) and in the trained state (Stepto *et al.*, 2009).

Studying whole-body protein turnover or mixed skeletal muscle protein turnover gives an overall picture of what is happening to protein metabolism at any given time. These measurements can tell us whether protein accretion or excretion is occurring, either at the whole body or skeletal muscle level. What these measures do not tell us, however, is what the fate of the protein being accumulated is or from what protein pool the excreted protein originated. Mixed muscle protein turnover can be indicative of whether the muscle as a whole is anabolic or catabolic but it does not tell us whether hypertrophy/atrophy or other metabolic adaptations are occurring. The turnover of particular protein pools is a more specific measure of adaptations that are occurring in the muscle. The myofibrillar and mitochondrial protein pools play a crucial role when studying adaptations to exercise.

II.II Myofibrillar Protein Turnover with Exercise

The myofibrillar proteins are the contractile element of the muscle and make up 60% of the proteins in skeletal muscle (Brooks *et al.*, 2004). Myofibrillar proteins are mostly made up of myosin (50%) and actin (20%), along with accessory and regulatory proteins that form the sarcomere and allow for contractile activity (Brooks *et al.*, 2004). These proteins turnover at low rate of about <1.5% per day (Jaleel *et al.*, 2008) but show a broad responsiveness to feeding and exercise (Moore *et al.*, 2009). Therefore, this pool is very dynamic and able to adapt to an exercise stimuli.

A muscle fibre can often be classified as a particular type based on the expression of myosin heavy chain (MHC) isoforms within the muscle cell. Slow twitch type I muscle fibres express mostly type I MHC, tend to have high oxidative capacity, and are ideally for low intensity contractions with a high endurance (i.e., fatigue resistance). In contrast, fast twitch type II fibres express IIa and/or IIx MHC have lower oxidative capacity (in the case of IIx) and are ideal for high intensity, high force contraction (Brooks *et al.*, 2004). A positive net protein balance in the myofibrillar protein pool would be indicative of skeletal muscle hypertrophy (Phillips, 2004; 2004; Hartman *et al.*, 2007; 2007) while a negative net protein balance would be indicative of atrophy. Adaptation may also occur with no net change in the myofibrillar protein pool, as fibre type shifts can occur from exercise training; where the muscle fibre begins to express a different MHC isoform (Flück & Hoppeler, 2003). Studying turnover in myofibrillar proteins in the acute stage after exercise in humans has not been done as the current methods for measuring fractional breakdown of muscle protein does not allow for studying subcellular protein pools. However, post-

exercise myofibrillar protein synthesis along with hypertrophy and fibre shifts after training can be indicative of long-term myofibrillar protein turnover changes.

Synthesis of myofibrillar proteins has been extensively studied in response to resistance exercise and nutritional interventions. Myofibrillar protein synthesis increases in the acute 4 hours after resistance exercise due to increases in the translation of mRNA encoding myofibrillar genes (Hawley, 2009). This response improves the net protein balance, but is still negative in the post-absorptive state since protein breakdown is also stimulated as a result of resistance exercise (Biolo *et al.*, 1995). With adequate provision of amino acids after exercise, a positive net balance of muscle protein occurs (Biolo *et al.*, 1997; Levenhagen *et al.*, 2001) which presumably will result in a positive net balance of myofibrillar proteins as well. Overtime, these bouts of resistance exercise with increases in protein net balance expand the myofibrillar protein pool and result in hypertrophy (McCall *et al.*, 1996; Phillips, 2004).

Aerobic exercise training has also been shown to result in hypertrophy in particular populations such as in the elderly with traditional endurance training (Harber *et al.*, 2009*b*). Generally, however, hypertrophy is not observed with aerobic exercise training in a young healthy population (Glowacki *et al.*, 2004; Wilkinson *et al.*, 2008). This is not to say, however, that aerobic exercise does not have an effect on myofibrillar protein metabolism. While it is uncommon for hypertrophy to occur, aerobic exercise training can lead to an increase in muscle protein turnover (Pikosky *et al.*, 2006) which may contribute to the fibre type remodeling that occurs with aerobic exercise training (Baumann *et al.*, 1987).

II.III Mitochondrial Protein Turnover with Exercise

The mitochondria exist as a network of membranous organelles within the skeletal muscle but are generally classified into intermyofibrillar or subsarcolemmal mitochondria (Hood, 2001). The mitochondrial reticulum is responsible for aerobic metabolism, including synthesizing ATP through the electron transport chain (Brooks *et al.*, 2004). Mitochondrial biogenesis results in the expansion of this network, due to coordinated translation of nuclear and mitochondrial genes to synthesize mitochondrial proteins and increase mitochondrial protein import (Hood, 2001). Contractile activity can induce mitochondrial biogenesis, which leads to increased aerobic capacity. Measuring cytochrome C oxidase content in muscle tissue homogenates is often used to assess mitochondrial content (Rooyackers *et al.*, 1996). An increase in cytochrome C oxidase content and maximal activity after training is indicative that an expansion of the mitochondrial pool has occurred and an increase in aerobic capacity is observed in parallel (Gibala *et al.*, 2006). Similar to myofibrillar proteins, mitochondria are in a constant state of turnover as mitochondrial proteins become damaged by, for example, oxidation by reactive oxygen species (ROS), or other metabolic consequences. In this way, an increase in cytochrome C oxidase content would be, in essence, due to a net positive balance of mitochondrial proteins over time, which may be due to increases in synthesis or decreases in breakdown (Miller & Hamilton, 2012).

In the acute post-exercise time period, mitochondrial biogenesis can be directly measured by mitochondrial fractional synthesis rate (FSR), measuring the synthesis of new mitochondrial proteins (Miller & Hamilton, 2012). While studies directly measuring mitochondrial turnover have been limited to animal and cellular studies, these studies have

shown that contractile activity can affect both mitochondrial protein synthesis and degradation, having an impact on overall mitochondrial turnover (Connor *et al.*, 2000). Human studies have shown that aerobic exercise can affect mitochondrial protein synthesis in the acute phase (Wilkinson *et al.*, 2008) as well as increase resting rates of muscle protein synthesis with training (Short *et al.*, 2004). While true mitochondrial protein turnover rates have not been measured in humans, the overall effect of the net changes in mitochondrial protein turnover has been shown numerous times by observing an expansion of the mitochondrial pool with aerobic exercise training. Mitochondrial fusion also occurs, which expands the network and potentially increases the mitochondrial capacity for fuel utilization (Pich, 2005). Measurable increases in mitochondrial proteins have been found as early as 5 days (3 exercise sessions) after the onset of HIIT training (Perry *et al.*, 2010). While due mostly to transient increases in mRNA, as training continues the steady-state levels of mitochondrial protein mRNAs increase, allowing for an even greater translational capacity (Flück & Hoppeler, 2003). Endurance training has also been shown to increase mitochondrial protein import (Takahashi *et al.*, 1998). This is important to allow the increased amount of nuclear encoded mitochondrial proteins to enter into the mitochondria (Puntschart *et al.*, 1995). All of these adaptations are indications of changes in protein turnover within the mitochondrial protein pool, with the end result that protein balance favours a net accumulation of protein into this relatively small protein pool in skeletal muscle.

III Acute Protein Synthetic Response to Dynamic Exercise

Dynamic exercise induces adaptations that require protein remodeling and accretion. In the acute post-exercise phase, MPS and MPB are modulated to allow the muscle to adapt to the

stimuli. While hypertrophic responses are usually not evident from repeated bouts of dynamic exercise the protein synthetic response after exercise is still important when studying the cellular responses leading to adaptation. This response can be studied using stable isotope methodology to measure mixed muscle protein synthesis as well as that of individual subcellular protein pools.

III.I Mixed Muscle Protein Synthetic Response to Dynamic Exercise

Using stable isotope methodology, many studies have showed that aerobic exercise stimulates mixed MPS. Mixed MPS represents an aggregate synthesis rate of all muscle proteins representing their respective turnover rates and proportions. Generally, mixed MPS is considered to be an accurate reflection of the synthesis rates of all muscle proteins and protein fractions, but is dominated by the myofibrillar proteins, as they comprise the majority of muscle proteins (~60% of total).

Mixed MPS was found to be increased 57% in the 2-6 h post-exercise period after 60 min of cycling at 70% $VO_{2\ peak}$ in the fasted state (Harber *et al.*, 2010). A similar increase was seen with amino acid (0.37 g/kg) and carbohydrate (0.83 g/kg) provision (Harber *et al.*, 2010). This increase in MPS was also seen to persist 24 h after 45 min of treadmill running at 75% of $VO_{2\ peak}$ with amino acid provision (37% increase) (Harber *et al.*, 2009*a*). Less intense aerobic exercise, 45 min of treadmill walking at 40% $VO_{2\ peak}$, resulted in an increase in mixed muscle protein FSR in the first 60 min post-exercise; however, by 180 minutes MPS had returned to resting values (Sheffield-Moore, 2004). It is very likely that a 4 h isotope incorporation period for measuring MPS after the walking protocol would not have detected this short lived increase in MPS. With a longer duration

of treadmill walking at 40% $VO_{2\ peak}$ for 4 h in the fasted state, mixed MPS increased and was measurable in the 4 h post-exercise period (Carraro *et al.*, 1990).

Comparing the protein synthetic responses to exercise of differing intensity between studies poses problems in that many other factors can affect protein synthesis, which are not controlled between studies and so confound reliable interpretation. These factors include length and type of exercise, nutritional status, variations in time points studied, and the isotope methodology used in the study (Smith *et al.*, 1992; Rooyackers *et al.*, 1996). It would seem that the longer the duration of the exercise, the increase in mixed MPS is greater, as well as the duration of that increase; for example, the results of Sheffield-Moore et al. in which subjects completed 45 min of walking at 40% $VO_{2\ peak}$ (Sheffield-Moore, 2004) versus those of Carraro et al., in which subjects completed 4 h of walking at 40% $VO_{2\ peak}$ (Carraro *et al.*, 1990).

Mixed MPS measurements, which have been made more often in studies of protein turnover responses to aerobic exercise (Carraro *et al.*, 1990; Sheffield-Moore, 2004; Harber *et al.*, 2010) are somewhat difficult to interpret however, since this method measures global skeletal muscle protein metabolism and does not allow us to make conclusions on the specific protein response of the muscle to the exercise stimulus. It was previously thought that since myofibrillar proteins make up such a large component of the mixed muscle, that mixed muscle FSR was representative of this pool, but this is not always the case (Burd *et al.*, 2010*b*). The basal turnover rates of each protein pool vary from as low as 0.025%/h for myofibrillar proteins to 0.06%/h for sarcoplasmic proteins, which includes the mitochondrial proteins (Mittendorfer *et al.*, 2005), and thus measuring protein-specific responses yield more insight into adaptive processes. That mitochondrial protein FSR has

been found to be almost twice that of mixed muscle FSR can be attributed to higher rates of mitochondrial protein turnover (Rooyackers *et al.*, 1996), which when measured in a mixed MPS measurement would be diluted by the slower synthesis rate of the far more abundant myofibrillar proteins. Thus, mixed muscle FSR might not be sensitive enough to detect intensity-dependent differences in protein synthesis if the affected protein pools are not isolated and measured separately. This is especially important when studying exercise protocols that are known to affect the synthesis of relatively smaller protein pools of sarcoplasmic (~20% of total muscle protein) or mitochondrial (~5-10% of total muscle protein) proteins, such as with aerobic exercise (Cuthbertson, 2005; Wilkinson *et al.*, 2008).

III.II Subcellular Protein Pool Synthetic Response to Exercise

The acute changes in mitochondrial and myofibrillar FSR after aerobic exercise would likely reveal more subtle protein-specific adaptations that occur with repeated bouts of the exercise stimulus. It has been shown, for example, that the acute rise in mixed MPS after resistance exercise (Wilkinson *et al.*, 2007) is quantitatively predictive of the degree of training-induced hypertrophy that occurs with training that used the same exercise and nutritional intervention (Hartman *et al.*, 2007). This may not hold true for the relationship between aerobic adaptations and mitochondrial protein synthesis but nonetheless, subcellular protein synthetic responses can point towards protein pools that are affected by the exercise stimulus.

Table I. Summary of literature on myofibrillar protein synthesis response to dynamic exercise.

Type	Intensity	Duration	FSR (fold-change)	Nutrition	Reference
single-leg kicking	67% W_{max}	1 h	6 h – 2.5 24 h – 3.25	intermittent	(Miller *et al.*, 2005)
weighted single-leg stepping	274 W	12 min	3 h – no change 6 h – 3.0 24 h – 3.2	post-ex 45 g PRO 135 g CHO	(Cuthbertson, 2005)
single-leg cycling	75% $VO_{2\ peak}$	45 min	4 h – no change	intermittent	(Wilkinson *et al.*, 2008)
cycling	55% $VO_{2\ peak}$	40 min	4 h – no change	post-ex 20 g PRO	(Donges *et al.*, 2012)

Myofibrillar protein synthesis following aerobic exercise has been studied sparingly, often in combination with nutritional support (Table I). Single-leg cycling for 45 min at 75% $VO_{2\ peak}$ did not induce increases in myofibrillar FSR 4 h after exercise, but mitochondrial FSR did increase (Wilkinson *et al.*, 2008). Similarly, cycling at 55% of $VO_{2\ peak}$ for 40 min resulted in protein pool-specific FSR responses with mitochondrial FSR increasing and myofibrillar FSR showing no response 4 h after exercise (Biolo *et al.*, 1997; Donges *et al.*, 2012). It is important to note that these studies provided nutrients either as a bolus of protein after exercise (Levenhagen *et al.*, 2001; Donges *et al.*, 2012) or as repeated protein doses throughout the study (Wilkinson *et al.*, 2008). Undoubtedly, feeding would modulate the protein synthetic response of each subcellular protein pool. Protein provision post-aerobic exercise has been shown to increase myofibrillar FSR compared to carbohydrate alone (Breen *et al.*, 2011). In contrast, Donges *et al.* showed no feeding-induced increase myofibrillar protein synthesis after aerobic exercise (Donges *et al.*, 2012). Other studies incorporating feeding have found increases in myofibrillar protein synthesis

after exercises which are dynamic in nature but may not be classified as traditional endurance exercise, such as single-leg kicking (Miller *et al.*, 2005; Cuthbertson, 2005). By comparison to resistance exercise, there exists a large deficit in subcellular protein synthesis data in humans after aerobic exercise.

IV Signaling Pathway Responses in Protein Metabolism to Aerobic Exercise

The translation of the exercise stimulus, with or without nutrient provision, to the protein synthetic response involves activation and deactivation of numerous signaling pathways leading to modification of DNA transcription, mRNA stabilization, mRNA translation, and post-translational modification of proteins (Flück & Hoppeler, 2003). This is the result of mechano-chemical transduction where the contraction of the muscle fibre is translated to a chemical signal, alongside biochemical perturbations of the skeletal muscle metabolism (Toigo & Boutellier, 2006). Aerobic exercise may cause initial signaling events due to tension-sensing integrin molecules, changes in calcium flux, alterations in membrane potential, and changes in the ADP/ATP ratio (Hood, 2001). These events may occur to different degrees with varied intensity and duration of the exercise potentially resulting in altered acute responses of measurable downstream signaling events post-exercise. Several signaling pathways have shown to be involved in propagating the stimuli of exercise to effect protein synthesis and mitochondrial biogenesis (Hood, 2001; Hawley, 2009).

Studying the activation of signaling proteins through probing for phosphorylated proteins with Western blotting is a useful tool in supporting measures of protein synthetic responses with upstream molecular changes occurring in the muscle. However, comparing changes in signaling molecule activation/deactivation, which is presumably reflected in phosphorylation of a specific protein residue, between studies, can prove difficult given the

sensitive nature of these measures to exercise type, duration, and intensity, participant age and activity level, nutrient provision, and muscle sampling time points. This section will describe the effects of aerobic exercise on signaling pathways implicated in skeletal muscle protein synthesis and mitochondrial biogenesis from human studies.

IV.I Translational Regulation by the mTOR Pathway

Translation involves the coordination of many accessory proteins, which assist in ribosomal binding to the mRNA target and subsequent translation of the transcript. The regulation of these proteins is accomplished through phosphorylation of the accessory proteins, which changes their binding affinity to their targets. The mammalian target of rapamycin complex 1 (mTORC1) pathway is a major regulator of translation initiation. Many signals that lead to an increase in translation and protein synthesis converge at mTORC1 which is composed of three proteins: mTOR, G-protein β-subunit-like protein (GβL), and raptor (Hood, 2001; Drummond *et al.*, 2009*a*). These signals include insulin, extracellular amino acids, hypoxia, and energy status (Figure I) (Brooks *et al.*, 2004; Drummond *et al.*, 2009*a*). Many of these signals are present during an exercise stimulus and therefore contribute to the control of translation via mTORC1. During exercise, the ratio of AMP to ATP increases, which is sensed by AMP-activated kinase (AMPK). In a state of energy stress, AMPK is activated and may help to reduce cellular processes which have high-energy requirements, such as protein synthesis (Rooyackers *et al.*, 1996; Kimball, 2006). Numerous cell culture experiments have shown that AMPK activation reduces mTOR signaling and subsequently reduces protein synthesis (Hood, 2001; Kimball, 2006). Another upstream regulator of mTOR is Akt (protein kinase B) which is activated indirectly by PI3K when insulin binds to its receptor (Tato *et al.*, 2011). Akt can activate mTOR directly through phosphorylation,

or indirectly by deactivating tuberous sclerosis complex (TSC) 1 and 2, which are inhibitors of mTOR (Winter *et al.*, 2011). Amino acids can also activate mTOR through Akt, MAP4K3 or vacuole protein sorting 34 (Vps34) (Drummond *et al.*, 2009*a*; Tato *et al.*, 2011).

The phosphorylation of mTOR at Ser2448 activates this molecule to subsequently phosphorylate downstream targets such as 4EBP-1 at Thr37/46 and p70S6 kinase 1 (p70S6K1) at Thr389, which are negative and positive regulators of the formation of the translation initiation complex, respectively (Kimball, 2006; Miller & Hamilton, 2012). When phosphorylated and active, p70S6K1 phosphorylates eukaryotic initiation factor 4B (eIF4B), which increases its binding affinity to eIF4E to promote translation. At the same time, mTOR phosphorylation of 4E-BP1 prevents this protein from binding to eIF4E allowing eIF4B to associate with eIF4E and promote translation (Connor *et al.*, 2000; Kimball, 2006). Skeletal muscle gains with resistance exercise have been shown to be correlated to p70S6K1 phosphorylation at Thr389 (Terzis *et al.*, 2007), which speaks to the importance of this pathway in translational regulation and protein synthesis. It should be noted, however, that this is not a universal finding (Mitchell *et al.*, 2012).

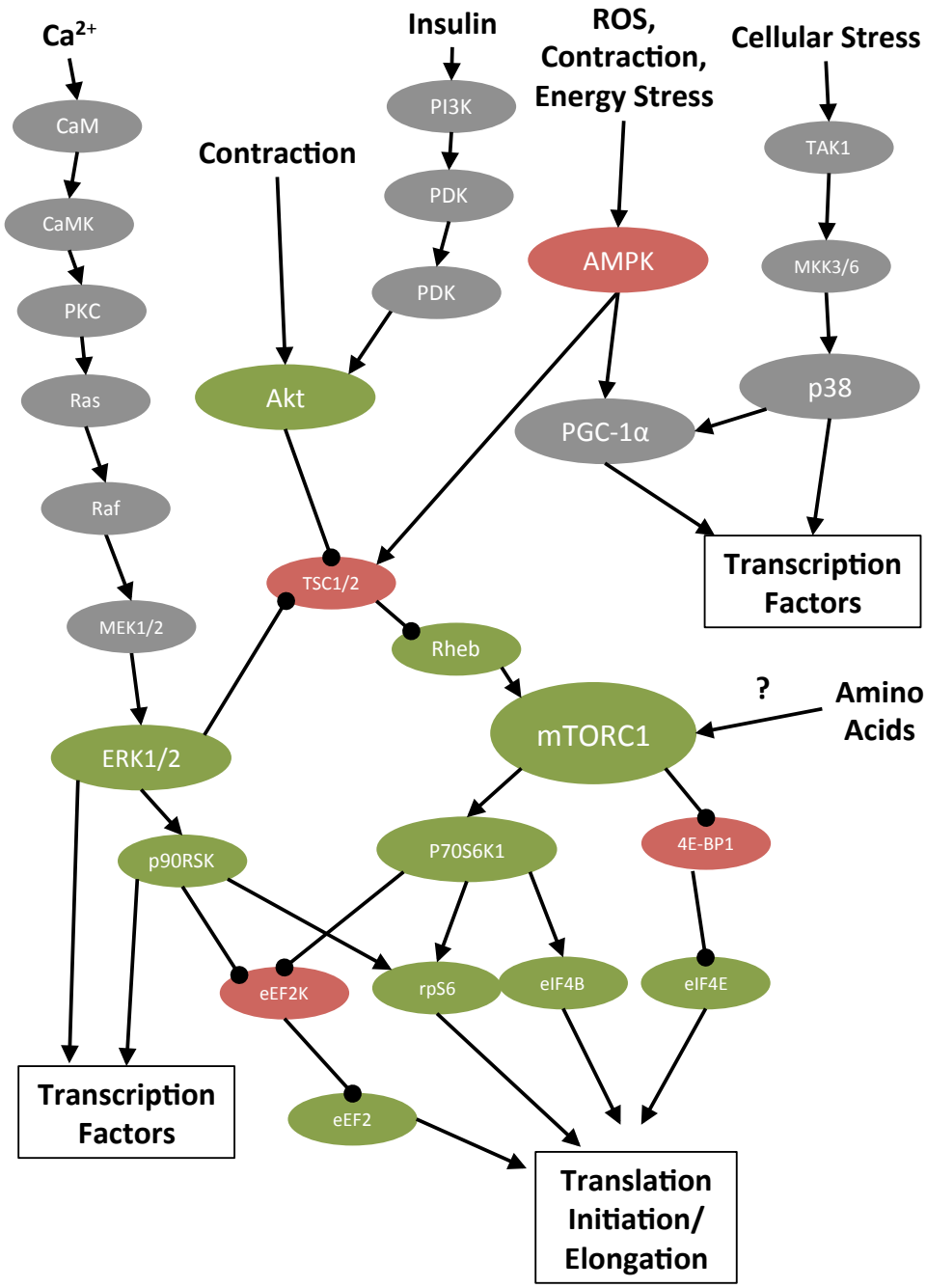

Figure I. A simplified schematic integrating mTORC and MAPK signaling pathways involved in transcriptional and translational regulation with exercise. Green represents molecules that are positive regulators of protein synthesis and red represents molecules that are negative regulators. (Widegren *et al.*, 2001; Baar, 2006; Kimball, 2006; Drummond *et al.*, 2009*a*).

While mTOR is generally implicated in hypertrophy with resistance exercise, mTOR has also been found to be phosphorylated after aerobic exercise (Mascher *et al.*, 2007; Wilkinson *et al.*, 2008). However, other studies have not been able to find changes in phospho-mTOR^{Ser2448} after aerobic exercise (Coffey *et al.*, 2010) unless nutrients are provided (Coffey *et al.*, 2010; Donges *et al.*, 2012). This may be due to the differing nature of the aerobic exercise (continuous versus repeated sprints). Nutrition can have an effect on mTOR phosphorylation (Fujita *et al.*, 2007) and therefore studying the effect of aerobic exercise *per se* on phospho-mTOR^{Ser2448} is difficult in studies with nutritional input. Phosphorylation of p70S6K1^{Thr389} is heavily influenced by nutrition as well and increases post-exercise with protein feeding. In aerobic exercise models, phosphorylation at Thr389 does not increase acutely between 0-3 h post-exercise (Flück & Hoppeler, 2003; Mascher *et al.*, 2007; Camera *et al.*, 2010; Coffey *et al.*, 2010). Phosphorylation of p70S6K1$^{Ser424/Thr421}$ has been found to increase after aerobic exercise (Mascher *et al.*, 2007) but it is thought that phosphorylation at Thr389 is the critical phosphorylation site for inducing activity of p70S6K (Pearson *et al.*, 1995).

Commonly measured upstream targets of mTOR include Akt and TSC2. Camera and colleagues found that phospho-Akt^{Thr308} and phospho-Akt^{Ser473} were increased 30 minutes after both resistive and aerobic exercise, with phospho-TSC2^{Thr1462} only increasing after aerobic exercise (Camera *et al.*, 2010). Alongside these upstream regulators, phospho-mTOR^{Ser2448} increased immediately after exercise, returning to resting levels at 15 min post-exercise and then increased 95% at 30 and 60 minutes post-exercise (Camera *et al.*, 2010). These data illustrate the importance of the timing of muscle sampling with these types of analyses. Donges and colleagues found that phospho-Akt^{Ser473} and phospho-

Akt^{Thr308} did not increase after cycling exercise of 55% $VO_{2\ peak}$ for 40 minutes with nutrient feeding (Donges *et al.*, 2012). While seemingly contradictory to Camera and colleagues, the former study utilized higher intensity cycling at 70% $VO_{2\ peak}$ for one hour, potentially indicating intensity and volume dependent Akt phosphorylation (Sheffield-Moore, 2004; Camera *et al.*, 2010). Confirming the impact of intensity on Akt phosphorylation, cycling for one hour at 75% $VO_{2\ peak}$ resulted in a 5-fold increase in phospho-Akt^{Ser473} (Sheffield-Moore, 2004; Mascher *et al.*, 2007) indicating Akt phosphorylation requires high intensity exercise.

While mTORC1 has been found to be important in muscle hypertrophy and protein translation (Carraro *et al.*, 1990; Bodine *et al.*, 2001), redundancies exist within the signaling pathway, making it difficult to rely on signaling data alone in predicting phenotypic adaptations to exercise, as increases in protein synthesis can occur without increased phosphorylation of mTOR (Smith *et al.*, 1992; Donges *et al.*, 2012). In addition, increases in phospho-$mTOR^{S2448}$ and phospho-$p70^{T389}$ have been observed with no increases in myofibrillar protein synthesis, but with increases in mitochondrial protein synthesis (Sheffield-Moore, 2004; Wilkinson *et al.*, 2008). It is also not known what role the mTOR pathway has, if any, in regulating protein synthesis of individual subcellular protein pools.

IV.II MAPK Signaling Pathway in Transcriptional and Translational Regulation

At the onset of exercise, calcium concentrations increase within the cell to allow for contractile activity. This increase in calcium activates calmodulin-activated protein kinase (CaMK) (Baar, 2006). Activation of CaMKII occurs in contracting muscle and has been

found to be dependent on the intensity of contractile activity (Rose, 2006). Activation of CaMK is linked to the mitogen activated protein kinase (MAPK) signaling pathway which can influence transcriptional activity as well as translational regulation through interaction with eIF-4E (Chen *et al.*, 2001). This pathway has three main lineages that are affected by exercise, the extracellular signal regulated kinase 1 and 2 (ERK1/2), p38 MAPK, and c-Jun NH_2-terminal kinases (JNK) (Chau Long *et al.*, 2004). Very little is known about the regulation and specificity of MAPK pathways in skeletal muscle, but these important signaling molecules have been extensively studied in other tissues (Chau Long *et al.*, 2004; Zehorai *et al.*, 2010). This section will focus on ERK1/2 and p38 MAPK which are implicated in mechanically-induced signaling (Martineau & Gardiner, 2001), influencing both transcriptional and translational regulation (Figure I) (Chau Long *et al.*, 2004).

The ERK1/2 pathway is initiated through growth factor signaling as well as mechanical stress, increased intracellular calcium, ROS and hypoxic conditions, which are all present during exercise (Widegren *et al.*, 2001). Phosphorylation of ERK1/2 at Thr202/Tyr204 activates this kinase, propagating the signal downstream to p90RSK and TSC1/2, influencing transcription and translation, respectively (Widegren *et al.*, 1998). The p38 MAPK pathway is known as a stress-activated pathway that may respond to both systemic and local factors (Widegren *et al.*, 1998). Transcription factors such as myocyte-enhancing factor (MEF2) and peroxisome proliferator-activated receptor 1 coactivator α (PGC-1α) are affected by p38 MAPK activation (phoshp-p38$^{Thr180/Tyr182}$) enhancing transcription (Chau Long *et al.*, 2004). Both ERK1/2 and p38 are activated during and immediately after aerobic exercise (Widegren *et al.*, 1998; Yu *et al.*, 2002). While p38 activation is sustained 15 min after exercise, ERK1/2 activation is short lived and begins to

decrease by 15 min post exercise (Widegren *et al.*, 1998). Training status can influence MAPK activation, with training reducing this large response to exercise (Yu *et al.*, 2002). Phosphorylation of ERK1/2 during exercise is also dependent on the intensity of the exercise bout, with increased intensity increasing the level of activation of both ERK1/2 and its upstream regulator MAPK kinase (MEK1/2) (Widegren *et al.*, 2000). It has been shown that while exercise at 40% $VO_{2\ peak}$ increases phospho-ERK1/2$^{Thr202/Tyr204}$ by 11-fold, exercise at 75% $VO_{2\ peak}$ increases phosphorylation by 39-fold during exercise (Widegren *et al.*, 2000). It can therefore be speculated that higher intensity exercise would mediate greater changes to translation and transcription, mediating greater changes to protein synthesis and subsequent adaptations.

IV.III PGC1-α and Mitochondrial Biogenesis

PGC-1α has been implicated as a major regulator of mitochondrial biogenesis through transcriptional regulation (Winder *et al.*, 2006). The level of PGC-1α mRNA is often measured to determine the genetic expression of PGC-1α and therefore an activation of this signaling cascade. Aerobic exercise induces an increase in PGC-1α mRNA within 2 hours after exercise, which returns to resting levels by 19 hours (Mathai *et al.*, 2008; Egan *et al.*, 2010; Perry *et al.*, 2010). This is then followed by an increase in PGC-1α protein content 24 hours after the exercise stimulus (Perry *et al.*, 2010). While the mechanisms of PGC-1α transcription induction after exercise are not yet known, some signaling molecules have been implicated to be involved in this role such as AMPK, CaMK, activating transcription factor-2 (ATF-2), p38 MAPK, ROS and cyclic GMP (Baar, 2004; Egan *et al.*, 2010; Lira *et al.*, 2010). Aerobic exercise increases PGC-1α mRNA in an intensity dependent manner

with high intensity exercise resulting in a 2.5-fold higher increase at 3 h post-exercise than low intensity exercise (Egan *et al.*, 2010).

PGC-1α affects the transcriptional activity of mitochondrial transcription factor A (Tfam), nuclear respiratory factors (NRFs), and peroxisome proliferator-activated receptors (PPARs) which assist in coordinating the transcription of mitochondrial and nuclear DNA for mitochondrial biogenesis (Perry *et al.*, 2010). Increases in mitochondrial proteins can occur before the observed increase in PGC-1α highlighting that increases in PGC1-α are not necessary for the initial changes in transcription after aerobic exercise (Perry *et al.*, 2010). PGC-1α is activated by phosphorylation by a number of molecules including AMPK, and then subsequent deacetylation by histone deacetylases (HDACs) and sirtuin 1 (SIRT1) (Wright *et al.*, 2007; Jager *et al.*, 2007; Lira *et al.*, 2010). It is thought that these post-translational modifications are also signals for nuclear translocation of PGC-1α (Wright *et al.*, 2007; Lira *et al.*, 2010). Little and colleagues found that the nuclear content of PGC-1α increased immediately after 90 min of cycling at 65% $VO_{2\ peak}$ (Little *et al.*, 2010*b*) and 3 hours after HIIT exercise with no change in whole muscle PGC-1α content until 24 h after exercise (Little *et al.*, 2011). This was preceded by increases in phosphorylation of signaling molecules such as p38 MAPK and acetyl CoA carboxylase (ACC), and followed by increased mitochondrial gene transcription 3 hours post-exercise and increased key mitochondrial enzyme protein content 24 hours after exercise (Little *et al.*, 2011). PGC-1α has also been shown to translocate to the mitochondria 3 hours after exercise (Safdar *et al.*, 2011), suggesting that the translocation of PGC-1α to the nucleus and mitochondria is a signal for promoting mitochondrial biogenesis, likely in an effort to coordinate both mitochondrial and nuclear transcription (Safdar *et al.*, 2011).

V Summary and Rationale for Research

It is thought that the acute response to exercise as well as the adaptations with training are related to the quantity of muscle fibres recruited for the exercise (Burd *et al.*, 2010*b*) and the pattern in which they are recruited and made to contract (Hood, 2001). In addition, the degree of metabolic perturbation in the muscle, changes in calcium concentrations (Rose, 2006), the degree of mechanical strain (Martineau & Gardiner, 2001), and many other signaling events (Widegren *et al.*, 2000; Egan *et al.*, 2010) would also affect the acute and presumably chronic responses to exercise. Exercise of differing intensities can create diverse responses in each of these metabolic indicators altering protein turnover to elicit a specific adaptive response. Many of the signaling molecules discussed in this chapter have been found to have intensity dependent activation (Wojtaszewski *et al.*, 2000; Widegren *et al.*, 2000; Rose, 2006; Egan *et al.*, 2010) and are specific to particular modes of exercise (Atherton, 2005). They do not work in isolation however, as all of these pathways are intricately interconnected together and mediate a response to the exercise stimulus (Figure I). However, it is unclear the role that translational regulation pathways have in directing subcellular protein synthesis.

Most of the research conducted in protein metabolism with aerobic exercise has focused on the post-exercise responses of mixed MPS. While initially of importance, these studies give a relatively non-specific idea of how the muscle is responding to the stimuli and newer methods now exist to dig deeper into the protein-specific responses and mechanisms of activation. The study of subcellular protein synthesis is required to begin to elucidate how aerobic exercise effects the synthesis of the proteins directly responsible for the adaptations leading to a new phenotype with aerobic exercise training. This becomes

even more important when studying aerobic exercise intensity as the individual subcellular protein pools, namely myofibrillar and mitochondrial, respond differently with different types of exercise and also following training (Wilkinson *et al.*, 2008).

Much of the existing research on the impact of aerobic exercise on mitochondrial protein synthesis has involved protocols that incorporated a nutritional intervention into the equation, perhaps with the knowledge that nutrient, and specifically protein, provision stimulates myofibrillar FSR following resistance exercise (Moore *et al.*, 2009). While of great practical significance, nutrition heavily influences protein metabolism and the effects of the exercise stimulus *per se* cannot be elucidated. This is especially evident when studying subcellular muscle protein fractions. Amino acid provision can increase both myofibrillar and mitochondrial protein synthesis in the rested state (Bohé *et al.*, 2003) as well as further enhance myofibrillar protein synthesis after exercise, in the absence of a further increase in mitochondrial or sarcoplasmic protein synthesis (Moore *et al.*, 2009; Coffey *et al.*, 2010; Breen *et al.*, 2011). Therefore, if one is to study the effect of exercise per se on subcellular protein synthesis then nutritional interventions must be kept to a minimum.

VI Statement of Research Question and Hypothesis

The purpose of this investigation was to study the effects of aerobic exercise intensity on myofibrillar and mitochondrial protein synthesis in the post-absorptive early (4 h) and late (24 h) recovery phase. The effects of resistance exercise on protein metabolism have shown to be persistent, lasting for at least 24 h post-exercise and beyond, and therefore we aimed to study protein synthesis in this time-period in addition to the immediate acute post-exercise time-period. In addition to subcellular protein synthesis, another aim of this work

was to examine proteins involved in contraction dependent signaling and translational regulation to explore the potential mechanism for intensity-dependent differences in protein synthesis.

It was hypothesized that myofibrillar protein synthesis will be stimulated after high intensity exercise due to the increased fibre recruitment with an absence of a response after low intensity exercise. It was also hypothesized that measurement of phosphorylation of signaling molecules such as p38 MAPK, ERK1/2, mTOR, and p70S6K would be consummate with their proposed mechanism(s) of action in stimulating protein synthesis and thus allow for discussion of a potential mechanism for exercise intensity dependent differences.

CHAPTER II

AEROBIC EXERCISE INTENSITY AFFECTS SKELETAL MUSCLE MYOFIBRILLAR PROTEIN SYNTHESIS AND ANABOLIC SIGNALING IN YOUNG MEN

1. INTRODUCTION

Adaptations to aerobic exercise are primarily characterized by increased aerobic capacity due to increases in mitochondrial content and metabolic enzyme expression (Gollnick & Saltin, 1982), while adaptations to resistance exercise are characterized by increased muscle fiber size due to hypertrophy (McCall *et al.*, 1996). Muscle protein turnover is an important aspect of adaptation to both modes of exercise for both hypertrophy and remodeling. Protein turnover consists of the balance between two processes, which in skeletal muscle are termed muscle protein synthesis (MPS) and muscle protein breakdown (MPB). The small transient changes in protein turnover after an acute bout of exercise, often interacting with nutrition, accumulate over repeated bouts and can make changes to the skeletal muscle protein pools that are specific to the exercise stimuli, leading to stimuli-specific adaptation. Expansion of protein pools such as the mitochondria, occur when skeletal muscle is repeatedly placed under high energy demand induced by repetitive low load contractions for prolonged periods (i.e., aerobic exercise) (Phillips *et al.*, 1996) or repetitive slightly higher load contractions for shorter but repeated bouts (i.e., sprinting) (Burgomaster *et al.*, 2008).

Mixed MPS in response to aerobic exercise has been found to increase after aerobic exercise in both the fasted (Harber *et al.*, 2010) and the fed state (Wilkinson *et al.*, 2008; Harber *et al.*, 2010). Low intensity aerobic exercise also increases mixed MPS but the effect does not persist beyond 1 h post exercise (Sheffield-Moore, 2004) unless a longer duration of exercise is performed (Carraro *et al.*, 1990). Mixed MPS measures global skeletal MPS although is dominated by the myofibrillar proteins, as they comprise the majority of muscle proteins (~60% of total) (Brooks *et al.*, 2004). This does not allow

conclusions to be made on the specific protein response of the muscle to the exercise stimulus, as the response of smaller protein pools tend to be diluted by that of the abundant myofibrillar proteins. When studying adaptations to dynamic and resistive exercise, mitochondrial and myofibrillar proteins are of greatest interest as they are responsible for mitochondrial biogenesis and skeletal muscle hypertrophy, respectively, and respond differently to different types of exercise (Wilkinson *et al.*, 2008). The study of subcellular protein synthesis is required to begin to elucidate how aerobic exercise affects the synthesis of the proteins directly responsible for the adaptations leading to a new phenotype with aerobic exercise training.

Moderate to high intensity cycling has resulted in subcellular specific FSR responses with mitochondrial FSR increasing and myofibrillar FSR showing no response 4 h after exercise with nutrition potentially modulating the subcellular protein synthetic response (Wilkinson *et al.*, 2008; Donges *et al.*, 2012). Amino acid provision can increase both myofibrillar and mitochondrial protein synthesis in the rested state (Bohé *et al.*, 2003) as well as further enhance myofibrillar protein synthesis after exercise (Moore *et al.*, 2009; Coffey *et al.*, 2010; Breen *et al.*, 2011). Therefore, if one is to study the effect of aerobic exercise *per se* on subcellular MPS then nutritional factors must be kept to a minimum.

The signaling mechanisms influencing subcellular protein synthesis are not well understood. Aerobic exercise may cause initial signaling events due to tension-sensing integrin molecules, changes in calcium flux, alterations in membrane potential, and changes in ADP/ATP levels (Hood, 2001). These events each may occur to different degrees with varied intensity and duration of the exercise potentially resulting in altered acute responses of measurable downstream signaling events post-exercise. Calcium plays a major role in

contraction and is therefore a major signaling molecule with downstream targets in the MAPK pathway. The p38 MAPK and ERK1/2 proteins are involved in adaptation to exercise and are implicated in mechanically induced signaling. Phosphorylation of ERK1/2 at Thr202/Tyr204 activates this kinase propagating signals downstream to p90RSK and TSC1/2, influencing transcription and translation, respectively (Widegren *et al.*, 1998). The p38 MAPK pathway is known as a stress-activated pathway that may respond to both systemic and local factors (Widegren *et al.*, 1998). Transcription factors such as myocyte-enhancing factor 2 (MEF2) and peroxisome proliferator-activated receptor 1 coactivator α (PGC-1α) are effected by p38 MAPK activation (phoshp-p38$^{Thr180/Tyr182}$), enhancing transcription (Chau Long *et al.*, 2004). One of the master regulators of mitochondrial biogenesis, PGC-1α, affects the transcriptional activity of mitochondrial transcription factor A (Tfam), nuclear respiratory factors (NRFs), and peroxisome proliferator-activated receptors (PPARs) which assist in coordinating the transcription of mitochondrial and nuclear DNA for mitochondrial biogenesis (Perry *et al.*, 2010).

As exercise intensity increases, more fibers are recruited to perform the work (Sale, 1987), and a new metabolic stress occurs in the muscle, contributing to greater and potentially divergent adaptations between relative high and low exercise intensity (Hood, 2001). Thus, the purpose of this study was to study the effects of work-matched aerobic exercise intensity on myofibrillar protein synthesis in early (4 h) and late (24 h) recovery and to explore potential mechanisms for intensity-dependent differences in subcellular protein synthesis by studying key signaling molecules involved in MPS. It is hypothesized that high intensity exercise will elicit acute responses closer to that of resistance exercise

due to the increase in muscular force required to complete the task. Additionally, we propose that signaling molecule activation will reflect intensity dependent differences.

2. METHODS

2.1 Participants and Ethics

Eight healthy, recreationally active males (mean ± SEM, 21 ± 1 years, 82.5 ± 3.8 kg, 180.7 ± 1.6 cm, $VO_{2\ peak}$: 46.7 ± 2.0 mL kg^{-1} min^{-1}) were recruited to participate in the study. Participants completed aerobic exercise 2-3 times per week and did not perform lower body resistance exercise more than once per week. All participants were informed of the purpose of the study, experimental procedures and associated risks prior to participation and exercise testing. All participants gave verbal and written consent to a protocol approved by the Hamilton Health Science Research Ethics Board, conforming to the standards for the use of human subjects in research as, outlined in the Declaration of Helsinki.

2.2 Experimental Design

This study consisted of 1) maximal aerobic capacity measures, 2) familiarization with the aerobic exercise intensity, and 3) infusion trials for resting and post exercise metabolic investigation. Each participant completed both exercise intensity trials making this study a within subject design.

Maximal Aerobic Capacity Measurements

Two weeks prior to the metabolic study, participants reported to the Exercise and Metabolism Research Laboratory and completed a $VO_{2\ peak}$ test on a Lode cycle ergometer (Groningen, Netherlands) with continuous oxygen uptake measurements (Ergocard Professional, Medisoft, Sorinnes, Belgium). The test began at 50 W and increased 1 W every 2 s until volitional exhaustion, which occurred between 8 and 12 min into the test. Exhaustion was defined by a respiratory exchange ratio of greater than 1.1 and the inability to maintain 60 rpm on the cycle ergometer. Peak power output in Watts (W_{max}), maximum

heart rate (HR_{max}), and average cadence were recorded. Participants were asked to maintain a constant cadence (within 5-10 rpm) between 70 and 100 rpm. The position of the saddle and handlebars was noted for each participant and was repositioned accordingly for each subsequent exercise bout. Each subjects' W_{max} was used to determine the workload for the relative high (HIGH, 60% W_{max}) and low (LOW, 30% W_{max}) intensity exercise bouts for each participant.

Familiarization

A familiarization session was carried out with each participant with the exercise intensity that was be performed on the days of metabolic investigation, and also to confirm the relative intensity of the exercise based on HR and VO_2. One week prior to the metabolic study, participants completed a 5 min bout of exercise at LOW and then HIGH intensity. Heart rate was measured throughout the familiarization. Oxygen consumption was also measured in the last 2 min of both HIGH and LOW. The participants were asked to maintain the same constant pedaling cadence that was comfortable for them during the maximum aerobic capacity test, which was also maintained during exercise trials.

Metabolic Investigation and Infusion Protocol

Participants underwent two experimental infusion trials on sequential days for both high and low intensity exercise (4 trials total) to study 4 and 24 h post-exercise muscle protein metabolism. At least 10 days separated the trials for the two intensities. Participants were asked to keep a diet record for the 48 h period preceding the first infusion protocol (day 1), and they were asked to repeat this diet in the 48 h period preceding the third infusion protocol (day 3). A standardized meal representing 30% of each subjects' energy requirements (64% CHO, 17% PRO, 19% fat) was provided and consumed in the evening

before day 1 and 3 before 2000 h on the evening prior to the trial. After an overnight fast and after refraining from physical activity for 2 days prior to the trail, participants reported to the laboratory at 0600 h for day 1 (Figure 1, Figure 2). A 20-guage catheter was inserted into an antecubital vein of one arm and a baseline blood sample was obtained. The catheter was kept patent with 0.9% saline drip for repeated blood sampling (every 0.5 to 1 h). A second catheter was then inserted into the other arm for the primed constant infusion of L-[*ring*-$^{13}C_6$]phenylalanine (prime: 2 μmol kg^{-1}; infusion: 0.05 μmol kg^{-1}; Cambridge Isotope Laboratories, Cambridge, MA, USA) which passed through a 2 μm filter. The participant rested in the bed until 3 hours into the infusion where a biopsy (~100-150 mg) was obtained from the *vastus lateralis* for fasted rested measurements. Muscle biopsies were obtained under local anesthesia (2% xylocaine) using a 5 mm Bergström needle modified for manual suction. Tissue obtained was blotted, freed of visible connective tissue and fat, and immediately frozen in liquid N_2 and stored at -80 °C until analysis. After the resting biopsy, participants began the exercise protocol on the same Lode cycle ergometer as used in the $VO_{2\ peak}$ test. Participants were randomized to complete the HIGH or LOW intensity protocol during trial 1. The HIGH protocol consisted of 30 min at 60% W_{max}. The LOW protocol consisted of a warm-up 60 min at 30% W_{max}. Measurements of HR were taken throughout the entire protocol, and VO_2 measurements were obtained three times during each ride. The participants returned to a bed to rest until 30 minutes after exercise when the second biopsy was obtained. After another 4 h of tracer infusion, a third biopsy was obtained and then the infusion was terminated. The diet was standardized between days 1 and 2 by providing participants with a meal immediately after the trial representing 50% of their daily caloric requirements (64% CHO, 17% PRO, 19% fat) and then providing a meal

of identical composition to consume in the evening before 2200 h. The next day participants returned to the laboratory after an overnight fast at 0700 h to undergo day 2 (Figure 1, Figure 2). The infusion protocol was carried out as in day 1, with biopsies obtained at 1.5 h and 5.5 h into the infusion to obtain 24 – 28 h post-exercise measurements. Approximately 2 weeks later, the participants returned to the laboratory to complete the same protocol with the opposite exercise intensity to their first trial. All blood samples were collected in heparinized evacuated containers and kept on ice until they were centrifuged to obtained plasma which was subsequently aliquoted, frozen, and stored at -20 °C until further analysis.

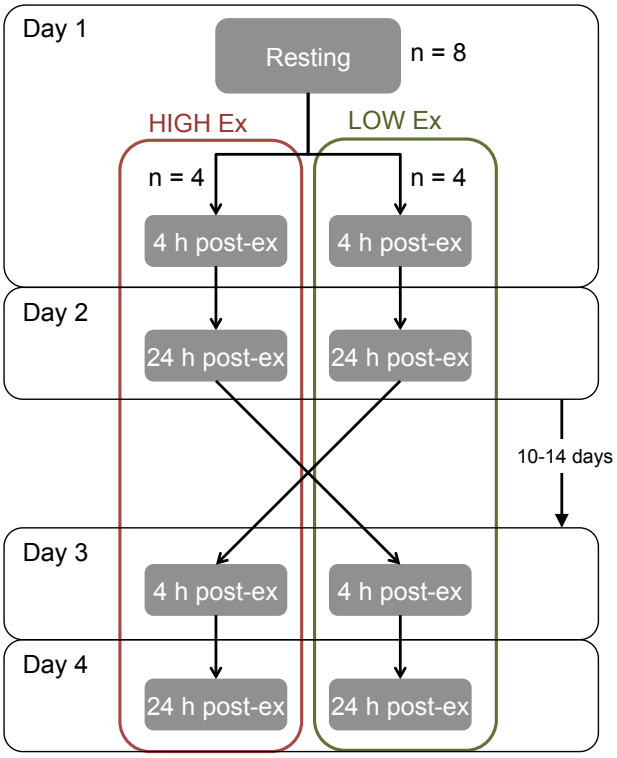

Figure 1. Schematic of study design and infusion trials.

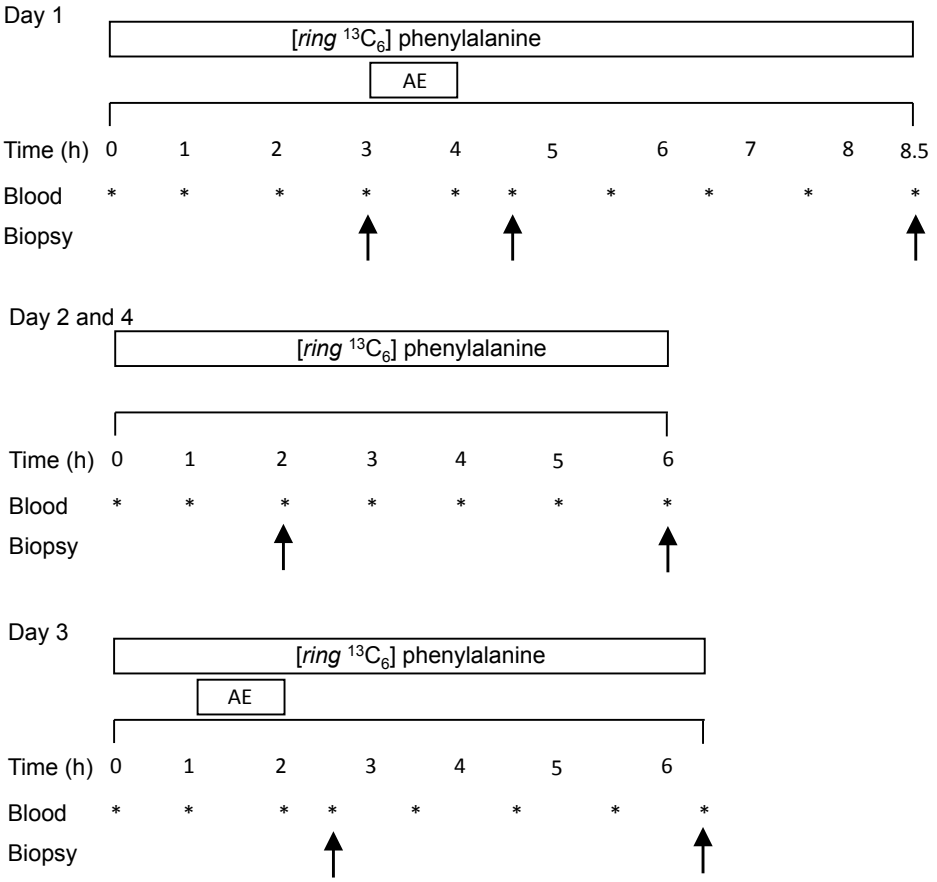

Figure 2. Schematic of infusion trials utilized in the study design. Timing is representative of performing low intensity exercise. Asterisk represent blood draws and single arrow represent muscle biopsies.

2.3 Blood and Muscle Analysis

Plasma [*ring*-$^{13}C_6$]phenylalanine enrichments were determined as previously described (Glover *et al.*, 2008*b*). Muscle intracellular (IC) free amino acids were extracted from a 10-15 mg piece of wet muscle with ice cold 0.6 M perchloric acid (PCA) and purified as previously described (Burd *et al.*, 2011*a*). Purified free amino acids were then converted to their heptafluorobutyrate (HFB) derivatives and analyzed for [*ring*-$^{13}C_6$]phenylalanine enrichment by a GC-MS (GC:#6890, MS:#5973, Hewlett-Packard, Palo Alto, CA, USA) as previously described (Moore *et al.*, 2009).

A ~100 mg piece of wet muscle was homogenized using a glass Dounce homogenizer in ice-cold homogenization buffer (10 μL mg^{-1}; 0.067 M Sucrose, 0.05 M Tris/HCl, 0.05 M KCl, 0.01 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 700x *g* for 15 min at 4 °C to pellet myofibrillar proteins. The supernatant was transferred to another Eppendorf tube and centrifuged at 12,000x *g* for 20 min at 4 °C to pellet mitochondria. The resulting supernatant (sarcoplasmic extract) was transferred to an Eppendorf tube and a 100 μL 1/10 dilution was made for use in a BCA assay. Both the extract and the dilution were frozen at -80 °C until further analysis. Amino acids were obtained from the mitochondrial pellet as described previously(Burd *et al.*, 2011*a*). Briefly, the pellet was washed twice with ice-cold homogenization buffer, once with ethanol and then dried under vacuum. Proteins were hydrolyzed by adding 6 N HCl and heating at 110 °C for 18 h. From the myofibrillar enriched pellet, nuclear proteins were extracted. The myofibrillar-enriched pellet was washed with ice-cold homogenization buffer and centrifuged at 700x *g* for 10 min at 4 °C. Three times, the pellet was washed with ice-cold PBS with added protease and phosphatase inhibitors and centrifuged at 15,000x *g* for 5 min at 4 °C. The pellet was fully resuspended in 4 μL of high salt buffer (HSB; 0.05 M Tris/HCl, 0.4 M NaCl, 0.001 M DTT, 0.001 M EGTA, 0.001 M EDTA, 0.1% SDS; added protease and phosphatase inhibitors) for every 1 mg of original wet tissue weight. The resuspended pellet was incubated on ice for 20 min, and was vortexed twice throughout. The Eppendorf tube was then placed in a sonication bath for 20 min at 4 °C followed by vortexing. The resuspension was again incubated on ice for 20 min, vortexing every 10 min and then was centrifuged at 15,000x *g* for 10 min at

4 °C. The resulting supernatant (nuclear extract) was transferred to an Eppendorf tube and a 100 μL 1/10 dilution was made for use in a BCA assay. Both the extract and the dilution were frozen at -80 °C until further analysis. The myofibrillar enriched pellet was washed with H_2O and centrifuged at 15,000x *g* for 5 min at 4 °C. Myofibrillar proteins were further extracted and hydrolyzed as described previously (Burd *et al.*, 2011*a*). The free amino acids from the mitochondrial and myofibrillar enriched fractions were purified using cation-exchange chromatography (Dowex 50WX8-200 resin, Sigma-Aldrich Ltd, St. Louis, MO, USA) and converted to their *N*-acetyl-*n*-propyl ester derivatives for gas chromatography combustion isotope ratio mass spectrometry (GC: #6890, Hewlett Packard, Palo Alto, CA, USA; IRMS: Delta Plus XP, Thermo Finnigan, Waltham, MA, USA).

2.4 Immunoblot Analysis

Both sarcoplasmic and nuclear extracts were used for immunoblot analysis for presence and/or phosphorylation of signaling molecules. The protein concentration of the extracts was determined using the BCA assay (Thermo Fisher Scientific, Rockford, IL, USA). Samples were prepared to the same concentration by diluting with H_2O and denatured with Laemmli sample buffer and heated to 95 °C. On a 10% SDS-PAGE gel, 20-40 μg of protein (depending on the target) was loaded and ran at 120 V for 1-1.5 h. Proteins were transferred onto a PVDF membrane using Fast Semi-Dry Transfer (Thermo Fisher Scientific Inc., Rockford, IL, USA). Membranes were blocked at RT for 1 h using 5% w/v milk or BSA in Tris-buffered saline with 0.1% Tween 20 (TBST). Membranes were incubated in primary antibody in TBST at 4 °C overnight: from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) rabbit polyclonal phospho-p70 (Thr389; 1:1,000 in TBST; #SC11759-R), from Abcam (Cambridge, MA, USA) rabbit polyclonal histone 2B (0.1 μg/mL in TBST,

#ab1790) and from Cell Signaling Technology (Danvers, MA, USA) rabbit polyclonal phospho-mTOR (Ser2448; 1:1,000 in TBST, #2971), rabbit monoclonal phospho-p38 MAPK (Thr180/Tyr182; 1:1,000 in TBST; #4511), rabbit polyclonal phospho-ERK1/2 (Thr202/Tyr204; 1:1,1000 in TBST, #9101), and rabbit monoclonal alpha-tubulin (1:2,000 in TBST, #2125). Membranes were washed with TBST and then incubated with secondary anti-rabbit HRP-linked antibody (1:10,000 in TBST; GE Healthcare Life Sciences Ltd., Baie D'Urfe, QC, Canada; NA934) at RT for 1 h. After washing, membranes were visualized using chemiluminescence (Supersignal West Dura Extended Substrate, Thermo Fisher Scientific Inc., Rockford, IL, USA), and imaged using Fluorochem SP Imaging system (Protein Simple: Alpha Innotech, Santa Clara, CA, USA). Images were quantified using National Institute of Health ImageJ software. Alpha-tubulin and Histone 2B were used as a loading control in sarcoplasmic and nuclear protein samples respectively.

2.5 Calculations

The fractional synthetic rates (FSR) of myofibrillar proteins were calculated using the precursor-product equation previously described, FSR (%/h) = $(E_{2b} - E_{1b})/E_p \times 1/t \times 100)$, where E_{2b} and E_{1b} are the bound protein enrichments from the muscle at time 2 and 1 respectively and E_p is the average enrichment of the precursor, intracellular phenylalanine (Moore *et al.*, 2009). Since participants were 'tracer naïve', the baseline pre-infusion blood sample enrichment represents the naturally abundant ^{13}C enrichments, and was used for E_{1b} to determine resting FSR (Burd *et al.*, 2011*b*).

2.6 Statistical Analysis

Aerobic exercise trial data was analyzed using two-tailed paired-samples *t*-test for between exercise intensities. Data within an exercise bout was analyzed using a repeated measures

one-way (factor: time) analysis of variance (ANOVA), with planned contrasts. Since a within-subject design was utilized, protein synthesis and signaling data were analyzed using a repeated measures two-factor (time x intensity) ANOVA. Relevant planned contrasts were used for protein synthesis data. Where appropriate, further *post hoc* analysis was performed with pairwise comparisons using Tukey's HSD test. Correlations were 2-tailed Pearson correlations. All statistical analyses were performed using SPSS Statistics (Version 20, IBM, Armonk, NY, USA). All data are presented as means ± SEM. Statistical significance was accepted at $p \leq 0.05$.

3. RESULTS

3.1 Aerobic Exercise Trial

All participants except for one completed the exercise at the prescribed intensity. For this one participant, the workload was reduced in the high intensity trial and therefore the lower intensity trial workload was reduced to remain energy matched within the participant. The average $\%VO_{2\ peak}$ and $\%HR_{max}$ was significantly higher in the HIGH trail (76 ± 3, 90 ± 1, respectively) than in the LOW trial (48 ± 1, 66% ± 2), ($p < 0.001$; Table 1). During the HIGH trial $\%VO_{2\ peak}$ was higher near completion of the bout than during the first VO_2 measure at 30% completion (n = 6, $p < 0.05$). This was also observed for $\%HR_{max}$ during the HIGH exercise bout (n = 8, $p < 0.05$). No change in $\%VO_{2\ peak}$ was observed during the LOW exercise bout. Energy expenditure was the same for both HIGH (369 kJ ± 19) and LOW (367 kJ ± 19).

Table 1. Characteristics of low and high intensity exercise during the trials.

	Low Intensity	High Intensity
Workload (Watts)	99 ± 4	198 ± 7
Time (min)	60	30
Average $\%V_{O2\ peak}$	48 ± 1	76 ± 3*
Average $\%HR_{max}$	66 ± 2	90 ± 1*
Energy Expenditure (kJ)	367 ± 19	369 ± 19

Values are means ± SEM. * Significantly different than low intensity, $p < 0.001$

3.2 Plasma Enrichment

The free plasma tracer enrichment was constant and was not different between the 0.5 – 4.5 and 24 – 28 h post-exercise incorporation times; 30-270 and 120-360 min respectively (Figure 3).

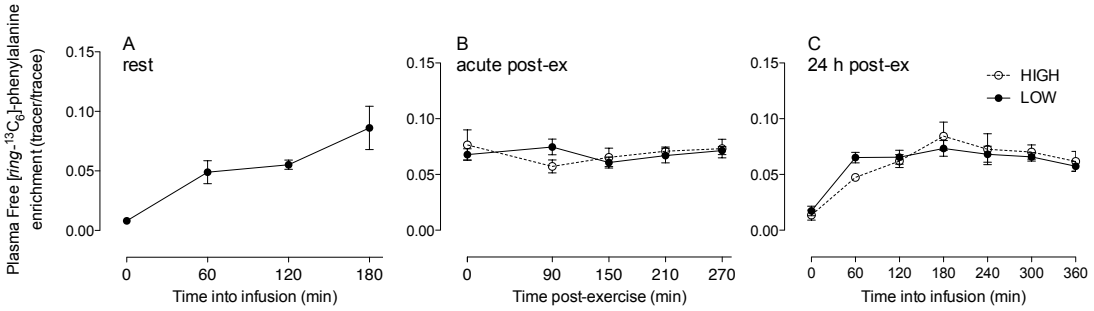

Figure 3. Plasma free [*ring*-$^{13}C_6$]-phenylalanine enrichment during rest (A), acute post-exercise (B), and 24 h post-exercise (C) tracer infusion. In B, time is expressed as minutes post-exercise as infusion times differed between participants due to the randomized design.

3.3 Myofibrillar Protein Synthesis

Myofibrillar FSR was increased in early recovery compared to rest ($p < 0.01$; Figure 4). In the LOW condition myofibrillar FSR returned to rest 24 h post-exercise, while Tukey's HSD test revealed that in the HIGH condition the 24 h myofibrillar FSR remained elevated ($p < 0.05$).

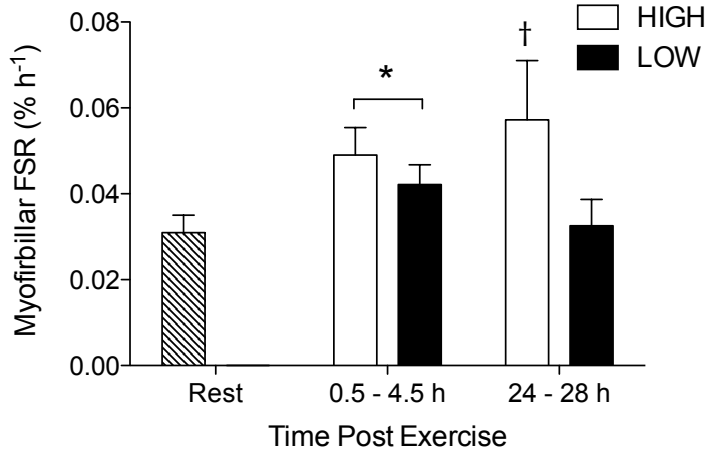

Figure 4. Myofibrillar fractional synthesis rate (% h^{-1}) at rest, and during early and late recovery from HIGH and LOW exercise. *different than rest ($p < 0.01$), †different than rest ($p < 0.05$).

3.4 Cell Signaling

There was a strong trend for a time × intensity interaction for phospho-mTOR^{Ser2448} (p = 0.056), with a significant difference at 0.5 h post exercise between HIGH and LOW (p < 0.005; Figure 5A). This effect is no longer seen at 4.5 h post exercise when phospho-mTOR^{Ser2448} begins to return to resting levels. At 24 and 28 h post-exercise, phospho-mTOR^{Ser2448} remains at resting levels. Downstream of mTOR, there was no effect of the exercise on phospho-p70^{Thr389} (Figure 5B). Aerobic exercise also did not have an effect on the phosphorylation of the MAPK protein ERK1/2$^{Thr202/Tyr204}$ (Figure 5C). However, there was an effect of time on phospho-p38$^{Thr180/Tyr182}$ (p < 0.005; Figure 5D) where phosphorylation was significantly higher at 4.5 h post-exercise compared to 0.5, 24, and 28 h post-exercise (p < 0.05). There was no effect of the exercise intensity on phospho-p38$^{Thr180/Tyr182}$.

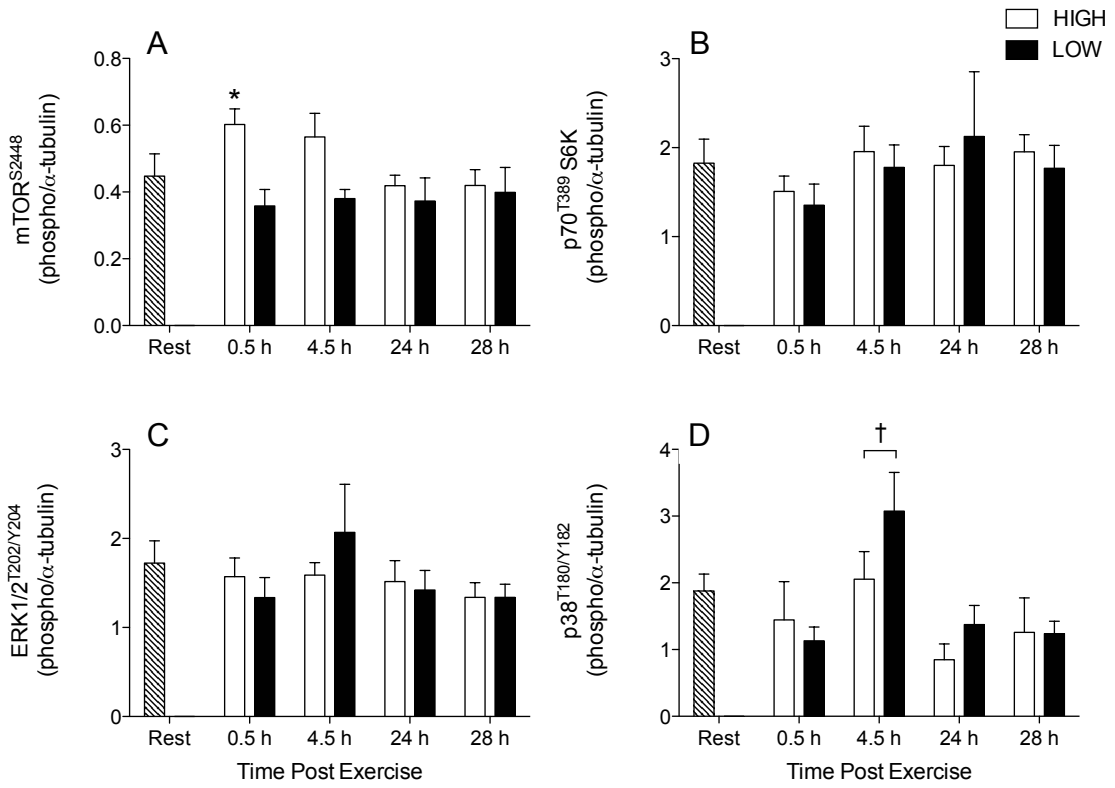

Figure 5. mTOR^{Ser2448} (A), p70^{Thr389} (B), ERK1/2$^{Thr202/Tyr204}$ (C), and p38$^{Thr180/Tyr182}$ (D) phosphorylation expressed as phosphorylated protein normalized to α-tubulin content. *different than LOW at time point, †different than 0.5, 24, and 28 h, ($p < 0.05$).

Myofibrillar FSR in early and late recovery after exercise was positively correlated with phospho-mTOR^{S2448} at 0.5 h post exercise (r = 0.698, $p < 0.005$ and r = 0.674, $p < 0.005$, respectively; Figure 6). No relationship was observed between phospho-p70^{Thr389} and myofibrillar FSR (data not shown).

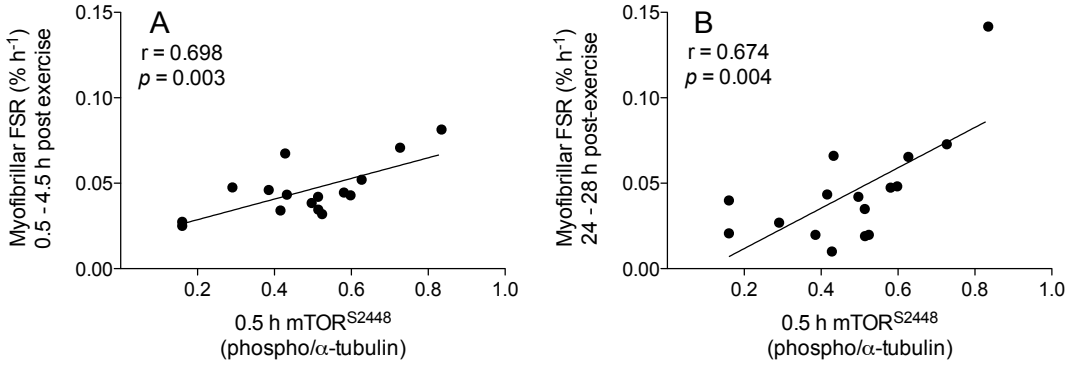

Figure 6. Correlation of myofibrillar FSR in early (A) and late (B) recovery with phospho-mTOR^{Ser2448} at 0.5 h post-exercise (r = 0.698, $p < 0.005$ and r = 0.674, $p < 0.005$, respectively).

3.5 Nuclear PGC-1α Content

There was no effect of aerobic exercise on nuclear PGC-1α content (Figure 7). Resting level of nuclear PGC-1α content was positively correlated with $VO_{2\ peak}$ (r = 0.864, $p <$ 0.01; data not shown).

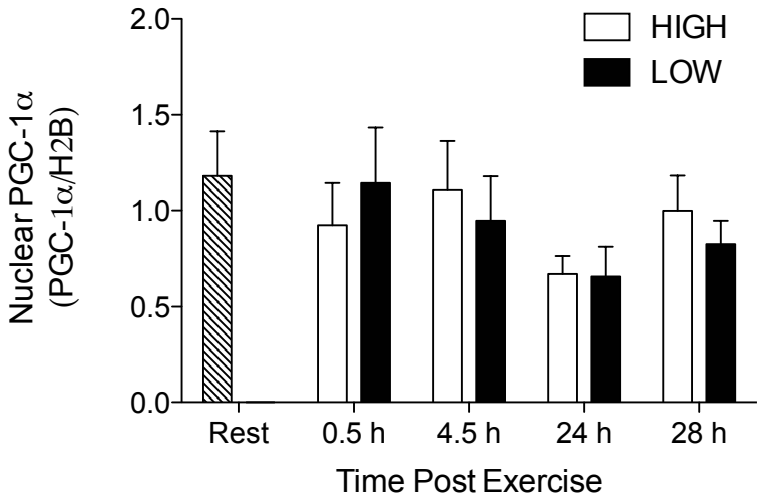

Figure 7. Nuclear content of PGC-1α expressed as PGC-1α normalized to histone 2B content.

4. DISCUSSION

The purpose of this investigation was to determine the effect of aerobic exercise intensity on myofibrillar protein synthesis in early and late post-exercise recovery. In addition, we aimed to study the activation of signaling molecules involved in contraction induced signaling and anabolic processes. We report that aerobic exercise stimulates myofibrillar protein synthesis in early recovery and exercise intensity may influence the length that this response persists. Additionally, increased mTOR phosphorylation was observed only after high intensity exercise and that the extent of phosphorylation of $mTOR^{Ser2448}$ was positively correlated with the early and late myofibrillar FSR, confirming the role that the mTOR pathway has in mediating myofibrillar protein synthesis.

4.1 Early (0.5 – 4.5 h) Recovery Myofibrillar FSR Response

The main finding of the current study is that myofibrillar protein synthesis was increased in early recovery after aerobic exercise in the fasted state (1.5-fold; Figure 4). Few studies have addressed the effect of aerobic exercise on myofibrillar protein synthesis, and those that have, have often included a feeding intervention into the study design which would presumably be additive to the stimulus of exercise. For example, Donges et al. found that cycling at 55% $VO_{2\ peak}$ did not result in increases in myofibrillar FSR (Donges *et al.*, 2012), while other more intense exercise protocols such as single leg kicking at 67% W_{max} (Miller *et al.*, 2005) and loaded single leg stepping (Cuthbertson, 2005), resulted in myofibrillar FSR increases both at 6 and 24 h post-exercise (Miller *et al.*, 2005; Cuthbertson, 2005), even above the rested fed state FSR (Miller *et al.*, 2005). Prior work from our group, which also measured myofibrillar FSR in the rested fed and post-exercise fed state, did not see increases in FSR post-exercise even with a relatively intense aerobic

exercise of single leg cycling at 75% $VO_{2\ peak}$ (Wilkinson *et al.*, 2008). In this case, the aerobic exercise was not able to induce an increase in myofibrillar FSR beyond that of the protein dose as was seen by Miller *et al*. Both of these studies utilized intermittent feeding throughout the investigation and therefore these differences in myofibrillar FSR may be due to the intensity of the exercise. Single leg kicking at 67% W_{max} was likely to induce a higher VO_2 and a greater relative 'exercise stress' as well as require greater forces in more isolated muscle groups to complete the dynamic movement (Richardson *et al.*, 1995; 1998). While the current study did not reveal a significant effect of intensity acutely, differences were seen 24 h post-exercise.

4.2 Late (24 – 28 h) Recovery Myofibrillar FSR Response

Myofibrillar FSR remained elevated 24 h post HIGH while resting values are observed in 24 h post LOW exercise (Figure 4). There was some variability in this 24 h post HIGH exercise response with 2 subjects returning to rest, while 6 out of 8 subjects maintaining or heightening the myofibrillar FSR rate measured in early recovery. It has been observed previously that for 24 h after resistance (Burd *et al.*, 2010*a*; 2010*b*) and high-intensity aerobic exercise (Miller *et al.*, 2005; Cuthbertson, 2005) in the fed state, that myofibrillar FSR is increased above rest. With some exercise models, such as resistance exercise 30% 1RM to failure the 24 h myofibrillar FSR response exceeds the early FSR response (Burd *et al.*, 2011*c*); however, those FSR measures 24 h post-exercise were in the fed-state and therefore may also be measuring heightened sensitivity to amino acids that results after exercise. The current study is the first to measure myofibrillar FSR 24 h after cycling in the fasted state and find increased myofibrillar FSR 24 h after high intensity aerobic exercise. It has been proposed that the extended protein synthesis response after exercise is dependent

on the volume of the exercise performed and possibly, type II fiber recruitment (Burd *et al.*, 2010*b*). The observation that HIGH exercise induces a 24 h myofibrillar FSR response with no response with LOW exercise, further adds to the speculation that increased fiber recruitment is required for sustained protein synthetic responses.

4.3 mTOR Signaling

We found that at 0.5 h after HIGH exercise, phospho-mTOR^{Ser2448} had increased from rest, but did not increase after LOW exercise (Figure 5A). The importance of the mTOR pathway in protein translation has been demonstrated numerous times but perhaps most convincingly with rapamycin treatment in humans (Drummond *et al.*, 2009*b*) and animals (Bodine *et al.*, 2001), and with knockout-like models in animals (Risson *et al.*, 2009). Increases in mTOR phosphorylation after cycling exercise has been observed previously (Mascher *et al.*, 2007; Wilkinson *et al.*, 2008; Coffey *et al.*, 2009; Camera *et al.*, 2010), however this is the first report of an aerobic exercise intensity effect on mTOR phosphorylation.

A downstream target of mTOR, p70S6K is a regulator of translation initiation and elongation (Kimball, 2006). This investigation did not report an effect of aerobic exercise on phospho-p70S6K1^{Thr389} not unlike the results of other studies of aerobic exercise with biopsies taken at similar time points (Mascher *et al.*, 2007; Camera *et al.*, 2010; Coffey *et al.*, 2010). Work from our lab shows that p70S6K1 is also highly responsive to nutrition (Glover *et al.*, 2008*a*) and therefore, given that this study was performed in the fasted state, it is perhaps not surprising that we did not see increases in phoshp-p70S6K1^{Thr389} post exercise. This signaling molecule is thought to be crucial in the propagation of mTOR signaling (Ohanna *et al.*, 2005) and has been shown on many occasions after resistance

exercise to correlate to muscle protein synthesis (Kumar *et al.*, 2009; Burd *et al.*, 2010*a*) and mass gains with training (Terzis *et al.*, 2007). This finding is inconsistent however, which may be due to the timing of the tissue sampling used when measuring the 'response' of p70S6K1 (Mitchell *et al.*, 2012).

Since no changes in phosho-p70S6K1^{Thr389} were observed post-exercise there was no relationship between the acute increase in phosphorylation and the myofibrillar FSR response. However, we did observe a positive correlation between acute 0.5 h post-exercise phospho-mTOR^{Ser2448} and both early and late recovery myofibrillar FSR (Figure 6), which explains similar variance previously reported relationship between phospho-p70S6K1^{Thr389} and myofibrillar FSR (Burd *et al.*, 2010*a*) and between phospho-4EBP-1 and myofibrillar FSR (Burd *et al.*, 2010*b*) after resistance exercise. The relationship between phospho-mTOR^{Ser2448} and myofibrillar FSR speaks to the importance in the mTOR pathway in regulating protein synthesis, and potentially, myofibrillar protein synthesis specifically.

4.4 MAPK Signaling

MAPK signaling is involved in both transcriptional and translational regulation, and is known to be involved in contraction dependent signaling after exercise. Molecules such as ERK1/2 and p38 MAPK are well-characterized molecules in the MAPK signaling pathway, both of which have been shown to respond to aerobic exercise. This study did not show an effect of aerobic exercise on phospho-ERK1/2$^{Thr202/Tyr204}$ at the time points studied. While ERK1/2 activation has been shown to be exercise dependent (Widegren *et al.*, 2000), the response of phospho-ERK1/2$^{Thr202/Tyr204}$ to similar cycling exercise has been shown to be immediately following exercise and short lived (Widegren *et al.*, 1998; Williamson *et al.*,

2003). Therefore, the lack of response seen can be attributed to the late 30 min post-exercise time point.

There was a main effect of time for phospho-p38$^{Thr180/Tyr182}$ where at 4.5 h post-exercise, phosphorylation was significantly higher than at 0.5 h and the resting levels achieved at 28 h post-exercise (Figure 5D). We do not report any effect of intensity (p = 0.30), which is in agreement to other groups who failed to find intensity dependent effects on p38$^{Thr180/Tyr182}$ phosphorylation where increases were seen immediately after exercise (Widegren *et al.*, 1998; Egan *et al.*, 2010; Bartlett *et al.*, 2012) subsiding by 3 h post-exercise (Benziane *et al.*, 2008; Egan *et al.*, 2010; Bartlett *et al.*, 2012) in exercise models of similar intensities to that of this investigation.

4.5 Nuclear PGC-1α Content

PGC-1α is known as a major regulator of mitochondrial biogenesis, coordinating both nuclear and mitochondrial genetic expression to induce adaptations to aerobic exercise over time. While increases in whole muscle PGC-1α content have been found to increase 24 h post-exercise (Baar *et al.*, 2002; Little *et al.*, 2011), generally acute increases in PGC-1α mRNA are measured to quantify the response of this signaling cascade to exercise. In addition to the genetic expression of PGC-1α, nuclear localization of PGC-1α is thought to mediate some acute responses to exercise (Little *et al.*, 2011). We found that nuclear content was not increased after exercise (Figure 7) in contrast to what other groups have shown after HIIT (4 × 30 sec all-out sprints) (Little *et al.*, 2011) and traditional endurance exercise (90 min at 65% $V_{O2\ peak}$) (Little *et al.*, 2010*b*). These models of exercise are higher in intensity and duration, respectively, from that utilized in this study potentially inducing a greater signal for PGC-1α nuclear localization that what we imposed with our subjects here.

While exercise intensity-dependent PGC-1α nuclear localization has yet to be reported, the PGC-1α mRNA post-exercise response is affected by intensity, with high intensity exercise inducing a 2.5-fold greater increase in mRNA 3 h post-exercise than a lower intensity exercise (Egan *et al.*, 2010). Another factor in observing nuclear PGC-1α is the timing of biopsy sampling. Nuclear localization has been observed immediately after traditional endurance exercise (further time points post-exercise have not been studied) (Little *et al.*, 2010*b*), however, is delayed after HIIT exercise with increases at 3 h post-exercise (Little *et al.*, 2011). Therefore, it is also possible, while less likely, that the time points utilized did not capture an increase in PGC-1α between 0.5 and 4.5 h post-exercise. It has been reported that increases in nuclear mitochondrial gene expression after exercise can occur without increases in PGC-1α content, and therefore nuclear localization of PGC-1α may be mediating these immediate effects (Wright *et al.*, 2007). It is unknown whether these initial responses have been observed in the absence of increases in PGC-1α nuclear localization.

A relationship between mitochondrial content and $VO_{2\ peak}$ exists, whereby training increases both mitochondrial content and $VO_{2\ peak}$ (Hoppeler *et al.*, 1985). With aerobic exercise training, parallel increase in both mitochondrial content (Hoppeler *et al.*, 1985) and enzyme activity (Freyssenet *et al.*, 1996) also occur. Endurance training in the form of HIIT has also been shown to increase nuclear PGC-1α content at rest (Little *et al.*, 2010*a*), highlighting that the role of nuclear PGC-1α may have in mediating mitochondrial adaptations. In support of this, we have reported that the resting nuclear PGC-1α content is positively correlated with $VO_{2\ peak}$. This was a strong correlation ($r = 864$), which highlights that molecular measures can reflect physiological outcomes and that nuclear PGC-1α is important in supporting mitochondrial content. At this time it is not possible to determine

the exact causal nature of this correlation, but it may be related to the subjects training status and thus propensity for mitochondrial biogenesis.

4.6 Aerobic Exercise Intensity Methodology

In resistance exercise models, intensity of exercise is generally defined by a relative measure as a percentage of 1 RM (%1RM). With dynamic exercises, which are often categorized as aerobic in nature, exercise intensity can be defined by a relative workload ($\%W_{max}$) similarly to resistance exercise, or as a relative aerobic load ($\%VO_{2\ peak}$). In this study, we chose to use $\%W_{max}$ as opposed to $\%VO_{2\ peak}$ to quantify intensity to keep the force required to perform the movement constant throughout the exercise bout and also to be able to have a linear relationship between the force required between the two intensities. Thus, in this study, HIGH was twice the workload of LOW. By modifying the duration we were able to match the exercise bouts for energy expenditure. Therefore, by comparing these two exercise intensities we are able to isolate the effects of the increase in force required to complete the same amount of work within a shorter period of time in the HIGH versus LOW exercise.

4.7 Conclusions

In conclusion, the present study confirmed that myofibrillar protein synthesis is stimulated in early recovery from aerobic exercise in the fasted state, and that exercise intensity may affect how long the MPS response is sustained for. We found that the increase in MPS was positively correlated with the phosphorylation of $mTOR^{Ser2448}$, highlighting the role of this pathway in mediating myofibrillar protein synthesis. While there was no effect of the exercise on nuclear localization of PGC-1α, we found a strong positive correlation between

resting nuclear PGC-1α content and aerobic capacity ($VO_{2\ peak}$), adding to the evidence for the importance of nuclear PGC-1α in supporting mitochondrial function.

4.8 Future Directions

The present study aimed to answer a basic mechanistic question as to what the effects of aerobic exercise intensity were on subcellular protein synthesis and related signaling cascades. Therefore, the trials were performed in a fasted state, eliminating the effects of nutrition on these measures. Nutrition has a major role in regulating protein synthesis and augments the effects of exercise. It would be of practical interest to study the effects of nutrition in addition to high intensity exercise, which could potentially yield greater increases in myofibrillar protein synthesis. With the intensity dependent results obtained in this study on myofibrillar protein synthesis, it is therefore plausible that aerobic exercise models, which use even greater force applications during movement such as HIIT, could have similar or even greater effect on myofibrillar protein synthesis.

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Appendix A

SUBJECT CHARACTERISTICS

Subject characteristics

Subject	Age (yr)	Height (cm)	Weight (kg)	$V_{O2\,peak}$ (mL kg^{-1} min^{-1})	W_{max} (W)
S1	20	179.0	74.4	43.1	290
S2	20	182.0	81.5	46.5	345
S3	20	174.0	76.6	56.0	356
S4	22	182.0	97.3	43.4	381
S5	19	179.5	101	41.9	338
S6	21	185.0	79.3	40.0	277
S7	23	176.5	70.2	53.7	339
S8	21	187.5	79.4	48.8	318
Mean	21	180.7	82.5	46.7	330
SD	1.3	4.391	10.9	5.75	34.2
SEM	0.45	1.552	3.86	2.03	12.1

Appendix B

EXERCISE DATA AND ANOVA TABLES

High intensity exercise data

Subject	Workload (W)	% $V_{O2\ peak}$ 1	2	3	Avg	% HR_{max} 1	2	3	Avg	Time (min)	Energy Expenditure (kJ)
S1	174	79	83	83	82	93	94	96	95	36.98	334
S2	207	63	65	65	64	82	89	92	88	37.65	388
S3	214	71	67	74	71	88	94	97	93	35.92	410
S4	229	80	83	88	84	83	84	86	84	35.23	432
S5	203	-	-	-	-	79	87	93	86	34.75	383
S6	166*	80	-	71	76	93	88	87	89	34.50	257
S7	203	75	77	78	76	89	95	97	94	34.50	384
S8	191	73	77	78	76	87	91	95	91	34.50	361
Mean	198	74	75	77	75	87	90	93	90	35.50	369
SD	20.7	6.2	7.7	7.6	6.7	5.1	3.9	4.3	3.9	1.232	54.0
SEM	7.31	2.3	3.2	2.9	2.5	1.8	1.4	1.5	1.4	0.4356	19.1

High intensity exercise %$VO_{2\ peak}$ ANOVA results (n = 6)

Source of Variation	SS	df	MS	F	*p*
Time	0.523	2	0.262	7.072	0.012*
Error(Time)	0.370	10	0.0370		

*significant effect

Planned contrasts for factor: time

Contrast	SS	df	MS	F	*p*
1 vs. 2	0.202	1	0.202	2.249	0.194
1 vs. 3	1.04	1	1.04	22.810	0.005*

*significant contrast

High intensity exercise %HR_{max} ANOVA results (n = 8)

Source of Variation	SS	df	MS	F	*p*
Time	1.51	2	0.755	7.613	0.006*
Error(Time)	1.39	14	0.0992		

*significant effect

Planned contrasts for factor: time

Contrast	SS	df	MS	F	*p*
1 vs. 2	0.980	1	0.980	5.277	0.055
1 vs. 3	3.00	1	3.00	8.115	0.025*

*significant contrast

Low intensity exercise data

Subject	Workload (W)	% $V_{O2\ peak}$ 1	2	3	Avg	% HR_{max} 1	2	3	Avg	Time (min)	Energy Expenditure (kJ)
S1	87	52	52	50	51	69	77	76	74	67.08	325
S2	104	44	47	45	45	58	64	58	60	67.98	389
S3	107	44	43	50	46	75	74	75	74	67.27	402
S4	114	47	54	50	50	59	61	61	61	67.23	428
S5	101	48	57	49	51	54	64	61	60	69.50	388
S6	83*	61	50	49	53	68	74	62	68	68.60	259
S7	102	44	47	46	46	59	66	69	65	68.50	386
S8	95	44	47	49	47	61	62	68	64	67.12	361
Mean	99.1	48	50	48	49	63	68	66	66	67.91	367
SD	10.3	6.0	4.5	1.9	3.0	7.0	6.3	6.8	5.8	0.8898	53.1
SEM	3.64	2.1	1.6	.68	1.1	2.5	2.2	2.4	2.0	0.3146	18.8

Low intensity exercise %VO2 peak ANOVA results

Source of Variation	SS	df	MS	F	*p*
Time	0.111	2	0.0554	0.380	0.691
Error(Time)	2.04	14	1.46		

Low intensity exercise %HR_{max} ANOVA results

Source of Variation	SS	df	MS	F	*p*
Time	0.998	2	0.499	4.069	0.040*
Error(Time)	1.72	14	0.123		

*significant effect

Planned contrasts for factor: time

Contrast	SS	df	MS	F	*p*
1 vs. 2	1.90	1	1.90	13.193	0.008*
1 vs. 3	0.911	1	0.911	3.257	0.114

*significant contrast.

Appendix C

MYOFIBRILLAR FRACTIONAL SYNTHESIS RATE (FSR) RAW DATA AND ANOVA TABLES

Myofibrillar protein fractional synthesis rate (FSR; % h^{-1})

		High Intensity Exercise		Low Intensity Exercise	
Subject	**Rest**	**0.5 - 4.5 h**	**24 - 28 h**	**0.5 - 4.5 h**	**24 - 28 h**
S1	0.01959	0.03405	0.04343	0.04203	0.03489
S2	0.02021	0.03456	0.01910	0.02741	0.02068
S3	0.03525	0.08139	0.1417	0.03849	0.04208
S4	0.04354	0.04459	0.04738	0.04607	0.01984
S5	0.05003	0.05206	0.06545	0.06738	0.01007
S6	0.03194	0.04293	0.04820	0.04751	0.02696
S7	0.01950	0.03196	0.01985	0.02509	0.03994
S8	0.02773	0.07075	0.07283	0.04328	0.06602
Mean	0.03098	0.04904	0.05725	0.04216	0.03257
SD	0.01151	0.01817	0.03904	0.01311	0.01726
SEM	0.00407	0.00642	0.01380	0.00464	0.00614

Myofibrillar protein FSR ANOVA results

Source of Variation	SS	df	MS	F	*p*
Time	0.002178	2	0.001089	3.407	0.062*
Error(Time)	0.004474	14	0.0003196		
Intensity	0.001328	1	0.001328	3.425	0.107
Error(Intensity)	0.002715	7	0.0003879		
Time × Intensity	0.001298	2	0.0006491	2.574	0.112
Error (Time × Intensity)	0.003531	14	0.0002522		

*strong trend for an effect ($p = 0.062$).

Planned contrasts for factor: time

Contrast	SS	df	MS	F	*p*
Rest vs 0.5 - 4 h	0.01711	1	0.001711	21.09	0.003*
Rest vs 24 – 28 h	0.01552	1	0.001552	2.607	0.150

*significant contrast

Pairwise comparisons with Tukey's HSD test for myofibrillar FSR factor: time × intensity

$HSD = q_{(k,df)} \sqrt{(MS_E/n)}$ $p < 0.05$

$k = 6$ $df_E = 14$ $n = 8$

$q_{(10,28)} = 4.64$

$HSD = 0.02605$

Pairwise Comparison	Mean Difference	Significance MD > HSD
Rest vs. 0.5 – 4.5 h HIGH	0.01806	N
Rest vs. 24 – 28 h HIGH	0.02627*	Y
Rest vs. 0.5 – 4.5 h LOW	0.01118	N
Rest vs. 24 – 28 h LOW	0.00159	N
0.5 – 4.5 h, HIGH vs. LOW	0.00688	N
24 – 28 h, HIGH vs. LOW	0.02468	N

*significant difference between means.

Appendix D

WESTERN BLOTTING RAW DATA AND ANOVA TABLES

Sarcoplasmic p-mTOR^{S2448} raw data (phospho/α-tubulin)

		High Intensity				Low Intensity			
Subject	**Rest**	**0.5 h**	**4.5 h**	**24 h**	**28 h**	**0.5 h**	**4.5 h**	**24 h**	**28 h**
S1	0.3480	0.4157	0.4253	0.4238	0.5092	0.5134	0.3943	0.5131	0.2688
S2	0.2327	0.5139	0.4647	0.4229	0.2212	0.1601	0.4299	0.6625	0.7721
S3	0.3805	0.8349	0.4767	0.4176	0.2937	0.4968	0.3957	0.3718	0.4994
S4	0.7314	0.5807	0.4154	0.3843	0.3786	0.3855	0.3928	0.2183	0.4694
S5	0.4587	0.6267	0.9866	0.6103	0.5238	0.4281	0.2134	0.1293	0.0863
S6	0.4356	0.5979	0.4378	0.4076	0.6311	0.2906	0.3421	0.4828	0.4485
S7	0.2705	0.5243	0.7268	0.3978	0.3696	0.1603	0.4845	0.4781	0.4404
S8	0.7241	0.7268	0.5848	0.2861	0.4326	0.4324	0.3878	0.1264	0.2071
Mean	0.4477	0.6026	0.5648	0.4188	0.4200	0.3584	0.3801	0.3728	0.3990
SD	0.1887	0.1307	0.2000	0.08951	0.1327	0.1401	0.07863	0.1966	0.2104
SEM	0.0667	0.0462	0.0707	0.0317	0.0469	0.0495	0.0278	0.0695	0.0744

Sarcoplasmic p-mTOR^{Ser2448} ANOVA results

Source of Variation	**SS**	**df**	**MS**	**F**	***p***
Time	0.09006	4	0.02251	0.644	0.636
Error(Time)	0.9786	28	0.03495		
Intensity	0.1967	1	0.1967	4.353	0.075
Error(Intensity)	0.3164	7	0.04520		
Time × Intensity	0.1885	4	0.04713	2.620	0.056*
Error (Time × Intensity)	0.5036	28	0.01799		

* strong trend for an interaction of time × intensity ($p = 0.056$).

Pairwise comparisons with Tukey's HSD test for p-mTOR^{Ser2448} factor: time × intensity

$HSD = q_{(k,df)} \sqrt{(MS_E/n)}$ $p < 0.05$

$k = 10$ $df_E = 28$ $n = 8$

$q_{(10,28)} = 4.92$

$HSD = 0.233$

Pairwise Comparison	Mean Difference	Significance MD > HSD
0.5 h HIGH vs. 0.5 h LOW	0.2442*	Y
4.5 h HIGH vs. 4.5 h LOW	0.1847	N
24 h HIGH vs. 24 h LOW	0.04601	N
28 h HIGH vs. 28 h LOW	0.02098	N

* significant difference between means.

Sarcoplasmic p-p70^{Thr389} raw data (phospho/α-tubulin)

		High Intensity				Low Intensity			
Subject	**Rest**	**0.5 h**	**4.5 h**	**24 h**	**28 h**	**0.5 h**	**4.5 h**	**24 h**	**28 h**
S1	0.6763	1.1501	3.3770	2.9759	2.7952	0.5259	0.8984	0.8707	1.1513
S2	1.9742	1.7046	1.9485	1.8561	2.6713	1.2543	2.5424	0.9799	1.1474
S3	1.1303	1.1379	2.0596	2.0972	1.6756	2.7269	2.2601	1.9245	0.8227
S4	1.8294	1.3370	1.1846	0.9702	1.2315	1.6583	2.1460	2.3718	2.6513
S5	3.0101	2.4618	2.3356	1.6391	2.1691	0.9783	0.6223	0.6577	2.6268
S6	2.5966	1.2147	0.8046	1.4961	1.6115	0.9011	2.4441	2.1501	2.4367
S7	1.9947	1.9342	1.4799	2.0142	1.7297	1.6606	1.7201	1.0673	1.8525
S8	1.4024	1.1374	2.4581	1.3666	1.7416	1.1311	1.6054	6.9790	1.4553
Mean	1.827	1.510	1.956	1.802	1.953	1.355	1.780	2.125	1.768
SD	0.7589	0.4852	0.8083	0.5994	0.5452	0.6722	0.7110	2.064	0.7299
SEM	0.2683	0.1715	0.2858	0.2119	0.1928	0.2377	0.2514	0.7297	0.2580

Sarcoplasmic p-p70^{Thr389} ANOVA results

Source of Variation	SS	df	MS	F	*p*
Time	2.729	4	0.6823	0.819	0.524
Error(Time)	23.32	28	0.8328		
Intensity	0.02988	1	0.02988	0.019	0.895
Error(Intensity)	11.16	7	1.594		
Time × Intensity	0.7455	4	0.1864	0.230	0.919
Error (Time × Intensity)	22.67	28	0.8095		

Sarcoplasmic p-p42/44$^{Thr202/Tyr204}$ raw data (phospho/α-tubulin)

		High Intensity				Low Intensity			
Subject	**Rest**	**0.5 h**	**4.5 h**	**24 h**	**28 h**	**0.5 h**	**4.5 h**	**24 h**	**28 h**
S1	1.2807	1.2261	2.1715	0.9787	1.5121	1.5044	1.0700	1.6424	2.0695
S2	2.8110	2.9449	1.4374	2.2299	2.0816	0.7108	0.9744	0.7772	1.0157
S3	1.2315	1.6825	1.5205	2.8245	0.8197	2.2286	1.9410	2.6941	0.6550
S4	2.8029	1.6798	1.4181	1.2525	1.0112	1.3827	1.6853	1.0138	1.1091
S5	1.4721	1.2348	1.2025	1.3143	0.7576	0.4031	5.7041	0.8682	1.4209
S6	1.8554	1.2982	1.9668	1.2140	1.7017	0.8762	1.2894	1.1881	1.5318
S7	1.2510	1.2485	1.0787	1.4289	1.2162	1.5491	1.8094	1.6401	1.5734
S8	1.0907	1.2640	1.9242	0.8799	1.6052	2.0289	2.0782	1.5491	1.3253
Mean	1.724	1.572	1.590	1.515	1.338	1.335	2.069	1.422	1.338
SD	0.7061	0.5876	0.3893	0.6681	0.4651	0.6353	1.524	0.6181	0.4239
SEM	0.2496	0.2077	0.1376	0.2362	0.1644	0.2246	0.5387	0.2185	0.1499

Sarcoplasmic p-p42/44$^{Thr202/Tyr204}$ ANOVA results

Source of Variation	SS	df	MS	F	*p*
Time	2.697	4	0.6743	0.923	0.464
Error(Time)	20.45	28	0.7305		
Intensity	0.01749	1	0.01749	0.025	0.879
Error(Intensity)	4.872	7	0.6961		
Time × Intensity	1.160	4	0.2900	0.696	0.601
Error (Time × Intensity)	11.67	28	0.4167		

Sarcoplasmic p-p38$^{Thr180/Tyr182}$ (phospho/α-tubulin)

		High Intensity				Low Intensity			
Subject	**Rest**	**0.5 h**	**4.5 h**	**24 h**	**28 h**	**0.5 h**	**4.5 h**	**24 h**	**28 h**
S1	2.7120	0.6443	2.1373	1.2443	1.2491	1.8914	1.9316	0.9305	1.2091
S2	1.0740	1.4498	1.4316	0.4717	0.7650	1.0528	5.4942	0.7479	1.6616
S3	1.2510	5.1482	1.7910	0.0166	0.7834	0.2116	2.6964	2.6055	0.5906
S4	1.9133	1.0437	1.4261	0.7225	4.6494	1.4587	1.2451	0.4285	1.1084
S5	1.8223	0.2023	1.3312	1.8368	0.3919	0.9917	5.4526	1.1022	1.7289
S6	1.2292	1.0373	1.9052	1.4763	1.7411	0.5558	3.6674	0.8743	1.5394
S7	3.0859	1.9396	1.5536	1.0055	0.2815	1.0115	1.8753	2.3091	0.3481
S8	1.9436	0.1073	4.8612	0.0229	0.2111	1.8714	2.2431	2.0102	1.7086
Mean	1.879	1.447	2.055	0.8496	1.259	1.131	3.076	1.376	1.237
SD	0.7195	1.615	1.167	0.6627	1.464	0.5916	1.638	0.8108	0.5293
SEM	0.2544	0.5710	0.4126	0.2347	0.5176	0.2092	0.5792	0.2867	0.1871

Sarcoplasmic p-p38$^{Thr180/Tyr182}$ ANOVA results

Source of Variation	SS	df	MS	F	p
Time	23.34	4	5.864	4.967	0.004*
Error(Time)	33.06	28	1.181		
Intensity	1.170	1	1.170	1.242	0.302
Error(Intensity)	6.595	7	0.9422		
Time × Intensity	4.510	4	1.128	0.770	0.554
Error (Time × Intensity)	40.98	28	1.463		

* significant effect

Pairwise comparisons with Tukey's HSD test for p-p38$^{Thr180/Tyr182}$ factor: time

$HSD = q_{(k,df)} \sqrt{(MS_E/n)}$ $p < 0.05$

$k = 5$ $df_E = 28$ $n = 16$

$q_{(5,28)} = 4.17$

$HSD = 1.133$

Pairwise Comparison	Mean Difference	Significance MD > HSD
Rest vs. 0.5 h	0.590	N
Rest vs. 4.5 h	0.686	N
Rest vs. 24 h	0.766	N
Rest vs. 28 h	0.631	N
0.5 h vs. 4.5 h	1.277*	Y
0.5 h vs. 24 h	0.176	N
0.5 h vs. 28 h	0.041	N
4.5 h vs. 24 h	1.452*	Y
4.5 h vs. 28 h	1.317*	Y
24 h vs. 28 h	0.135	N

* significant difference between means

Nuclear PGC-1α (PGC-1α/H2B)

		High Intensity				Low Intensity			
Subject	**Rest**	**0.5 h**	**4.5 h**	**24 h**	**28 h**	**0.5 h**	**4.5 h**	**24 h**	**28 h**
S1	0.6671	0.4441	1.0036	1.1366	1.2247	0.8778	0.2996	0.6419	0.6944
S2	1.1151	0.8222	0.9942	0.9919	0.4519	0.5097	0.8527	0.2932	1.4889
S3	2.4134	0.8400	1.3787	0.5779	0.2968	3.0201	2.0683	1.4914	0.3976
S4	0.3683	0.9445	0.7642	0.4904	0.7467	1.2077	1.4612	1.0774	0.8186
S5	0.8568	2.4133	2.6846	0.6143	0.7845	0.3708	0.1591	0.5730	0.9243
S6	1.0732	0.8381	0.7690	0.6892	1.8369	1.0071	0.4645	0.5127	0.6479
S7	1.8415	0.5185	0.1988	0.3719	1.4740	0.9725	0.8235	0.5707	0.5464
S8	1.1225	0.5718	1.0781	0.4955	1.1760	1.2043	1.4484	0.1035	1.0888
Mean	1.182	0.9241	1.109	0.6710	0.9990	1.146	0.9472	0.6580	0.8259
SD	0.6551	0.6282	0.7215	0.2632	0.5226	0.8147	0.6622	0.4387	0.3441
SEM	0.2316	0.2221	0.2551	0.09305	0.1848	0.2881	0.2341	0.1551	0.1217

Nuclear PGC-1α : histone-2B ANOVA results.

Source of Variation	SS	df	MS	F	p
Time	2.387	4	0.5967	1.767	0.164
Error(Time)	9.456	28	0.3377		
Intensity	0.01262	1	0.01262	0.021	0.889
Error(Intensity)	4.190	7	0.5985		
Time × Intensity	0.4100	4	0.1025	0.383	0.819
Error (Time × Intensity)	7.488	28	0.2674		

Appendix E

METHODS FOR ISOLATING SUBCELLULAR PROTEIN FRACTIONS FOR USE IN WESTERN BLOTTING AND DETERMINING MUSCLE PROTEIN SYNTHESIS

E-I Mitochondrial Protein Extraction for Determination of Protein Synthesis:

This section describes in detail the procedure used to isolate mitochondrial proteins for determining mitochondrial protein synthesis. It is ideal, when using [$^{13}C_6$]-phenylalanine as the isotope tracer, to use ~100 mg muscle tissue to yield enough mitochondrial proteins to achieve an adequate signal during GC-C-IRMS analysis. This protocol yields highly enriched mitochondrial proteins with some myofibrillar protein contamination as determined with western blotting (Figure E.1).

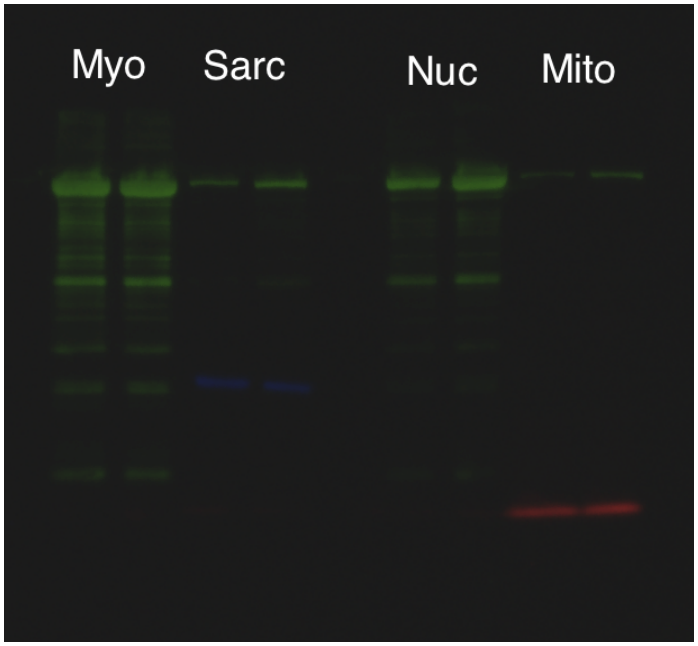

Figure E.1 – Western blot of mitochondrial, myofibrillar, nuclear and sarcoplasmic fractions of two tissue samples isolated using Appendix E-I and E-II protocol targeting MHC (green), LDH (blue), and COX IV (red), showing a highly enriched mitochondrial fraction.

Stock Solutions Required:

- 1 M Sucrose: 17.115 g sucrose in 50 mL ddH_2O, store at 4 °C.
- 1 M Tris/HCl: 6.07 g Tris Base in 30 mL ddH_2O, pH to 7.4 with HCl, bring solution to 50 mL with ddH_2O. Store at room temperature.
- 1 M KCl: 3.73 g KCl in 50 mL ddH_2O. Store at 4 °C.

- 0.5 M EDTA/Tris: 9.30 g EDTA in 30 mL ddH_2O, pH to 7.4 using Tris HCl powder, bring to 50 mL. Store at 4 °C.

- 0.1 M EGTA/Tris: 1.905 g EGTA in 30 mL ddH_2O, pH to 7.4 using Tris HCl powder, bring to 50 mL. Store at 4 °C

- 6 M HCl: 250 mL 12 M stock HCl into 250 mL cold ddH_2O

Buffer Recipes:

- Isolation Buffer 1 (IB1): 670 µL 1 M sucrose, 500 µL 1 M Tris/HCl, 500 µL 1 M KCl, 200 µL 0.5 M EDTA, bring to 5 mL with ddH_2O, pH to 7.4, bring to 10 mL with ddH_2O. Add 1 PhosSTOP (phosphatase inhibitor) and 1 cOmplete Mini (protease inhibitor) tablet.

- Isolation Buffer 2 (IB2): 2.5 mL 1 M sucrose, 300 µL 0.1 M EGTA/Tris, 100 µL 1 M Tris/HCl, bring to 5 mL with ddH_2O, pH to 7.4, bring to 10 mL with ddH_2O.

Protocol:

1. Weigh 80-100 mg of wet muscle into a labeled 1.5 mL Eppendorf tube.
2. Place Glass Dounce homogenizer in ice to cool.
3. Transfer wet muscle to Glass Dounce homogenizer.
4. Add 10 µL per mg wet muscle of cold IB1 buffer to muscle in glass homogenizer.
5. Homogenize thoroughly (5-10 minutes) until no fibrous pieces can be seen.
6. Pull up sample with a glass pipette and place in 1.5 mL Eppendorf tube labeled MYO.
7. Centrifuge the homogenate at 700x *g* for 15 min at 4 °C.
8. Transfer the supernatant into a 1.5 mL Eppendorf tube labeled MITO. MYO pellet can be used in E-II for nuclear and myofibrillar protein extraction, or can be frozen at -80 °C for future use.
9. Centrifuge MITO supernatant at 12,000x *g* for 20 min at 4 °C.
10. Transfer the supernatant into a 1.5 mL Eppendorf labeled WS (for western blotting)(F-II).
11. Aliquot 10 µL of WS into 90 µL of ddH_2O in a tube labeled BCA for BCA protein assay (F-I). Place both WS and BCA in -80 °C freezer for future use.
12. Resuspend MITO pellet in 500 µL of IB2.
13. Centrifuge at 12,000x *g* for 15 min at 4 °C.
14. Remove and discard supernatant.
15. Gently resuspend pellet in 500 µL of IB2.
16. Centrifuge at 12,000x *g* for 5 min at 4 °C.
17. Remove and discard supernatant.
18. Add 500 µL of 95% EtOH – DO NOT VORTEX.

19. Centrifuge at 12,000x *g* for 5 min at 4 °C.
20. Remove and discard supernatant.
21. Pierce a hole in the Eppendorf lid and dry under vacuum using lyophilizer.
22. Transfer pellet very carefully to a 4 mL screw top tube and add 1 mL of 6 M HCl. Cap vials tightly.
23. Hydrolyze overnight at 110 °C.
24. Purify hydrolyzed product over Dowex cation exchange resin (Appendix E-VI).

E-II Nuclear Protein Extraction for Use in Western Blotting:

This section describes in detail the procedure used to isolate nuclear proteins from the myofibrillar enriched protein fraction resulting from Appendix E-I. This protocol yields a nuclear enriched protein fraction contaminated with MHC but with no sarcoplasmic protein contamination, as determined with western blotting (Figure E.1, E.2).

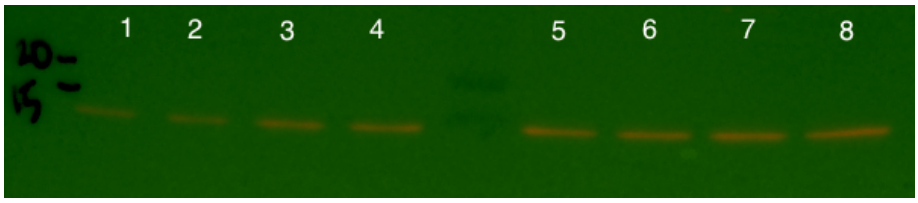

Figure E.2 – Western blot with ladder overlay of nuclear extract loaded in incremental qunantities 5 µg (lanes 1 and 2), 10 µg (lanes 3 and 4), 15 µg (lanes 5 and 6), and 20 µg (lanes 7 and 8) probed for H2B (red).

Stock Solutions Required:

- 10× PBS: 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4 (sodium phosphate dibasic), 2.4 g KH_2PO_4 (potassium phosphate monobasic), 1L ddH_2O, pH to 8.0.
- 1 M Tris/HCl: 6.07 g Tris Base in 30 mL ddH_2O, pH to 7.4 with HCl, bring solution to 50 mL with ddH_2O. Store at room temperature.
- 1 M DTT: 0.154 g DTT in 1 mL ddH_2O, vortex. Store as 100 µL aliquots at -20 °C.
- 0.1 M EGTA: 1.90 g EGTA in 30 mL ddH_2O, add 3 NaOH tablets to solubilize (~pH 8), pH to 7.6 with 1M HCl. Store at 4 °C.
- 0.5 M EDTA: 9.5 g EDTA dehydrate in 30 mL ddH_2O, add NaOH tablets to solubilize (~pH 8), pH to 7.6 with 1M HCl. Store at 4 °C.
- 10% SDS (w/v): 5 g in 50 mL ddH_2O. Store at room temperature.
- 1 M NaCl: 2.92 g NaCl in 50 mL ddH_2O. Store at 4 °C.

Buffer Recipes:

- 1× PBS + inhibitors: 3 mL 10× PBS, bring volume to 30 mL with ddH_2O, add ½ PhosSTOP and ½ cOmplete Mini tablet.

- High Salt Buffer (HSB) – for 10 mL: 500 µL 1 M Tris/HCl, 4 mL 1 M NaCl, 10 µL 1 M DTT, 100 µL 0.1 M EGTA, 20 µL 0. 5M EDTA, pH to 7.4 with 0.1 M HCl, bring volume to 10 mL with ddH_2O, add 1 PhosSTOP and 1 cOmplete Mini tablet, keep on ice and add 100 µL 10% SDS just before use.

Protocol:

1. Continue from MYO pellet obtained from mitochondrial extraction protocol (Appendix E-I, Step 8). Thaw on ice if pellet was frozen.
2. Add 400 µL IB1 to myo pellet.
3. Centrifuge at 700x *g* for 10 min at 4 °C.
4. Remove and discard supernatant.
5. Add 400 µL of ice cold 1× PBS+inhibitors to pellet and vortex to resuspend.
6. Centrifuge at 15,000x *g* for 5 min at 4 °C.
7. Remove and discard supernatant.
8. Add 400 µL of ice cold 1× PBS+inhibitors to pellet, vortex.
9. Centrifuge at 15,000x *g* for 5 min at 4 °C.
10. Remove and discard supernatant.
11. Add 400 µL of ice cold 1× PBS + inhibitors to pellet.
12. Centrifuge at 15,000x *g* for 5 min at 4 °C.
13. Remove and discard supernatant.
14. Add SDS to HSB to make 0.1% SDS.
15. Resuspend pellet in ice cold HSB at a ratio of 4 µL per mg of original tissue weight.
16. Break up pellet with 10 µL pipette tip by stirring then vortex on high for 10 s.
17. Incubate on ice for 20 min, vortexing every 10 min for 10 s.
18. Place in sonication bath at 4 °C for 20 min.
19. Vortex for 10 s.
20. Incubate on ice for 20 min, vortexing every 10 min for 10 s.
21. Centrifuge at 15,000x g for 10 min at 4 °C.
22. Transfer supernatant (nuclear extract) into Eppendorf labeled NUC and keep on ice. Pellet is MYO and can continue to E-III for further extraction.
23. Aliquot 10 µL of NUC into Eppendorf labeled BCA-NUC with 90 µL ddH_2O.
24. Store NUC and BCA-NUC in -80 °C until use for BCA protein assay (Appendix F-I) and western blotting (Appendix F-III).

E-III Myofibrillar Protein Extraction for Determination of Protein Synthesis:

This section provides in detail the procedure to extract myofibrillar proteins from the myofibrillar enriched fraction from Appendix E-II step 22.

Reagents Required:

- 0.3 M NaOH: 3 g NaOH into 250 mL ddH_2O
- 1 M PCA: 86.2 mL 70% stock perchloric acid into 1000 mL ddH_2O
- 70% ethanol: 294.8 mL 95% EtOH stock and bring volume to 400 mL with ddH_2O
- 6 M HCl: 250 mL 12 M stock HCl into 250 mL cold ddH_2O

Protocol:

1. Add 500 µL of ddH_2O to MYO pellet and vortex to fully suspend.
2. Centrifuge at 15,000x *g* for 5 min at 4 °C.
3. Remove and discard supernatant.
4. Add 1 mL 0.3 M NaOH to pellet and vortex thoroughly.
5. Heat at 50 °C for 30 min, vortexing for 10 s every 10 min.
6. Centrifuge at 10,000x *g* for 5 min at 4 °C.
7. Transfer supernatant to labeled 4 mL screw top vial.
8. Add 1 mL 0.3 M NaOH to pellet and vortex thoroughly.
9. Heat at 37 °C for 10 min.
10. Centrifuge at 10,000x *g* for 5 min at 4 °C.
11. Add supernatant to previous supernatant (step 7) in the labeled 4 mL screw top vial.
12. Pellet contains collagen and can be discarded if this fraction is not being studied.
13. Add 1 mL of 1 M PCA to supernatant in screw top vial. A white precipitate should be seen.
14. Centrifuge uncapped vials at 2000 RPM for 10 min at 4 °C using the blood tube rotor, being careful when lowering the tubes into the bucket using tweezers to prevent breakage.
15. Remove and discard supernatant.
16. Add 500 µL 70% EtOH. DO NOT VORTEX.
17. Spin at 2000 RPM for 10 min at 4 °C. See precaution at step 14.
18. Remove and discard supernatant.
19. Add 500 µL 70% EtOH. DO NOT VORTEX.
20. Spin at 2000 RPM for 10 min at 4 °C. See precaution at step 14.
21. Remove and discard supernatant.
22. Add 1.5 mL 6 M HCl. Cap vials tightly.
23. Hydrolyze overnight at 110 °C.
24. Purify hydrolyzed product over Dowex cation exchange resin (Appendix E-VI).

E-IV Intracellular Free Pool (IC) Extraction

This section describes in detail the procedure for obtaining the intracellular free pool (IC) of amino acids used to calculate the muscle protein FSR.

Reagents Required:

- 0.6 M PCA: 25.9 mL 70% stock perchloric acid into 500 mL ddH_2O.

Protocol:

1. Weigh out 10-20 mg of wet muscle and place in 1.5 mL Eppendorf tube on ice and add 500 µL 0.6 M PCA.
2. Homogenize thoroughly with Teflon pestle.
3. Centrifuge at 10,000 RPM for 3 min at 4 °C.
4. Transfer supernatant to a 2.0 mL Eppendorf tube labeled IC.
5. Add 500 µL 0.6 M PCA to pellet and further homogenize and vortex.
6. Centrifuge at 10,000 RPM for 3 min at 4 °C.
7. Transfer supernatant to IC tube from step 4.
8. Add 500 µL 0.6 M PCA to pellet and further homogenize and vortex.
9. Centrifuge at 10,000 RPM for 3 min at 4 °C.
10. Transfer supernatant to IC tube from step 4.
11. Purify combined supernatant over Dowex cation exchange resin (Appendix E-VI) or store at -80 °C to column at a later date.

E-V Mixed Plasma Protein Extraction

This section describes in detail the procedure for the extraction of mixed plasma proteins from plasma. This protocol is used on the baseline plasma sample (before beginning isotope prime and infusion) to determine the baseline enrichment in a tracer naïve participant.

Reagents Required:

- Acetonitrile (ACN)
- 70% ethanol: 294.8 mL 95% EtOH stock and bring volume to 400 mL with ddH_2O
- 6 M HCl: 250 mL 12 M stock HCl into 250 mL cold ddH_2O

Protocol:

1. Thaw baseline plasma samples on ice.
2. Add 500 µL ACN to labeled 1.5 mL Eppendorf.
3. Add 200 µL plasma directly to ACN in Eppendorf.
4. Centrifuge at 10,000 RPM for 5 min at 4 °C.
5. Remove and discard supernatant.
6. Add 500 µL ACN to pellet.
7. Centrifuge at 10,000 RPM for 5 min at 4 °C.
8. Remove and discard supernatant.
9. Add 500 µL ddH_2O and vortex.
10. Centrifuge at 10,000 RPM for 5 min at 4 °C.
11. Remove and discard supernatant.
12. Add 500 µL 70% EtOH and vortex.
13. Centrifuge at 10,000 RPM for 5 min at 4 °C.
14. Remove and discard supernatant.
15. Pierce a hole in the Eppendorf lid and dry under vacuum using lyophilizer.
16. Transfer dried pellet to a 4 mL screw top vial.
17. Add 1.5 mL 6 M HCl and hydrolyze overnight at 110 °C.
18. Purify combined supernatant over Dowex cation exchange resin (Appendix E-VI).

E-VI DOWEX Amino Acid Clean Up Procedure:

This section provides in detail the procedure to clean up hydrolyzed protein fractions or intracellular amino acids. This method utilizes a DOWEX cation exchange resin to clean up the free amino acids prior to GC-C-IRMS analysis.

Equipment Required:

- 3 mL syringe
- Glass wool
- Cation exchange resin: DOWEX 50W-X8, mesh size 200, Sigma-Aldrich, washed with ddH_2O, washed with 2 M NH_4OH, neutralized with ddH_2O and then stored in 1 M HCl.
- pH paper

Reagents Required:

- 2 M NH_4OH: 276 mL stock 14.5 M NH_4OH in 1.724 L ddH_2O. *Prepare and use in fume hood.*
- 1 M HCl: 83 mL stock 12 M HCl in 917 mL ddH_2O. *Prepare in fume hood.*
- 0.1 M HCl: 10 mL 1 M HCl in 90 mL ddH_2O.

Protocol:

Sample Preperation:

1. Dilute hydrolyzed samples to a 1:2 dilution (add 1 mL ddH_2O to mitochondrial hydrolysates, and 1.5 mL ddH_2O to myofibrillar and mixed plasma protein hydrolysates). Do not dilute intracellular amino acid samples.
2. Prepare two blank test column samples by creating a 3 M HCl solution to the same volume as the samples columning.

Column Preparation:

1. Pull out plunger of 3 mL syringe, one per sample plus two for test columns.
2. Wet glass wool and cut into pieces in an appropriate size to block syringe barrel at the exit point.

3. Use tweezers to put wool into barrel and use the plunger to push glass wool to the end of the barrel.
4. Arrange columns in a logical fashion according to sample labeling to prevent sample mix ups.
5. Pipette ~1 mL of DOWEX resin into the syringe on top of the glass wool, making sure the same amount of resin is in each syringe.
6. Add 2 mL ddH_2O to column until eluent is neutral and no longer acidic.
7. Add 1.5 mL 2 M NH_4OH to column to elute any contaminants. Repeat until eluent is basic.
8. Add 2 mL ddH_2O to column until eluent is neutral and no longer basic.
9. Add 4 mL 1 M HCl (2 mL increments) to column, ensuring that eluent is acidic. An acidic resin will retain amino acids.

Sample Columning:

1. Add test samples to test columns.
2. Add 2 mL ddH_2O to test columns until eluent is neutral.
3. Add 0.5 mL 2 M NH_4OH to test columns until the eluent turns basic. Record the amount of 2 M NH_4OH required (usually 1.5-2.5 mL).
4. Subtract 1 mL from the amount of 2 M NH_4OH required to turn test columns basic, and add this amount to sample columns. (*NOTE: Especially for mitochondrial hydrolysates with very low amino acid levels, it is best to just begin collecting eluent after first 2 M NH_4OH addition; i.e. skip this step*)
5. Place columns on top of labeled test tubes to collect eluent.
6. Add 4 mL 2 M NH_4OH (2 mL increments) to elute amino acids into the test tubes.
7. Dry samples down in rotor evaporator at 60 °C (~6-8 h).
8. Reconstitute intracellular samples in 300 µL, mitochondrial and mixed plasma samples in 500 µL, and myofibrillar samples in 700 µL 0.1 M HCl.