INFLUENCE OF PROTEIN NUTRITION AND EXERCISE ON

MUSCLE METABOLISM

By

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ABSTRACT

The influence of protein nutrition on endurance performance has received growing attention over the last several years. At present, there is no clear consensus as to whether protein feeding harnesses any ergogenic benefit for endurance athletes, due to methodological differences between the studies conducted to date. Whilst prior studies suggest that endurance capacity may be extended with additional protein, chapters in this thesis demonstrate no effect of protein on endurance performance. In addition to endurance capacity, protein ingestion during and following exercise has been advocated to enhance the recovery process. However, the impact of carbohydrate-protein co-ingestion on recovery from intense exercise is considered to be somewhat equivocal, due to the practical relevance of different tests of recovery and the lack of a clear physiological mechanism. Indeed, data presented herein indicates that protein co-ingestion does not enhance recovery at 24 hours post-exercise. Consequently, there is currently no basis on which to recommend protein feeding for endurance performance and recovery.

Nutrient strategies implemented in the post-exercise period can markedly alter the acute response of muscle protein synthesis. Thus, over an extended period of training appropriate post-exercise nutrient manipulations may modulate metabolic adaptations. Whereas the metabolic and molecular response to protein nutrition with resistance exercise has received increasing attention, protein feeding following endurance exercise has received considerably less. The combination of endurance exercise and protein ingestion increases the synthesis of mixed muscle protein via the phosphorylation of signalling intermediates implicated in mRNA translation initiation and elongation. Herein, we demonstrate that the response of mixed muscle proteins to post-endurance exercise protein feeding is due, in large part, to an increase in the synthesis of contractile proteins. Furthermore we provide evidence of altered signalling phosphorylation with protein feeding which may explain the protein synthetic response.

Insulin resistance that precedes Type II diabetes is characterized by blunted sensitivity of the pancreas to glucose and impaired glucose uptake in skeletal muscle. It is suggested that defective intracellular signalling, crucial for glucoregulation, may lead to hyperglycemia. However, lifestyle interventions including nutrient manipulations and exercise have the potential to improve glycemic control and can effectively restore glycemic control. Indeed, a

single bout of resistance exercise can improve insulin sensitivity and glycemic control for up to 48 hours post-exercise. In addition to resistance exercise, co-ingesting protein and/or amino acids with a carbohydrate load facilitates the release of insulin, thereby suppressing the prevailing plasma glucose response. The final experimental chapter in this thesis shows that the rate of glucose disposal from the circulation is elevated 24 hours after a single bout of resistance exercise in healthy normoglycemic males. It appears that prior exercise increased the basal and insulin-stimulated phosphorylation of proximal intracellular signalling intermediates regulating glucose uptake in skeletal muscle. Interestingly, protein ingestion did not augment the glucose-lowering effects of prior resistance exercise.

"Achievement is not always success while reputed failure often is. It is honest endeavour, persistent effort to do the best possible under any and all circumstances".

Orison Swett Marden, writer and physician (1850 - 1924)

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CHAPTER 1

GENERAL INTRODUCTION

1.1   Introduction

The quality and quantity of nutritional intake harnesses many benefits for athletic populations. Nutritional strategies implemented prior to and during exercise can profoundly alter exercise performance and prolong endurance capacity. Further, effective post-exercise nutritional strategies can replenish depleted energy stores, attenuate indices of muscle damage and augment the recovery of endurance capacity and muscle function. In addition to exercise performance and recovery, dietary modifications can profoundly alter the adaptive response to exercise. Although the major perturbations to cellular homeostasis and muscle substrate stores occur during exercise, the activation of intracellular signalling pathways important for chronic training adaptations are thought to occur during the first few hours of recovery, (114, 209). This has led to suggestions that many chronic training adaptations are generated by the cumulative effects of the transient events that occur during recovery from each (acute) exercise bout. Evidence is accumulating to suggest that nutrient intake can serve as a potent modulator of many of the acute responses to exercise training.

Protein, derived from the Greek word *proteos*, means primary or 'most important'. In the sporting environment, protein nutrition was initially favoured by bodybuilders, but it now a common consideration for the majority of athletes. However, the effectiveness of protein nutrition for endurance athletes is yet to be fully elucidated. In combination with methodological limitations that cloud our understanding of how protein feeding can impact

endurance performance and recovery, very little is known regarding the influence of protein nutrition on the metabolic and molecular adaptations to endurance exercise. Thus, improving our understanding of how nutritional strategies can optimize the adaptive response to exercise could provide the knowledge necessary to allow athletes to successfully modify their training and dietary habits and achieve their competitive goals. In light of evidence to suggest that protein nutrition could be important for endurance athletes, chapters in this thesis detail studies that have focussed on determining the effects of protein nutrition on endurance performance, indices of recovery and metabolic and cellular adaptations to endurance exercise.

Beyond athletic performance, lifestyle interventions such as exercise training and dietary manipulations have the potential to prevent or delay the incidence of metabolic disorders. Skeletal muscle mass accounts for ~50% of total body mass. Accordingly, skeletal muscle is a substantial contributor to basal metabolic rate (135). Furthermore, skeletal muscle represents the prime storage depot for body amino acids and is a critical metabolic tissue involved in glucose disposal and lipid oxidation, particularly during periods of exercise (2, 176, 274). As such, maintaining a large and metabolically active skeletal muscle mass through exercise, has some influence on the risk for metabolic disorders (e.g., Type II diabetes) as well as all-cause mortality in a variety of diseased states (274). Indeed it is known that a single bout of resistance exercise can improve glucose tolerance and insulin sensitivity for up to 48 hours post-exercise in humans (176, 205). At the level of skeletal muscle, the mechanisms facilitating this response are not fully understood.

In addition to contractile stimuli, nutritional modifications can be used to fulfil several distinct roles in the treatment of metabolic disorders, for example, by increasing weight-loss

and lowering glucose concentrations. Besides the application of energy restricted diets, it has been suggested that specific food components can be applied to directly modulate glycemic control. In this regard, cell culture and animal studies elegantly show that protein and/or branched chain amino acids can promote skeletal muscle glucose uptake (and removal from the circulation) via the activation of insulin-dependent and insulin-independent signalling pathways. In the absence of clear evidence in humans, that exercise and protein nutrition can improve metabolic health parameters, chapters in this thesis will also focus on examining the response of glucose metabolism to an acute bout of resistance exercise-alone or resistance exercise with protein feeding.

1.1.1   Metabolic basis of exercise training adaptations

Skeletal muscle is a malleable tissue capable of altering the type and amount of protein in response to disruptions to cellular homeostasis. The complex process of exercise-induced adaptation in skeletal muscle involves specific signalling mechanisms initiating replication of DNA genetic sequences (151) that enable subsequent translation of the genetic code into a series of amino acids to create new proteins (31). Skeletal muscles sense and distinguish contraction-specific signals to produce adaptations over time that are specific to the nature, intensity and duration of exercise (129, 167). However, it is thought that there is substantial overlap in the molecular response to different exercise modes (156). Contraction generates transient increases in the quantity of messenger RNA (mRNA) that for a multitude of genes typically peaks within 12 hours post-exercise and returns to basal levels within 24 hours (21, 209, 275). This directional change in mRNA is generally the same as the encoded protein during adaptation to a new steady-state level (30). Thus, contractile stimuli acutely increase transcriptional activity and the subsequent synthesis of new proteins and may be potentiated when appropriate nutrient strategies are implemented post-exercise. In summary, chronic

adaptations to training are thought to represent the summation of each discrete exercise bout, with these perturbations leading to cumulative alterations in gene and protein expression and ultimately distinct phenotypic changes (112, 167).

However, the ability to infer phenotypic changes in skeletal muscle from 'snapshots' of gene expression is complicated by the fact that multiple factors may be involved in the translation of mRNA species that can ultimately affect the expression of their protein products such as, for example, the post-transcriptional modification or stability (via action of chaperone proteins and RNAses) of mRNA transcripts and/or their translational regulation by other molecules such as microRNAs (271). In the absence of measured changes in protein abundance and/or the direct synthesis of their protein product it may be difficult to conclusively ascertain the functional outcome of changes in gene transcription.

1.1.2   Protein metabolism in skeletal muscle tissue

Skeletal muscle proteins are constantly and simultaneously synthesized and degraded. Net protein balance is defined as the difference between muscle protein synthesis and muscle protein breakdown. Thus, a significant rise in skeletal muscle protein synthesis (anabolism) and/or a reduction in muscle protein breakdown (catabolism), such that net protein balance remains positive can result in the accretion of skeletal muscle proteins. Conversely, a negative net protein balance, arising from a reduction in synthesis and/or increase in breakdown, will result in a loss of skeletal muscle proteins. Net protein balance is maintained by ingestion of protein-containing meals which results in systemic hyperaminoacidemia that is stimulatory for the synthesis of new proteins (18, 27, 28, 83). Therefore, in persons with consistent dietary habits who are not performing exercise, skeletal muscle protein mass remains relatively stable. However, the feeding-induced stimulation of muscle protein

synthesis is only transient and even in the face of available amino acids returns to basal levels (28). Exercise stimuli, aerobic and more so resistive, improve net protein balance. However, the ingestion of protein during post-exercise recovery is necessary to shift net protein balance in favour of muscle protein accumulation (23, 218). Thus, feeding and exercise must be utilized concurrently to manifest a positive net protein balance and increase protein mass when performed habitually.

In healthy individuals, changes in the rate of muscle protein synthesis are thought to represent the primary locus of control for regulating overall muscle protein balance (218). Evidence to support this contention is based on the following observations: i) the transition from the fasted to the fed state is associated with a 60-80% stimulation of muscle protein synthesis with no measurable change in breakdown (23, 260, 262); ii) the acute (i.e. 3 hours) feeding and exercise-induced increase in net protein balance is due primarily to changes in muscle protein synthesis and is additive to the daily net protein balance (242); iii) the acute change in muscle protein synthesis is qualitatively predictive of long term changes in muscle mass in response to both hypertrophic (i.e., resistance training) (82, 110, 116, 157, 268) and atrophic (i.e., immobilization) stimuli (94).

1.1.3   Intracellular signalling mechanisms regulating protein metabolism

Acute changes in muscle protein synthesis are regulated at the level of mRNA translation of which the primary rate controlling step is the initiation and formation of a translation-competent ribosome (147). The first stage of mRNA translation involves charging of initiator methionyl-tRNA, which is catalyzed by the guanine nucleotide exchange activity of the ε-subunit of eukaryotic initiation factor (eIF) 2B (eIF2Bε) and its subsequent

association with eIF2. This ternary structure then binds to the 40S ribosomal subunit to form the 43S pre-initiation complex. Following this, hyperphosphorylation of the negative regulator 4E-binding protein 1 (4E-BP1) results in its release of eIF4E, which is then permitted to bind to the mRNA and form the heterotrimeric eIF4F-mRNA complex. The eIF4F-mRNA complex then combines with the 43S pre-initiation complex to form the 48S pre-initiation complex, which subsequently binds to the 60S ribosomal subunit to form a translationally competent ribosome. Although initiation is the primary regulated step in mRNA translation, peptide chain elongation consumes the vast majority of energy during protein synthesis and also represents another level of control for the synthesis of proteins *in vivo* (263). The translocation of the ribosome along mRNA is catalyzed by eukaryotic elongation factor 2 (eEF2), which is negatively regulated by phosphorylation that is catalyzed by eEF2 kinase. Of particular importance to the regulation of protein synthesis appears to be the mTOR-mediated activation of p70S6K, which phosphorylates both eIF4B, to enhance the helicase activity of eIF4F complex and eEF2 kinase. Furthermore, hyperphosphorylation of p70S6K and downstream target ribosomal protein (rp) S6 facilitates translation initiation by increasing the affinity of ribosomes for binding 5'terminal oligopyridimine (5'TOP) mRNAs (131). Thus, p70S6K plays a role in both the initiation and elongation stages of mRNA translation (213). The signalling mechanisms regulating skeletal muscle protein synthesis are presented in Figure 1.1.

1.2   Influence of nutrition on muscle protein synthesis

Skeletal muscle is a heterogeneous tissue, composed of a variety of different proteins with distinct functions. From the standpoint of overall muscle mass and quality, the most important protein fraction within skeletal muscle is myofibrillar, which is composed

primarily of the contractile proteins actin and myosin. However, skeletal muscle contains many non-myofibrillar proteins and organelles (e.g., mitochondria, sarco- and endoplasmic reticula, ribosomal proteins, etc.) that are found within the cytoplasm of the muscle cell and therefore are collectively referred to as sarcoplasmic proteins. Mitochondrial proteins are involved in energy production and sarcoplasmic proteins contribute to anaerobic energy production, intracellular transport and numerous other functions. In addition, the external support lattice of muscle cells is comprised primarily of both fibrillar and non-fibrillar collagen protein. The majority of studies investigating the effects of nutrients on muscle protein synthesis measure changes in a mixed muscle protein fraction. This practice has provided the framework for our basic knowledge of how muscle responds to feeding but has the obvious disadvantage of representing an average synthetic rate of all muscle proteins. Measurement of the mixed muscle protein synthetic rate could mask potentially important physiological differences within specific muscle protein fractions. Therefore, the following sections of this chapter will discuss the changes in the synthesis of mixed muscle and specific muscle fractions in response to feeding.

1.2.1   Fate of dietary amino acids

A substantial portion (up to ~80% for some) of dietary amino acids absorbed by the small intestines, are subsequently catabolized or utilized for the synthesis of constitutive hepatic and secreted proteins within gut tissues, with the balance being transported to the liver to encounter a similar metabolic fate (174, 216). In addition to the synthesis of constitutive hepatic and exported proteins (especially albumin (61)), dietary amino acids can also be converted into glucose by the liver to provide a source of energy for other body tissues such as, for example, the brain. Because of its high turnover rate and large metabolic capacity, this first-pass splanchnic extraction, which includes both gut and liver metabolism,

represents a means for contending with large fluctuations in peripheral blood amino acid concentrations following ingestion of large protein containing meals (174). However, in order to support the synthesis of other body proteins there must be a net efflux of amino acids from the liver into the peripheral circulation. These amino acids bypass first-pass extraction and provide the substrates for the synthesis of a wide variety of body proteins, arguably the most important of which, based on their role in locomotion and amino acid storage as well as their collective contribution to whole body substrate metabolism, are skeletal muscle proteins. Dietary amino acids transported from the circulation into the skeletal muscle enter the free pool of amino acids whereby they can stimulate and support the synthesis of new muscle proteins. In addition to their roles as substrates for muscle protein synthesis, amino acids can also be deaminated and converted into a source of metabolic fuel for muscle and other tissues of the body. This process occurs either directly via the production of tricarboxylic acid (TCA) cycle intermediates, or indirectly via the production of precursors for hepatic gluconeogenesis. Under conditions in which the delivery of dietary amino acids to the muscle and other body tissue exceeds their ability to be incorporated into new proteins there is a marked increase in amino acid catabolism (179). Therefore, it is apparent that, in addition to the well defined role of stimulating and supporting muscle protein synthesis, the disposal of dietary amino acids can also occur through other metabolic pathways within different tissues of the body.

1.2.2   Muscle protein synthesis after feeding

It has been well established that the transition from a fasted to fed state is characterized by the inward transport of amino acids into skeletal muscle and a marked increase in the synthesis of muscle proteins (22, 217). The feeding-induced stimulation of protein synthesis, which is consistent across different muscles and fibre types (41, 180), functions to restore the

obligatory losses of muscle protein that occur during the fasting so that on a day-to-day basis muscle mass is maintained. The primary stimulatory effect of feeding arises almost exclusively from the presence of the essential amino acids (236, 244, 261), in particular the branched chain amino acid leucine (235). There is evidence to indicate that insulin can enhance the synthesis of muscle proteins when administered alone (89) or in the presence of a sub-optimal level of amino acids (i.e., a level below which protein synthesis is maximally stimulated) (99). The mechanism by which insulin facilitates the protein synthetic response in this situation is not direct but likely occurs via the enhanced delivery of nutrients to the muscle, secondary to an insulin-induced vasodilation and capillary recruitment (51, 85). With the provision of an optimal level of exogenous amino acids it appears that only basal insulin concentrations are required to allow the full stimulation of muscle protein synthesis (95, 99). These data suggest the role of insulin in healthy individuals is permissive rather than stimulatory for the anabolic effect of amino acids. However, in the absence of amino acids the role of post-prandial insulin concentrations for muscle anabolism may be more important (89). Thus, the meal-induced stimulation of muscle protein synthesis is primarily regulated by the presence of dietary amino acids.

Muscle protein synthesis is rapidly (i.e. within 30-90 min) increased in response to a physiological increase in plasma amino acids (10, 28, 83). Interestingly, the stimulation of muscle protein synthesis during a constant infusion of amino acids subsides after ~3 hours despite the presence of a persistent hyperaminoacidemia (28). The presence of a refractory period for protein synthesis (28) combined with the observation that the synthesis of mixed muscle proteins is regulated by the extracellular rather than intracellular concentration of amino acids (27), suggests that muscle protein synthesis may be stimulated primarily by the acute change in plasma amino acid concentration in response to amino acid ingestion.

Subsequent studies have established that mixed muscle protein synthesis is rapidly stimulated after the ingestion of dietary amino acids (83). Furthermore, recent studies have begun to describe the temporal changes in the synthesis of specific muscle protein fractions after the ingestion of physiological protein ingestion (10, 181) and intravenous infusion of mixed amino acids (28).

1.2.3   Synthetic response of different protein fractions after feeding

It is thought that myofibrillar proteins are the prime storage depot for amino acids in the body. This notion is supported by evidence that during periods of reduced amino acid availability, myofibrillar proteins are preferentially targeted for degradation (272). Thus, in order to maintain muscle mass, protein feeding must stimulate the synthesis of myofibrillar proteins to counteract fasted-state losses. In spite of the critical role myofibrillar proteins play in amino acid storage, few studies have examined the response of myofibrillar proteins to alterations in nutrient status. Work by Bohé and colleagues (27, 28) showed that myofibrillar proteins display similar sensitivity to a mixed muscle protein fraction during the provision of amino acids, with regard to the magnitude and duration of synthesis. This is not surprising considering that myofibrillar protein accounts for ~60-65% of all skeletal muscle protein and would contribute substantially to rates of mixed muscle protein synthesis. However, it should be noted that early studies examining the response of myofibrillar protein synthesis to protein feeding infused amino acids intravenously, an approach which may not adequately describe a physiological situation in which amino acids are ingested orally. More recent studies demonstrate a similar amplitude and duration of myofibrillar and sarcoplasmic protein synthesis in response to physiological whey protein ingestion (10, 181). These data are in contrast to previous studies reporting that sarcoplasmic proteins are less sensitive to nutrient availability than myofibrillar proteins (59, 180). Accepting that muscle protein synthesis is

regulated by acute changes in extracellular amino acid concentration (27), it is possible that a bolus ingestion of 25g (181) and 48g (10) of protein supplied in these studies, enhanced the sensitivity of sarcoplasmic proteins to nutrients, as a result of a greater physiological increase in extracellular amino acid concentration compared with oral ingestion of 10g of crystalline amino acids (59), or a sustained infusion of amino acids (180).

Sarcoplasmic proteins, in which mitochondrial proteins are included, are thought to be less sensitive to nutrient availability than myofibrillar proteins (59, 180). Differences in the nutrient sensitivity of the two protein fractions has been hypothesized to represent a mechanism muscle composition to ensure that and the relative amount of myofibrillar and sarcoplasmic proteins are maintained, despite differences in the rates of turnover (14, 59, 180). Due to technical limitations, changes in mitochondrial protein synthesis rates in response to the ingestion of a physiological dose of protein have not been well characterized. However, Bohé et al. (27, 28) showed the synthetic response of myofibrillar, sarcoplasmic and mitochondrial proteins were similar in magnitude and duration following an amino acid infusion. In union with the findings of Moore et al. (181), these data imply that an accelerated increase in plasma amino acid concentration, typically found after the ingestion of rapidly digested protein such as whey, might maximize the synthesis of sarcoplasmic and mitochondrial proteins to match that of the myofibrillar fraction. Thus, to advance our understanding of how non-myofibrillar proteins respond to nutrients it would be valuable to characterize changes in the synthesis of mitochondrial muscle proteins to physiological nutrient ingestion. Further to this suggestion, Moore et al. (181) recently showed the combined effect of resistance exercise and whey protein feeding potentiated the synthetic response of myofibrillar proteins. Thus, the synergistic effect of nutrient and contractile

stimuli may be important for the synthesis of specific protein fractions and remains to be

fully elucidated.
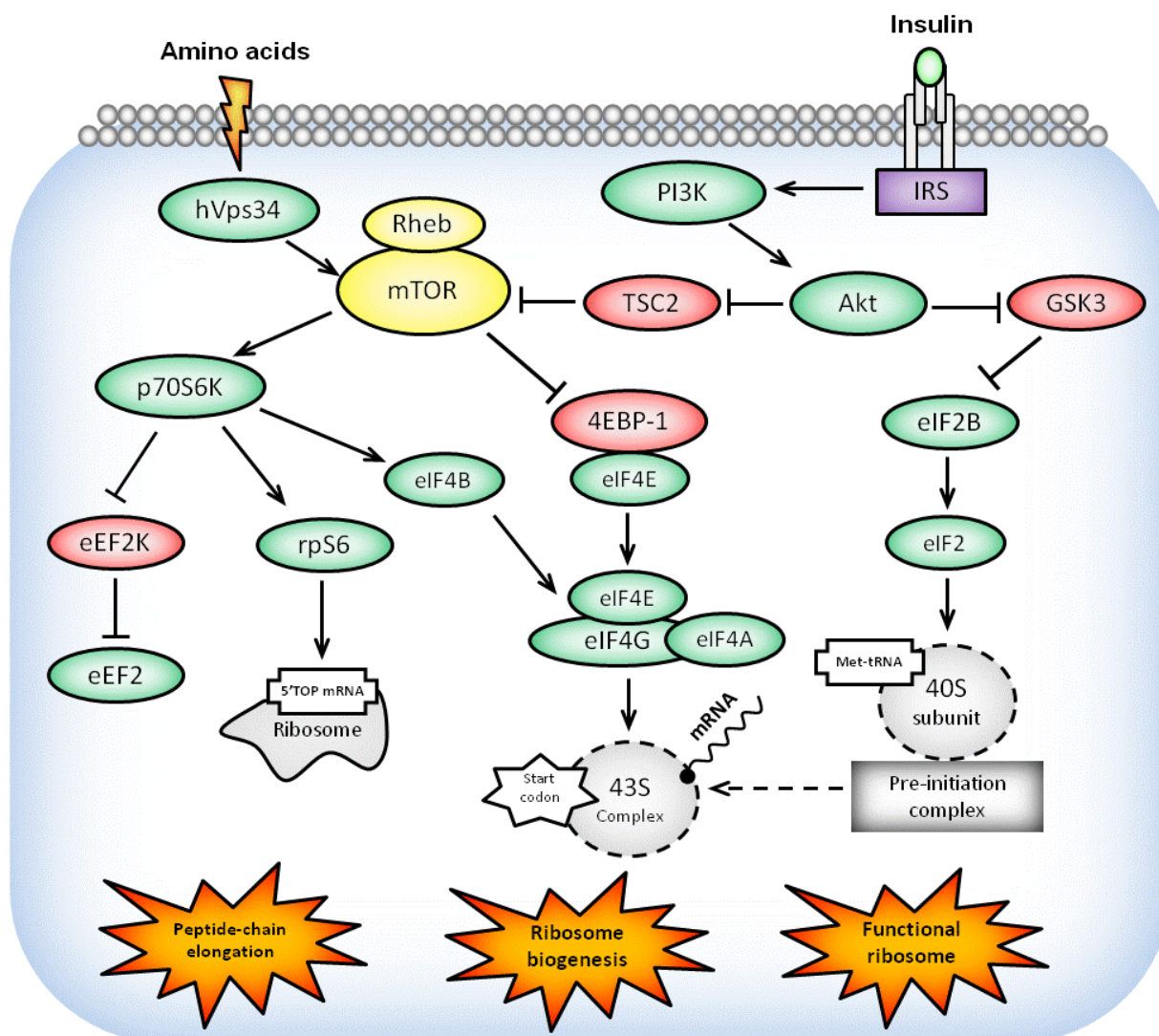
Figure 1.1 A simplified schematic representation of the intracellular signalling mechanisms controlling skeletal muscle protein synthesis by amino acids and insulin. Proteins have been labelled to designate them as positive (green) or negative (red) regulators of mTOR and muscle protein synthesis.

1.2.4   Intracellular signalling response to feeding

Considerable advances have been made toward improving our understanding of the

intracellular signalling events that contribute to the activation and regulation of mRNA

translation in skeletal muscle. Of critical importance to the activation of muscle protein

synthesis with nutrient ingestion appears to be proteins within the mTOR signalling cascade. For example, the feeding-induced increase in human skeletal muscle protein synthesis is accompanied by an increase in the phosphorylation of mTOR and its downstream effectors involved in mRNA translation initiation (p70S6K, 4E-BP1) and elongation (eEF2) (10, 60, 83, 93, 103, 234). The association between these signals and muscle protein synthesis was recently brought to light by Atherton and colleagues (10), who demonstrated that the 'upswing' in muscle protein synthesis following amino acid ingestion is echoed in the phosphorylation of several intermediates in the mTOR signalling pathway.

Although studies investigating the effect of amino acid feeding on intracellular signalling in humans typically provide additional carbohydrates that would enhance insulin-stimulated phosphorylation of mTOR by its upstream kinase Akt (83, 99), amino acids have been shown to activate mTOR *in vivo* directly with only a basal requirement for insulin (105, 119, 263). It has recently been shown that during the constant provision of amino acids, signalling molecule phosphorylation upstream and downstream of mTOR is enhanced by the presence of insulin (73, 119). However, despite the incremental activation (evidenced by greater phosphorylation) of signalling proteins in the mTOR cascade with graded insulin concentration, there was no concomitant elevation of muscle protein synthesis (119). These data suggest that a minimum level of phosphorylation is required to activate the signalling proteins involved in the regulation of mRNA translation with nutrients, after which, additional activation of the mTOR pathway, such as with hyperinsulinemia, fails to stimulate protein synthesis further. While most feeding studies in humans have investigated the role of the insulin phosphatidylinositol 3-kinase (PI3K) pathway in the activation of mTOR (73, 83, 93, 119), there is mounting evidence from cell culture studies indicating that amino acids can activate mTOR independent of insulin via a class of regulatory GTPase proteins (280) as well

as the type III PI3K vacuole protein sorting-34 (hVps34) (146); these pathways may represent a mechanism by which mTOR signalling molecule phosphorylation is enhanced by dietary amino acids in humans in the absence of hyperinsulinemia (59, 100, 163). Alternatively, the dephosphorylation of a novel and as yet unexplored in humans, site on the guanine nucleotide exchange factor eIF2B by amino acids has been shown to occur independently of mTOR activation *in vitro* (264). Finally, the role of 4E-BP1 in negatively regulating protein synthesis may be called into question based on the recent observation that over-expression of this protein in cultured cardiomyocytes has no effect on the rapamycin sensitive stimulation of global protein synthesis (123). Collectively, these data demonstrate the complexity regulation of mRNA translation and highlight the potential for multiple pathways for the activation of muscle protein synthesis with feeding in humans.

1.3   Metabolic and cellular adaptations to endurance exercise and nutrition

For competitive endurance athletes, the desired outcome of an endurance training regimen is to increase the ability to sustain a desired power output or speed of movement over a given time. Therefore, training for enhancement of endurance performance should aim to induce a myriad of physiological and metabolic adaptations that enable the athlete to: i) increase the rate of energy production from both aerobic and oxygen-independent pathways; ii) maintain tighter metabolic control (i.e. match ATP production with ATP hydrolysis); iii) minimize cellular disturbances; iv) increase economy of motion; and v) improve the resistance of the working muscles to fatigue during exercise. Key components of a training regimen are the duration, intensity, total volume and the frequency with which training sessions are conducted. The sum of these inputs can be termed the 'training stimulus' (12). When exercise training persists over an extended period of time, 'chronic' adaptations occur. Although chronic adaptations in skeletal muscle are thought to result from the

cumulative effect of repeated bouts of exercise, the initial signalling responses that lead to these chronic adaptations are likely to occur after each training session (266). It has been established that the process of converting a mechanical signal generated during contraction to a molecular event that promotes skeletal muscle adaptation, involves the up-regulation of primary and secondary messengers that initiate a cascade of events that result in activation and/or repression of intracellular signalling pathways regulating exercise-induced gene expression and protein synthesis and breakdown (270).

The extent to which acutely altering substrate availability might modify the training impulse has been a key research area among exercise physiologists and sport nutritionists for a number of years. Altering substrate availability affects not only resting energy metabolism and subsequent fuel utilization during exercise, but also the regulatory processes underlying gene expression (7, 109). To bring about such modifications, a number of highly coordinated processes occur, including gene transcription, RNA transport from the nucleus, protein synthesis and, in some cases, post-translational modification of the protein.

1.3.1   Endurance exercise adaptations

Prolonged endurance training elicits a variety of metabolic and morphological adaptations. These include; fast-to-slow fibre-type transformation (278), glycogen sparing effects due to alterations in substrate metabolism (115), enhanced lactate kinetics and increased mitochondrial density (125). Moreover, repeated bouts of endurance exercise alter the expression of a host of gene products that promote adaptation toward a fatigue resistant phenotype (3). Mitochondria are the main sub-cellular structures that determine the oxidative capacity and fatigue resistance to prolonged contractile activity in skeletal muscle (118). Endurance training has been shown to increase mitochondrial protein content by 50–100%

within ≈6 weeks, but a protein turnover half-life of ≈1 week means a continuous training stimulus is required to maintain elevated mitochondrial content (278). While enhanced oxygen kinetics, substrate transport and buffering capacity all contribute to enhanced endurance capability in skeletal muscle, improved endurance is due primarily to increased mitochondrial density and enzyme activity termed 'mitochondrial biogenesis'.

1.3.2   Muscle protein metabolism after endurance exercise

Measurement of mixed muscle protein synthesis in skeletal muscle represents a weighted average of the rates of synthesis of all proteins in the muscle, i.e. it does not distinguish the response of different proteins constituting the mixed fraction. Intuitively, given the divergent adaptive response to different types of training, it is safe to assume that resistance and endurance exercise should differentially influence distinct protein fractions. At present, relatively few studies have investigated the impact of endurance exercise on muscle protein turnover. This may be related to the general observation that endurance exercise does not typically result in significant gains in muscle mass. However, changes in muscle protein synthesis following endurance exercise are relevant for tissue repair and remodelling as well as changes in synthesis of non-contractile proteins (i.e. mitochondrial proteins) (35). Currently, differences in exercise mode and intensity utilized in previous studies, cloud our understanding of the protein synthetic response to endurance exercise (40, 178, 243).

It has been well documented that aerobic exercise increases whole-body (39) and mixed muscle protein synthesis le (40, 106-108, 178, 243) (Table 1.1). Initial studies examining the acute response of muscle proteins to aerobic exercise established that low load treadmill exercise (at 40% $\dot{V}O_{2max}$) stimulated an increase in muscle protein synthesis in untrained

subjects (40, 231). Although non-significant, Tipton et al. (243) also demonstrated that high intensity swimming increased muscle protein synthesis by ~40% in trained female swimmers. Studies investigating the protein synthetic response to endurance exercise have thus far yielded variable results which may be related to the muscle studied, the mode/intensity of exercise, or participant training status. Certainly, the latter may have considerable influence as it has been shown chronic endurance training increases basal rates of skeletal muscle protein turnover (208, 231). With regard to the synthesis of specific muscle protein fractions, Miller et al. (178) reported that a unique, one-legged kicking model of endurance exercise increased the synthesis of sarcoplasmic and myofibrillar proteins for 48 and 72 hours post-exercise, respectively. Endurance exercise, however, is not commonly characterized by skeletal muscle hypertrophy as would be expected with such a robust increase in myofibrillar protein synthesis. Therefore, the "aerobic" nature of this exercise model may be more appropriately labelled a low-intensity resistive type exercise. However, a recent study by Wilkinson et al. (267) examined the synthetic response of myofibrillar and mitochondrial proteins following single-leg cycling for 45 min (75% $\dot{V}O_{2max}$) in both the untrained and trained state. Following the cycle, a robust increase in mitochondrial protein synthesis occurred regardless of training status (267). Thus, mitochondrial and to a lesser extent sarcoplasmic proteins are the primary proteins contributing to the increase in mixed muscle protein synthesis after endurance exercise, potentially to counteract the suppression of muscle protein synthesis that occurs during endurance exercise (40). Whether this marked synthetic response of mitochondrial proteins can be enhanced with post-exercise nutrient provision remains to be elucidated.

In addition to muscle protein synthesis, muscle protein breakdown is also elevated following acute endurance exercise (231), whilst resting rates of breakdown increase

following long-term endurance training (208). Direct measurement of muscle protein breakdown with endurance exercise is rare and the results somewhat equivocal (74, 84, 231). Using arteriovenous blood sampling, other investigators have demonstrated that whole-body protein breakdown is transiently elevated after moderate intensity walking exercise, compared with rest (231), but others report no change following exercise (243). These studies utilized moderate to low-intensity exercise, rather than high intensity exercise. However, studies of high-intensity endurance exercise have reported an increase in indirect markers of muscle protein breakdown including 3-methyl-histidine excretion (40) and net amino acid efflux from the leg (26, 253) during exercise.

1.3.3   Intracellular signalling response to endurance exercise

Expansion of skeletal muscle aerobic capacity is determined by changes in mitochondrial protein content, observed after as little as 6-7 training sessions in humans (90). Adaptation of mitochondrial protein content in skeletal muscle is highly complex and involves the coordinated expression of a number molecular targets (125), the vast majority of which are located in the nucleus (96). Thus, an important aspect of mitochondrial biogenesis is the transport of nuclear precursor proteins into the organelle (125). Critical to the expression of genes promoting mitochondrial biogenesis are the principles of gene regulation; transcription initiation and interaction at the gene promoter (125). Mitochondrial biogenesis with repeated endurance training stems from an increase in the activity of transcriptional complexes that contain PPAR γ co-activator (PGC1α). A seminal study by Pilegaard et al. (210) showed that acute endurance exercise increases the transcription and mRNA content of PGC1α, an effect that is further potentiated after repeated training bouts.

Recently, control of mitochondrial oxidative function has been linked to transcriptional control of PGC1α by mTOR (56), a critical protein implicated in the regulation of muscle

protein synthesis through translation initiation. In support of this hypothesis, others report that endurance exercise mediates the phosphorylation of proteins implicated in mRNA translation initiation (38, 175, 267) (i.e. mTOR) elongation (175) (i.e. eEF2). Quantitatively, it appears that there is little difference in the extent of the acute signalling responses of leg muscles working in different modes of exercise (38, 267). Thus, the notion that endurance and resistance training adaptations occur through divergent molecular signalling pathways *in vitro* (9, 49) may not accurately reflect the adaptive process in humans. These data suggest that any major increase in contractile activity or possibly fuel utilization in untrained muscle will result in the same global anabolic response.

Skeletal muscle protein breakdown is primarily mediated through the ATP dependent ubiquitin-proteosome pathway (UPP), which degrades myofibrillar and sarcoplasmic proteins in concert with the calpain system (13, 92). Atrogin-1 and muscle specific RING finger protein-1 (MuRF-1) are the primary atrogenes of the UPP and are induced through FOXO signalling (92). These factors are acutely responsive to endurance exercise, presumably as part of the muscle remodelling process (106, 164). Although the role of these proteolytic factors in regulating exercise-induced protein breakdown is not well defined, recent evidence indicates the mRNA expression of MuRF-1, calpain-2, atrogin-1 and MRF4 are increased following high-intensity resistance (164, 275) and endurance exercise (108, 164, 275).

In summary, endurance exercise elevates mitochondrial protein synthesis via enhanced translational activity. Subsequent to this process, the activity of transcription factors and transcriptional co-activators is critical in the regulation of mitochondrial biogenesis. However, our current understanding of how translational and transcriptional processes act

synergistically to control mitochondrial biogenesis is limited and further studies are warranted.

1.3.4   Muscle protein synthesis after post-endurance exercise feeding

Currently, few studies have investigated the response of muscle protein synthesis to post-endurance exercise feeding. Levenhagen and colleagues (162) were the first to show that the ingestion of carbohydrate plus protein immediately after a 1 hour cycle (60% $\dot{V}O_{2max}$), increased leg and whole-body protein synthesis; changes associated with a net protein gain. However, in the study of Levenhagen et al. (162) carbohydrate-only and carbohydrate plus protein beverages were not matched for energy intake, thus it was unclear whether the differences observed were due to the protein *per se* or differences in total energy intake. To remedy this, Howarth et al. (120) compared carbohydrate plus protein to carbohydrate-only beverages matched for total energy and carbohydrate content. In contrast to the single bolus feeding of Levenhagen et al. (162) beverages in this study were ingested at regular intervals during 3 hours of post-exercise (50-80% $\dot{V}O_{2max}$) recovery. The authors demonstrated that carbohydrate plus protein increased mixed muscle protein synthesis compared with both carbohydrate treatments, without any significant rise in insulin concentration. Despite strong evidence that mixed muscle protein synthesis is stimulated by protein feeding rather than any change in insulin following endurance exercise, doubt has been raised by a recent study showing no additive effect of feeding on mixed muscle protein synthesis following a 1 hour cycle (~72% $\dot{V}O_{2max}$) (108). However, the authors (108) measured muscle protein synthesis at 2-6 hours post-exercise, 1 hour after feeding had occurred. Thus, the peak anabolic response to exercise and feeding may have occurred earlier than was measured concealing any potential difference (157, 268). Importantly, none of these studies (108, 120, 162) have attempted to resolve the specific protein fractions contributing to changes in mixed muscle

protein synthesis. Furthermore, it is possible that the measure of mixed muscle protein synthesis may not be sensitive enough to reflect changes in the synthesis of specific proteins (108). Recently, Moore et al. (181) demonstrated that the synergism of protein ingestion and resistance exercise augmented myofibrillar protein synthesis. Thus, it seems that protein nutrition is more than merely a substrate, but instead is an input into a system that affects phenotype due to the influence it exerts in regulating muscle protein synthesis. These data have led Howarth et al. (120) to suggest that the increase in mixed muscle protein synthesis with protein ingestion following endurance exercise could be due, in large part, to an increase in the synthesis of mitochondrial proteins. To date, this thesis has yet to be confirmed. A summary of studies investigating the effect of a single bout of endurance exercise on protein metabolism are presented in Table 1.1.

1.3.5   Intracellular signalling response to post-endurance exercise feeding

Despite the permissive role of insulin on muscle protein synthesis, it has been suggested that elevated plasma insulin levels are required to fully activate the translation of mRNA, initiated following endurance exercise (158). The suggestion is that insulin activates mTOR, via an upstream pathway involving PI3K and Akt (72, 147). Interestingly, relatively new findings from cell culture and drosophila experiments demonstrate that essential amino acids can directly stimulate mTOR via hVps34 (36) as well as through the Ste20 protein kinase, MAP4K3 (78). A family of small GTPases known as Rag proteins may also be important in promoting the intracellular localization of mTOR towards Rheb during amino acid stimulation, though this has only been observed in human embryonic kidney cell cultures to date (221). In summary, these data suggest that insulin and amino acids act independently to regulate mTOR. However, following carbohydrate plus protein feeding, amino acid-

induced mTOR activation appears to be crucial for increasing protein synthesis as opposed to the rise in insulin concentration (5, 6).

Relatively few studies have investigated the response of intracellular signalling to carbohydrate plus protein feeding following endurance exercise. Ivy et al. (126) compared the intracellular signalling response to carbohydrate plus protein feeding ingested over 45 minutes after a 1 hour variable intensity cycle. The authors demonstrated that carbohydrate plus protein activated mTOR and rpS6 compared with a non-energetic placebo. However, the study design of Ivy et al. (126) did not investigate the response of protein or carbohydrate feeding alone. Thus the authors could not say with any certainty whether the effects of the drink on intracellular signalling were due to the greater amino acid availability, elevated plasma insulin, or a combination of the two. The same research group, (186) had earlier conducted a more rigorous experiment in which they showed that ingesting carbohydrate or protein after exhaustive exercise transiently increased the phosphorylation of mTOR, 4E-BP1, rpS6 and p70S6K in rats. However, the phosphorylation of rpS6 and 4E-BP1 was sustained when carbohydrate and protein were co-ingested. Taken together, these data demonstrate that feeding either carbohydrate or protein alone following intense endurance exercise increases the phosphorylation of proteins implicated in translation initiation, but not to the extent of carbohydrate and protein provided together. On the other hand, recent evidence indicates that increasing levels of circulating insulin in the post-endurance exercise period through feeding interventions, may suppress the phosphorylation of proxy markers of muscle proteolysis (108). However, in the absence of muscle protein turnover data to support the intracellular signalling response, it difficult to make definitive conclusions regarding the precise role of post-endurance exercise nutrient ingestion on muscle protein breakdown.

Table 1.1 Comparison of studies examining muscle protein metabolism and intracellular signalling following endurance exercise

Study	Endurance protocol	Exercise duration	Subject training status	Protein synthesis	Protein breakdown	Intracellular signalling
Carraro *et al.* 1990 (40)	40% VO_{2max}	4 hours	Healthy young males	• 96% ↑ in mixed MPS **	• 85% ↑ in 3MH post-ex *	N/A
Tipton *et al.* 1996 (243)	~85-90% HR_{max}	~1.5 hours	Trained female swimmers	• 41% ↑ in mixed MPS	• ↔ in WBPB at 3 h post-ex	N/A
Sheffield-Moore *et al.* 2004 (231)	40% VO_{2max}	45 min	Untrained elderly and young males	• ~ 65-75% ↑ in mixed MPS *	• ↑ in WBPB at 10 min post-ex *	N/A
Miller *et al.* 2005 (178)	~67% W_{max}	1 hour	Healthy young males	• 52% ↑ in SARC MPS * • 70% ↑ in MYO MPS *	N/A	N/A
Mascher *et al.* 2007 (175)	75% VO_{2max}	1 hour	Healthy young males	N/A	N/A	•↑ Akt, mTOR, p70S6K, AMPK, •↓ eEF2 *
Ivy *et al.* 2008 (126)	40-90% VO_{2max}	1 hour	Healthy young males	N/A	N/A	•↑ rpS6, p70S6K *
Wilkinson *et al.* 2008 (267)	75% VO_{2max}	45 min	Healthy young males	• ↑ Mitochondrial MPS *	N/A	•↑ mTOR, p70S6K, FAK, eIF4E, AMPK *
Harber *et al.* 2010 (108)	~72% VO_{2max}	1 hour	Healthy young males	• 57% ↑ in mixed MPS *	N/A	• ↑ MuRF-1, MRF4, calpain-2 *
Harber et al. 2010 (107)	75% VO_{2max}	45 min	Trained males	• ~37% in mixed MPS *	N/A	N/A

$\dot{V}O_{2max}$; maximal oxygen uptake, HR_{max}; maximum heart rate, W_{max}; maximal work load, MPS; muscle protein synthesis, SARC; sarcoplasmic, MYO; myofibrillar, WBPB; whole-body protein breakdown, 3MH; 3-methyl histidine, MuRF-1; muscle specific RING finger-1, MRF4; muscle regulatory factor 4, post-ex: post-exercise, ↑; increase, ↓; decrease, ↔; no change, *; $P < 0.05$, **; $P < 0.01$.

1.4   Influence of protein nutrition on endurance performance and recovery

Nutritional strategies that improve performance and assist recovery are unique to each sporting event and individual. Although most athletes can satisfy their nutritional requirements before and/or after exercise, long-duration activities require that participants also address their nutritional needs during exercise. Endurance exercise promotes vast increases in energy utilization, with significant increases in carbohydrate and fat oxidation rates (32, 53). Sizeable losses of fluid and electrolytes from sweat may also occur, particularly during prolonged exercise in the heat (192). As a result, inadequate fluid and nutrient intake during endurance exercise can lead to dehydration, hyponatremia, glycogen depletion, hypoglycemia and impaired performance. In addition, nutritional deficiencies during prolonged activity may limit the capacity for rapid recovery after exercise, which may affect subsequent performance. Numerous studies have investigated nutritional approaches to minimize these issues, resulting in the emergence of strategies that elicit positive effects for endurance athletes. For endurance athletes, carbohydrate feeding is the most commonly used nutritional strategy. It is generally agreed that carbohydrate beverages are effective in promoting fluid balance and euglycemia and augmenting performance during prolonged endurance activities (50, 52, 133, 248). Typical guidelines suggest ingesting sports beverages with 4 - 8% carbohydrate at regular intervals during exercise to provide approximately 600 - 1400 mL of fluid and 30 - 60 g of carbohydrate per hour (4, 50, 52). The traditional role of carbohydrate sports beverages has been to optimize performance by delaying dehydration and hypoglycemia and potentially influencing glycogen depletion and central fatigue. However, nutrient intake during prolonged exercise may also have important implications for recovery from exercise.

Recent interest has centred on the use of protein nutrition in sports beverages for endurance athletes. Specifically, it has been suggested that the co-ingestion of carbohydrate with protein during exercise, improves endurance performance. Moreover, recent studies indicate that carbohydrate-protein co-ingestion during and following exercise may reduce indices of muscle damage, thus enhancing the recovery of subsequent performance and muscle function.

1.4.1   Carbohydrate-protein co-ingestion for endurance exercise performance

Several studies have shown that carbohydrate plus protein ingestion can extend endurance time-to-exhaustion in the range of 13-36% (127, 222, 223). More recently, Saunders et al. (224) suggested the addition of protein to carbohydrate specifically improved late-exercise time-trial performance. However, a number of methodological differences between studies make it difficult to discern whether any benefits observed for carbohydrate plus protein are the result of a protein-mediated effect.

First, there are discrepancies in the way exercise performance has been assessed in previous studies. The ecological validity of exhaustive exercise protocols (used in (127, 222, 223)) is limited, as endurance athletes do not compete in events that require sustaining a fixed power output for as long as possible. Further, exhaustive exercise tests display a large variation between repeated bouts of ~26% (132), which may be exacerbated in lesser trained participants (57). Moreover, claims that the addition of protein improves late-exercise performance were based on a time-trial protocol that was not specifically designed to record power output and measure late-stage exercise performance (224). On the contrary, time-trial

cycle tests have been found to be highly reproducible and sensitive to small changes in exercise performance (132, 160, 201). Secondly, additional energy consumed when protein is added to a carbohydrate containing beverage may explain the performance benefits observed by others. Indeed, studies in which endurance capacity was extended with additional protein, fed a carbohydrate dose that may have been too low (<50 g·h^{-1}) to attain peak exogenous oxidation rates (134). Thus, if carbohydrate is consumed at a rate considered optimal for exogenous carbohydrate oxidation, the role of protein for improving endurance performance may be negligible (252). Finally, the control of external variables has varied greatly between previous studies. Knowledge of parameters including; i) time elapsed, ii) distance travelled and iii) heart-rate during exercise may compromise the blinding of treatments, creating a placebo effect. Early studies did not report the control of these conditions (127, 219, 222-224, 251). In contrast, investigations that implement a strictly controlled exercise environment reported no effect of protein co-ingestion on time-trial performance (199, 252).

In addition to methodological considerations, there is currently no plausible mechanism to explain the reported performance benefits when protein is added to a carbohydrate beverage. It has been suggested that the addition of protein to carbohydrate may enhance endurance performance through one or a combination of several putative mechanisms. These include; i) altering substrate utilization in a glycogen depleted state (i.e. during late-exercise) (224); ii) offsetting the decline in TCA cycle intermediates during prolonged exercise iii) delaying the onset of central fatigue and iv) facilitating fuel transport via an increase in fluid absorption. Currently, the efficacy of adding protein to carbohydrate for endurance performance remains unclear.

1.4.2 Carbohydrate-protein co-ingestion for indirect markers of muscle damage and recovery

The traditional role of carbohydrate feeding in close proximity to exercise has been to optimize performance by delaying dehydration and hypoglycemia as well as potentially influencing glycogen depletion and central fatigue. However, nutrient intake during prolonged exercise is thought to be effective in ameliorating indices of muscle damage, thought to impair the rate of recovery. Thus, a number of studies have reported that carbohydrate plus protein co-ingestion during and following endurance exercise improves several indices of recovery. Specifically, these studies demonstrate that the addition of protein to carbohydrate prolongs subsequent time-to-exhaustion in the range of 40-55% (222, 233, 269). Others have found additional protein results in small increases in the recovery of muscle function, on the order of 1-2 knee extension lifts (251) and 1-2cm in vertical jump height (251). In contrast to these data, several studies failed to find improvements in recovery of subsequent performance, assessed using various methods (19, 98, 165, 177, 219). Thus, the impact of adding protein to carbohydrate on recovery from intense exercise is considered to be somewhat equivocal.

Reductions in the concentration of proxy markers of muscle damage such as plasma creatine kinase (91, 165, 177, 219, 222, 223, 233, 251) and lactate dehydrogenase (165, 219) are thought to play a key role in aiding the recovery process when protein is added to carbohydrate. Moreover, several studies suggest that the addition of protein to a carbohydrate beverage may improve recovery through attenuating subjective ratings of muscle soreness (165, 177, 219, 233). However, evidence to support an effect of additional protein for improving recovery is inconsistent with several investigations finding no difference in the post-exercise rise in plasma creatine kinase (20, 98), muscle soreness (20, 177) and the

recovery of endurance capacity (19) when additional protein is ingested. Thus, although many endurance athletes now considered it a 'necessity' to consume protein following exercise, the efficacy of protein supplementation as a tool to improve recovery is unclear, due in large part, to the applicability of different recovery assessments to the athletic environment and a limited understanding of the mechanisms facilitating these purported improvements. A comparison of studies investigating the impact of carbohydrate-protein co-ingestion on endurance performance and indices of recovery is highlighted in Table 1.2.

Table 1.2 Comparison of studies examining endurance performance and recovery following carbohydrate-protein co-ingestion

Study	Endurance protocol	Beverages	Timing	CHO $(g \cdot h^{-1})$	PRO $(g \cdot h^{-1})$	Fluid $(mL \cdot h^{-1})$	Performance outcome	Recovery outcome
Ivy *et al.* 2003 (127)	• TTE at 85% $\dot{V}O_{2max}$.	CHO C+P	During Ex	45 45	- 4	600	• 36% ↑ in TTE for C+P *	N/A
Saunders *et al.* 2004 (222)	• TTE at 75% $\dot{V}O_{2max}$ • TTE at 85% $\dot{V}O_{2max}$ 12-15 h later	CHO C+P	During and immediately post-Ex	37* 37*	- 9*	500*	• 29% ↑ in 1st TTE for C+P * • 40% ↑ in 2nd TTE for C+P *	• 83% ↓ in plasma CK for C+P *
Romano-Ely *et al.* 2006 (219)	• TTE at 70% $\dot{V}O_{2max}$ • TTE at 80% $\dot{V}O_{2max}$ 24 h later	CHO C+P	During and immediately post-Ex	56 * 45 *	- 11*	560*	None	• 65% ↓ in CK for C+P * • 66% ↑ in MS for CHO *
Luden *et al.* 2007 (165)	• 5km race after 6 days of supplementation •	CHO C+P	Immediately post-training	102* 102*	- 26*	1400*	None	• ↓ MS and CK for C+P *
van-Essen & Gibala.2006 (252)	• 20km cycle time-trial	CHO C+P	During Ex	60 60	- 20	1000	None	N/A
Osterberg *et al.* 2008 (199)	• Pre-loaded cycle time-trial	CHO C+P	During Ex	60 60	- 20	1000	None	N/A
Valentine *et al.* 2008 (251)	• TTE at 75% $\dot{V}O_{2max}$	CHO CHO C+P PLA	During Ex	78 100 78 -	- - 12 -	1000	• C+P and CHO ↑ TTE * • CHO TTE similar to PLA	• ~20% ↑in LE for C+P * • ~50% ↓ in CK for C+P *
Saunders *et al.* 2007 (223)	• TTE at 75% $\dot{V}O_{2max}$	CHO C+P	During and immediately post-Ex	41* 41*	- 10*	560*	• 13% ↑ TTE for C+P *	• ~45% ↑ in CK for CHO *

TTE; time-to-exhaustion test, $\dot{V}O_{2max}$; maximal oxygen uptake, Ex; exercise, CHO; carbohydrate, PRO; protein, C+P; carbohydrate plus protein, PLA; placebo, *; average values based on 70kg adult, CK; plasma creatine kinase, MS: muscle soreness, LE; leg extension strength, *; $P < 0.05$.

1.5 Glucose metabolism in skeletal muscle

Insulin stimulates the uptake of glucose from the circulation into many body tissues, of which, skeletal muscle accounts for ~75-80% of whole body insulin-stimulated glucose uptake (63, 232). Thus, skeletal muscle is considered as the primary tissue for regulating glucose homeostasis. At the cellular level, insulin stimulates glucose uptake into skeletal muscle by promoting glucose transporter protein (GLUT4) translocation to the cell membrane through the activation of an insulin-dependent signalling pathway.

1.5.1 Intracellular regulation of glycemic control in skeletal muscle

Appropriate signalling through the insulin pathway is critical for the regulation of plasma glucose levels in skeletal muscle. The binding of insulin to its receptor leads to the tyrosine phosphorylation of the insulin receptor (IR), the insulin receptor substrate-1 (IRS-1) and hence, to the activation of PI3K. The activation of PI3K leads to the production of phosphatidylinositol-(3, 4, 5)-P3 (PIP3), a key secondary messenger, which recruits 3-phosphoinositide-dependent protein kinase (PDK) to the plasma membrane (257). PI3K activity also induces the activation of downstream signalling intermediates; Akt and atypical protein kinase C (PKC) (257). In skeletal muscle, Akt mediates the insulin-stimulated inactivation of glycogen synthase kinase-3 (GSK3) by phosphorylation, thereby allowing glycogen synthase (GS) to become dephosphorylated and activated (55). Activation of PI3K is known to result in ribosomal protein S6 kinase (p70S6K) activation, a mechanism involving mTOR (212), which is inhibited by the blocking agent rapamycin. p70S6K can also be directly phosphorylated by PDK in a rapamycin-independent manner (11) and may play an important role in mediating GSK3, thus promoting the activation of glycogen synthase. The role of Akt in the regulation of insulin stimulated glucose transport is strengthened by the

recent discovery of AS160, a 160-kDa protein which is phosphorylated by Akt (140). AS160 contains a GTPase activating domain for Rabs, which are small G proteins required for membrane trafficking (including GLUT4 translocation). It has been shown that pAS160 in rat skeletal muscle is rapidly increased when exposed to a physiological increase in insulin (34). Furthermore, insulin-stimulated phosphorylation of AS160 occurs in a PI3K-Akt-dependent manner (65, 141).

1.5.2   Type II diabetes and insulin resistance

The prevalence of diabetes mellitus has reached global epidemic proportions. It is predicted that by 2025, 300 million people will have diabetes (279). The majority of diabetic patients (over 90%) suffer from Type II diabetes, a progressive metabolic disorder with a slow and insidious onset. The incidence of Type II diabetes and associated metabolic conditions stems from societies adopting ever more sedentary lifestyles in the face of an excessive energy intake. Consequently, diabetes imparts major health consequences at both individual and public health levels (136). Although several pharmacologic approaches to the treatment of Type II diabetes are available, there is growing interest in developing non-pharmacological interventions to improve glycemic control. Lifestyle interventions such as exercise and nutrition that delay, or even prevent, Type II diabetes have the potential to improve the health of the population and reduce the economic burden on healthcare services.

The pathophysiology of Type II diabetes develops primarily from i) defects in skeletal muscle sensitivity to insulin ii) defects in liver sensitivity to insulin and iii) pancreatic β cell dysfunction. Collectively, these defects are referred to as the Triumvirate (64). Under normal

circumstances, insulin binds to insulin receptors on target organ cells, resulting in a series of cellular events that promote intracellular glucose transport and metabolism (206). Insulin resistance describes the diminished sensitivity of cells to the signalling effects of insulin. It is generally accepted that resistance to insulin in target tissues, primarily skeletal muscle, precedes the development of Type II diabetes (204). In insulin resistant states, the pancreas compensates by secreting increased amounts of insulin to maintain normoglycemia. This resistance is followed by a decrease in insulin secretion as a result of progressive pancreatic β-cell dysfunction, leading to the onset of overt diabetes with fasting hyperglycemia. Comprehensive discussion of the mechanisms underpinning insulin resistance in liver tissue and β cell dysfunction is beyond the scope of this thesis chapter. Instead, the interested reader is referred to the following literature (62, 64). Thus, in the current chapter, the mechanisms responsible for insulin resistance will be discussed within the context of skeletal muscle tissue.

1.5.3   Mechanisms of insulin resistance in peripheral tissues

The intracellular signalling defects underlying insulin resistance in skeletal muscle are now becoming apparent. Signalling defects at the level of the insulin receptor are unlikely to fully account for the impairments in glucose transport in skeletal muscle from Type II diabetic patients (46, 154). Downstream of the insulin receptor, impaired insulin-stimulated PI3K activity in skeletal muscle has been confirmed in studies of obese (154, 249) and Type II diabetic patients (58, 144, 145). Defects in insulin signalling at the level of IRS-1 and PI3K probably account for the impaired insulin action on PKC reported by others (17, 145). Current investigations regarding cellular defects at the level of Akt in insulin resistant states have yielded conflicting results (17, 145, 155). These discrepant findings are likely due to

fundamental differences in cell culture and human study models. Thus, a combination of relatively minor defects downstream of IRS phosphorylation may act synergistically to cause insulin resistance and impair glucose tolerance, defined as the ability of skeletal muscle and the liver to remove glucose from the circulation (137).

Along with skeletal muscle, adipose tissue is also an important site for insulin-stimulated glucose uptake (47). In addition to Type II diabetes, insulin resistance is also commonly associated with obesity, hypertension and cardiovascular disease. Many studies have revealed an association between increased lipid availability and insulin resistance, suggesting that there is a causative link between the two. Studies demonstrate that high-fat feeding is closely associated with the accumulation of intramuscular triacylglycerol (IMTG) (68, 143, 198, 237). Moreover, insulin resistance can occur through short term (physiologically relevant) increases in lipid availability. However, it appears unlikely that the IMTG accumulation *per se* directly impairs insulin action (33). This suggestion is further supported by the observation that endurance trained (highly insulin sensitive) individuals have elevated IMTG content. Instead, it is believed that intermediates in fatty acid metabolism, such as fatty acyl-CoA (198), ceramides (104) or diacylglycerol (DAG) (241) and an elevated inflammatory state, link obesity to compromised insulin signalling in the muscle. DAG is identified as a potential mediator of lipid-induced insulin resistance (113, 214). Increased DAG levels are associated with PKC activation and a reduction in both insulin-stimulated IRS-1 tyrosine phosphorylation and PI3K activity (276). Recently, Newgard et al. (187) demonstrated the accumulation of a number of acylcarnitines in skeletal muscle of high-fat fed rats supplemented with additional branched chain amino acids (BCAA's) compared with non-supplemented rats. Thus, in the context of high-fat consumption, supplementation of protein and/or amino acid may contribute to obesity-

induced insulin resistance. The efficacy of protein ingestion on FFA metabolism during a period of reduced energy intake provides an interesting avenue for future studies.

1.6   Influence of exercise on glucose homeostasis

Insulin resistance and Type II diabetes are strongly associated with a decline in physical inactivity (70, 153). However, despite the underlying metabolic and molecular defects the precipitate insulin resistance, skeletal muscle contraction can effectively restore glycemic control in a manner independent of insulin (111). Moreover, these properties are preserved in individuals with Type II diabetes (97, 142). Thus, at best, exercise represents an effective means of preventing insulin resistance and Type II diabetes, but perhaps more realistically, represents a means by which to attenuate the metabolic symptoms associated with these conditions. Long-term intervention studies (3-5 years) show that Type II diabetes 'risk reduction' is further reduced when physical activity is implemented in combination with dietary modifications (75, 202). More importantly, increased physical activity and reduced energy intake are associated with a greater reduction in the incidence of diabetes compared with pharmacological interventions such as metformin (a suppressor of endogenous glucose production) (250).

1.6.1   Resistance exercise and glycemic control

Physical inactivity induces a wide variety of changes in both whole-body functional capacity and muscle metabolism (220), with most of the metabolic effects of physical inactivity on insulin resistance being rapid in onset and of relatively short duration (97). Studies investigating the role of physical activity as a means to improve the insulin sensitivity have generally applied endurance exercise as a model. Indeed, a single bout of moderate-to-

high intensity endurance exercise has been shown to acutely improve insulin sensitivity and/or glucose tolerance (66, 67, 176, 205). This effect has been reported to persist for a period ranging from 2 hours (176), 4–6 hours (273), 12–16 hours (66, 67) to 48 hours post-exercise (176, 205). In contrast to endurance exercise, limited information is available regarding the impact of resistance exercise on glucose metabolism. It has been demonstrated that 6-12 weeks of resistance training lowers the percentage of glycosylated haemoglobin and increases glucose disposal in Type II diabetics due, in large part, to muscle hypertrophy and an increase in the protein content of insulin-signalling intermediates (54, 77, 117). In contrast, the effectiveness of a single bout of resistance exercise on glycemic control and the mechanisms regulating this response are less well understood. Whereas some authors have reported an improved glucoregulatory response 12–24 hours after a single bout of resistance exercise (77, 81, 150, 258), others have failed to observe any change in insulin sensitivity (44, 166). These discrepant findings may be related to the method utilized to determine glucose and insulin kinetics (i.e. oral glucose tolerance tests vs. insulin tolerance test) or differences in the exercise stimulus and participant training status. Nevertheless, these data strongly suggest that a single bout of resistance exercise of sufficient volume and intensity can improve insulin sensitivity and glycemic control in humans.

1.6.2   Intracellular signalling regulating glucose uptake with acute exercise

The molecular mechanisms for enhanced glucose uptake and insulin sensitivity with exercise are related to the increased expression and/or activity of key enzymes and signalling proteins involved in skeletal muscle glucose and fat metabolism (69, 277). Prolonged improvements in insulin sensitivity and glucose transport may reflect changes in protein expression (i.e. enhanced or suppressed) that occur following each acute exercise bout. The immediate effects of resistance exercise on skeletal muscle glucose transport have long been

thought to occur via AMPK regulation of GLUT4 trafficking to the cell membrane (71, 240) rather than through any enhancement of proximal insulin signalling steps (122, 273). However, it has recently been demonstrated that contractile activity can promote GLUT4 translocation irrespective of AMPKα2 (159). In this regard, recent work has implicated sucrose non-fermenting AMPK-related kinase (SNARK) protein as an novel and potentially important mediator of skeletal muscle glucose uptake (149). Currently, the precise signalling events that promote glucose uptake in skeletal muscle are unclear.

The recent discovery of the most distal signalling proteins implicated in insulin-stimulated GLUT4 transport, AS160 and TBC1D1 (from the same family domain of proteins), has provided us with a clue as to how contraction-induced AMPK activity regulates GLUT4 translocation. Phosphorylation of AS160/TBC1D1 is thought to lead to inhibition of AS160's activation of Rab-GTPase proteins associated with the GLUT4 vesicle and/or cause AS160 to disassociate from the GLUT4 vesicle. This mechanism of action thereby allows for the accumulation of Rab GTP, thus favouring GLUT4 translocation (42, 140). Several studies, using specific antibodies for individual phospho-sites on AS160, have reported an increase in AS160 phosphorylation following resistance exercise (71, 121, 246). Furthermore, when isolated skeletal muscle is treated with AICAR (an AMPK activator), or subjected to contraction *in situ*, both AS160 and TBC1D1 phosphorylation are increased (34, 152, 239). Conversely, when AMPK is inhibited (via knockout of AMPKα2), contraction-stimulated AS160 phosphorylation is significantly reduced (152). Recently it has been hypothesized that a large proportion of the contraction-stimulated increase in glucose transport may be attributed to TBC1D1, whereas AS160 may play a more prominent role in regulating insulin-stimulated glucose uptake (42). To date, this thesis has yet to be confirmed in humans. At present, methodological limitations regarding non-specific antibody detection of

AS160/TBC1D1 cloud our understanding of the phospho-specific response of these two proteins to insulin and contraction stimuli (42). Indeed, it has recently been demonstrated in mice that contraction-stimulated skeletal muscle glucose uptake is regulated by site-specific phosphorylation of TBC1D1 (259).

In summary, it has been hypothesized that AS160 and TBC1D1 act as a nexus for insulin-dependent and insulin-independent (i.e. contraction) signalling pathways on GLUT4 translocation and skeletal muscle glucose uptake (43). Consequently, the intracellular signals emanating from contracting muscle during exercise may interact with the insulin signalling pathway at the level of AS160/TBC1D1, thereby acting synergistically to enhance glucose uptake (43). In support of this suggestion, Treebak et al. (245) recently confirmed that AS160 phosphorylation was increased by prior exercise, both before and immediately after insulin stimulation.

1.7   Influence of protein nutrition on glucose homeostasis

Concepts regarding dietary changes that prevent or assist in the management of Type II diabetes are beginning to emerge. In the last decade the role of protein feeding as an intervention to reduce hyperglycemia has received considerable attention. Previously, when dietary recommendations for the general population, or for persons with diabetes were considered, the major focus was the relative amounts and types of carbohydrate and fat (1). Protein nutrition is generally considered in the context of that necessary to maintain, or gain lean body mass, i.e., that needed to promote a positive net muscle protein balance. However, in the early 1900's, it was reported that the ingestion of egg white protein in a single-meal study did not elevate blood glucose concentration in healthy humans (130). In persons with

Type II diabetes, it has been suggested that protein ingestion lowers postprandial glucose concentrations (193, 196). Long-term, protein-rich diets are associated with better glycemic control and plasma lipid profile in obese and insulin resistant individuals and promote energy expenditure and greater relative fat reduction, compared with energy matched, high carbohydrate or high fat diets (15, 161, 265). Thus, it appears that protein nutrition could play an important role in glycemic control in humans.

1.7.1 Insulin-stimulated hypoglycemia with protein co-ingestion

Several decades ago Floyd and Fajans (76, 79, 80) showed that the infusion of high doses of amino acids potentiated the release of insulin. In contrast, oral ingestion of protein or amino acids, in an amount likely to be ingested in a high protein meal, does not alter the insulin response (87, 88, 138, 139, 194, 197). Alternatively, co-ingesting protein with carbohydrate does stimulate a greater insulin release than the ingestion of either macronutrient alone. The synergistic effect of protein plus carbohydrate on insulin release was first described in the 1960's (200, 215) and later confirmed by Nuttall and colleagues (195, 196). Studies conducted *in vitro* (24, 25, 168, 169, 227, 228, 230) and in humans (76, 226) demonstrate that amino acids co-ingested with carbohydrate potentiate insulin release from pancreas β cells. Increasing postprandial endogenous insulin release may lower blood glucose levels by assisting the process of skeletal muscle glucose uptake via the insulin-dependent signalling cascade that, ultimately, enhances glucose transporter protein translocation to the cell membrane. Thus, protein co-ingestion may alleviate many side effects of diabetes (e.g. cardiovascular disease risk, glycation of tissues/neurones, inflammation) and postponing an individuals' dependency on exogenous insulin therapy.

Several notable studies from the Dutch group of Luc Van-Loon have focussed on the insulinotropic effect of protein plus carbohydrate ingestion in Type II diabetics. Initially, this group demonstrated that the insulin response to carbohydrate intake can be nearly tripled by the addition of a free amino acid/protein mixture (254). Subsequently, the group showed that this free amino acid/protein plus glucose mix augmented insulin release and lowered plasma glucose excursions in Type II diabetics (173). With the use of a labelled glucose tracer infusion during drink ingestion, the authors attributed the reduction in plasma glucose to an increase in the rate of glucose disposal from the circulation, presumably taken up by skeletal muscle. However, recent work from this group showed that co-ingesting protein with each main meal (three times a day) did not reduce the prevalence of hyperglycemia over the course of the day in Type II diabetics (172). To explain this finding, the authors posit that the increase in total energy intake (~14%) when additional protein was co-ingested may have offset any hypoglycemic effect. Thus, protein ingestion may be best implemented in the diet in place of, rather than in addition to, other macronutrients. Nevertheless, these data provide overwhelming evidence that co-ingesting protein with carbohydrate promotes hyperinsulinemia. Thus, whilst the sensitivity of the pancreas to glucose is significantly impaired in persons with long-standing Type II diabetes (211), the capacity to secrete insulin when protein is co-ingested with carbohydrate remains functional (171, 173). However, the efficacy of protein co-ingestion as a non-pharmacological treatment for Type II diabetes in daily, free-living conditions is less than clear, although Manders et al. (172) hypothesized that the presence of free amino acids, leucine in particular, may be important in stimulating hyperinsulinemia (170).

1.7.2   Protein absorption and hydrolysis for glucose homeostasis

The co-ingestion of most common food sources of protein with glucose, promotes hypoglycemia in Type II diabetics (86). However, the insulinotropic response is dependent on the protein source consumed (148, 193, 195). Differences in insulinotropic and glycemic properties of various protein sources are likely to be associated with; i) the post-absorptive pattern of amino acid appearance in the circulation and the degree of hydrolysis of the protein source.

Casein proteins are digested slowly and induce a lower but more prolonged hyperaminoacidemia, whereas whey protein, which is digested quickly, induces higher postprandial retention of amino acids (29, 190). The greater insulinotropic response to whey protein feeding may be connected to more rapid digestion kinetics. The suggestion that whey-induced hyperaminoacidemia is important in glucose homeostasis is supported by studies showing that glycogen content in liver (184) and skeletal muscle tissue (185) of whey protein fed rats, was greater than rats fed casein. In addition to the absorption kinetics, the degree of hydrolysis of ingested proteins may be an important determinant for maintaining normoglycemia. It is generally accepted that di- and tri-peptides, remaining after the initial peptidase digestion, are absorbed intact (101). Indeed, whey, egg and casein hydrolysates, containing mostly di- and tri-peptides, are more rapidly absorbed than their respective intact protein sources (102). However, there was no difference in the glucose and insulin response between animal and vegetable protein hydrolysates co-ingested with glucose (37, 48). Collectively, these data imply that the rate of amino acid appearance in the circulation, dependent on the protein source and state of hydrolysis, is an important determinant of the insulinotropic and hypoglycaemic response.

1.7.3   Insulinotropic properties of free amino acids

In addition to protein co-ingestion with carbohydrate as a means to promote hyperinsulinemia, it is now clear that certain free amino acids act as direct insulin secretagogues (76, 225, 226). Nilsson et al. (190) first reported a correlation between the postprandial insulin and early increments in leucine, valine, lysine, and isoleucine after the consumption of different protein containing food sources. These data are further supported by studies demonstrating that the insulinemic response to BCAA's plus glucose co-ingestion mimics the glycemic and insulinemic response to ingesting intact whey protein with glucose (183, 189). Based on these data, it is apparent that the BCAA's are the most potent insulin secretagogues (45, 124, 255). Mechanistically, *in vivo* and *in vitro* work indicates that the BCAA leucine both stimulates and enhances pancreatic β cell insulin secretion through oxidative decarboxylation and allosterically activating glutamate dehydrogenase (188, 229). The precise mechanisms, by which amino acids promote insulin exocytosis from the pancreas, although not yet fully elucidated, likely involve depolarization of the plasma membrane via energy sensing Ca^{+2} and k^+ channels (188, 256). At present, the impact of BCAA on glucoregulation in healthy and Type II diabetics is still the subject of some debate. Manders and co-workers (170) fed a carbohydrate plus protein beverage with or without additional leucine to Type II diabetics and reported no difference in the response of insulin or plasma glucose.

1.7.4   Mechanisms of glucose uptake with amino acids

The signalling events that facilitate the glucose uptake in skeletal muscle are highly sensitive to a number of stimuli, including amino acids, and can be 'turned on' in the presence and absence of insulin. Surprisingly little is known about the mechanisms regulating glucose homeostasis following protein and amino acid ingestion in healthy and Type II diabetic humans. In contrast, the last decade has seen the emergence of rat and cell culture

studies in which glucose uptake and the associated regulatory signalling mechanisms have been determined following treatment with different proteins and free amino acids, ingested alone or with glucose. The main findings of these studies are summarized in Table 1.3.

Amino acids, particularly leucine, enhance the phosphorylation of mTOR and can act synergistically with insulin to fully activate mTOR (5, 105, 203). However, insulin and amino acids signal to mTOR via divergent pathways (105). Initially, Armstrong et al. (8) reported that amino acids promote glycogen synthesis and glucose uptake in cultured human muscle cells via non-insulin dependent pathways via transient inhibition of GSK3 through phosphorylation of p70S6K. Furthermore, amino acid-stimulated glycogen synthesis was blocked by the mTOR inhibitor rapamycin (8). Others (207) also demonstrate that the provision of leucine stimulates glycogen synthesis in L6 myotubes in an mTOR dependent manner. The importance of the mTOR-p70S6K pathway and the amino acid leucine for glucose uptake is strengthened by Nishitani et al. (191). In rats with cirrhosis, a condition characterized by impaired glucose metabolism, these authors demonstrated that leucine and isoleucine enhanced glucose uptake, thus suppressing plasma glucose excursions compared with saline fed controls. In soleus muscles from these rats, leucine and isoleucine were shown to increase GLUT4 translocation to the cell membrane in insulin-free conditions. Taken together, these studies demonstrate an important regulatory role of the mTOR-p70S6K pathway in amino acid-stimulated glucose uptake. Intracellular regulation of glycemic control via insulin and BCAA is summarized in Figure 1.2.

Insulin is a potent activator of muscle glycogen synthesis owing to its stimulatory effect on glucose transport. When BCAA are provided in the presence of an elevated insulin response, glucose uptake is potentiated through an increase in the phosphorylation of insulin-dependent signalling intermediates Akt, PI3K and PKC (182, 184). With regard to mTOR

signalling, evidence suggests that insulin and amino acids act synergistically to fully activate

this protein (105, 203) and that both stimuli signal to mTOR via divergent pathways (105).

Whereas amino acid feeding in insulin-free conditions appears to promote glycogen synthesis

via mTOR signalling, in the presence of insulin, BCAA-stimulated mTOR phosphorylation

has been shown to be rate-limiting for skeletal muscle glucose uptake through an enhanced

degradation of IRS-1 (16, 128, 238, 247). Thus, it appears that mTOR signalling may be an

important step in the regulation of glucose uptake in skeletal muscle cells. However, the

specific role of mTOR signalling remains to be tested in healthy or Type II diabetic humans.
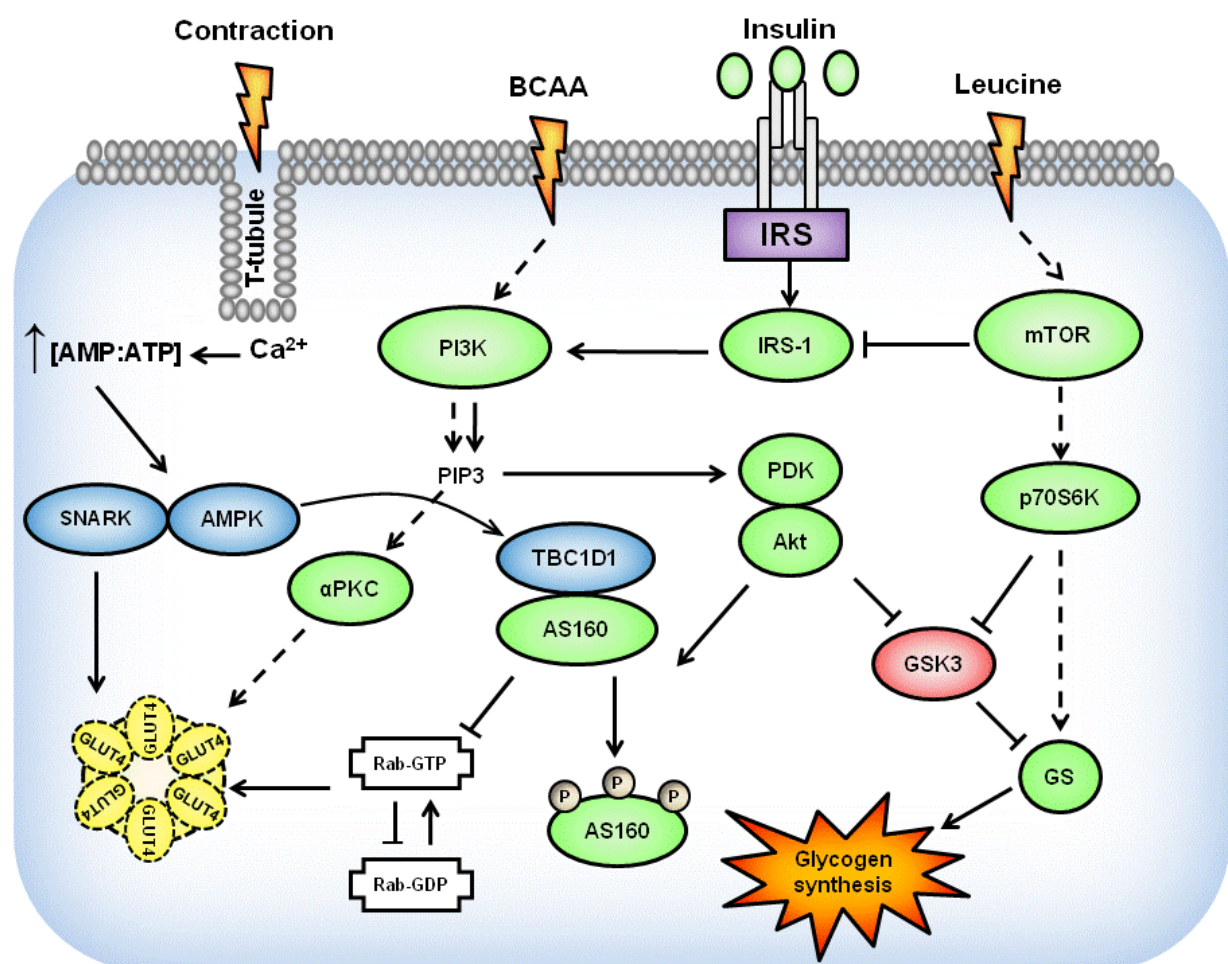
Figure 1.2 A simplified schematic representation of insulin-, BCAA- and contraction-stimulated signalling mechanisms regulating glucose uptake in skeletal muscle. Dashed arrows indicate BCAA-stimulated signalling activation. Solid arrows indicate insulin- and contraction-stimulated signalling activation. Proteins have been labelled to designate them as positive contractile (blue), positive feeding-induced (green) or negative (red) regulators of glucose uptake.

Table 1.3 Studies investigating glucose metabolism and intracellular signalling in response to protein/amino acids on in rat and cell culture models.

Study	Model	Method	Protein/AA source	Glucose metabolism	Signalling response	Dependent on insulin
Peyrollier *et al.* (2000)	• L6 myotubes	AA deprived muscle cells were incubated with AA.	Leu	• N/a	• AA ↑ **p70S6K** and ↓ **GSK3** * • Dependent on **PI3K** and **mTOR** but not Akt *.	• No
Armstrong *et al.* (2001)	• Cultured human myoblasts	Myoblasts were incubated in an amino acid medium.	Mixed AA medium	• AA medium ↑ GS activation **	• Dependent on **mTOR** and **PI3K** but not Akt **. • AA ↑ **p70S6K** and ↓ **GSK3** **	• No
Nishitani *et al.* (2002)	• Isolated rat soleus muscle	Isolated muscles incubated in leucine.	Leu	• Leu ↑ glucose uptake *	• Glucose uptake with AA was dependent on **PI3K** and **αPKC** but not mTOR *.	• No
Doi *et al.* (2003)	• Healthy rats • C$_2$C$_{12}$ myotubes	Post-OGTT BCAA feeding and glucose uptake in myotubes.	Leu, Ile, Val or Con	• Iso ↓ plasma glucose ** • Iso ↑ glucose consumption *	• Dependent on **PKC** and **PI3K but not** mTOR *	• No
Nishitani *et al.* (2005)	• Cirrhosis induced rats	Post-OGTT BCAA feeding and isolation of soleus muscles.	Leu, Ile , Val or control	• Leu and Iso ↑ glucose tolerance and uptake *	• Leu and Iso ↑GLUT4 and GS activity * • Leu phosphorylated **p70^{S6K}** and was dependent on **mTOR** *	• No
Morifuji *et al.* (2009)	• L6 myotubes • isolated rat epitrochlearis muscles	L6 myotubes and isolated muscle incubated with WPH dipeptides.	WPH dipeptides Iso+Leu.	• Dipeptides and Iso+Leu ↑ glucose uptake *	• Iso+Leu were dependent on **PI3K** and **αPKC **	• No
Morifuji *et al.* (2009)	• Exercise trained rats	Rats ingested glucose or glucose plus protein immediately post-exercise.	Glu alone or with WPH, CH, or BCAA	• WPH ↑ muscle glycogen levels at 2 hours post-ex *	• WPH ↑ phosphorylation of **Akt** and **PKC** *	• Yes
Iwanaka *et al.* (2009)	• Isolated rat epitrochlearis muscle	Isolated muscle treated with Leu following insulin or contraction	Leu	• Leu ↑contraction-induced glucose uptake * • Leu ↓ insulin-stimulated glucose uptake *	• Leu ↑ phosphorylation of **p70S6K** after contraction * • Leu ↑ phosphorylation of **IRS-1** and ↓ **Akt** with insulin *	• No • Yes

OGTT; oral glucose tolerance test, BCAA; branched chain amino acids, Leu; leucine, Ile: isoleucine, Val; valine, GLUT4; glucose transporter protein, GS; glycogen synthase, p70^{S6K}; ribosomal protein S6 kinase, mTOR; mammalian target of rapamycin, WPH; whey protein hydrolysate, PI3K; phosphatidylinositol 3-kinase, PKC; atypical protein kinase C, Akt; protein kinase B, AA; amino acids, GSK3; GS kinase-3, IRS-1; insulin receptor substrate-1, *; $P < 0.05$, **; $P < 0.01$.

1.8 Specific objectives of the thesis

This thesis describes a series of studies investigating the effectiveness of protein nutrition on exercise performance, indices of recovery and the metabolic/molecular adaptations to endurance and resistance exercise training. **Chapter 2** describes a study in which we determined whether the addition of protein to a carbohydrate beverage improved cycle time-trial performance. Furthermore, we tested the hypothesis that carbohydrate plus protein ingestion during exercise enhances recovery by measuring indirect markers of muscle damage and muscle function. Chapters 3 and 4 focus on the influence of protein nutrition on protein metabolism following acute endurance exercise and glucose metabolism following acute resistance exercise. **Chapter 3** describes a study in which we determined the synthetic response of myofibrillar and mitochondrial proteins to carbohydrate plus protein ingestion following prolonged cycling exercise. We simultaneously measured the phosphorylation of intracellular signalling proteins implicated in translation initiation and elongation phase of protein synthesis, in an attempt to provide a molecular mechanism to explain the divergent response of specific protein fractions to post-endurance exercise protein ingestion. **Chapter 4** describes a study in which we first determined the impact of a single bout of resistance exercise on the glycemic response to an oral glucose load at 24 hours post-exercise. Second, we tested the hypothesis that co-ingesting protein with the oral glucose load would augment resistance exercise-induced improvements in glycemic control. During this experiment, we utilized dual isotopic tracer methodology to measure endogenous and exogenous glucose kinetics in combination with muscle biopsies to determine the underlying intracellular mechanisms regulating glycemic control following resistance exercise and protein ingestion. **Chapter 5** discusses the results of the studies described in the previous chapters, providing an overview of the main conclusions. The practical implications of the research are discussed and recommendations for future research are provided.

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CHAPTER 2

NO EFFECT OF CARBOHYDRATE-PROTEIN ON CYCLING PERFORMANCE AND INDICES OF RECOVERY

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2.1 Abstract

Purpose: The aim of this study was to determine whether adding protein to a carbohydrate beverage would improve late-exercise cycle time-trial performance over carbohydrate alone. Furthermore, we examined the effects of co-ingesting protein with carbohydrate during exercise on post-exercise markers of sarcolemmal disruption and the recovery of muscle function. **Methods:** In a double-blind, cross-over design, 12 trained male cyclists performed 120 min of steady-state cycling (SS) at ~55% $\dot{V}O_2$max followed by a time-trial lasting ~1 h (TT). At 15 min intervals during SS exercise participants consumed either a carbohydrate (CHO) or carbohydrate-protein (CHO+Pro) beverage (providing 65 g·h^{-1} carbohydrate; or 65 g·h^{-1} carbohydrate plus 19 g·h^{-1} protein). Twenty-four hours following the onset of the SS cycle, participants completed a maximum isometric strength test. At rest and 24 h post-exercise, a visual analogue scale (VAS) was used to determine lower-limb muscle soreness and blood samples were obtained for plasma creatine kinase (CK) concentration. Dietary control was implemented 24 h prior to and during the time-course of each trial. **Results:** Average power output sustained during TT was similar for CHO and CHO+Pro with no effect of treatment on time-to-complete the time-trial (60:13 ± 1:33 min:sec and 60:51 ± 2:40 min:sec for CHO and CHO+Pro, respectively). Post-exercise isometric strength significantly declined for CHO (15 ± 3%) and CHO+Pro (11 ± 3%) compared with baseline (486 ± 28 N). Plasma CK concentrations and VAS soreness significantly increased at 24 h post-exercise, with no difference between treatments. **Conclusions:** The present findings suggest CHO+Pro co-ingestion during exercise does not improve late-exercise time-trial performance, ameliorate markers of sarcolemmal disruption or enhance the recovery of muscle function at 24 h post-exercise over carbohydrate alone.

Key words: Time-trial, endurance exercise, sarcolemmal disruption, muscle function.

2.2 Introduction

The quality and quantity of nutritional intake plays a critical role in response to training and in athletic performance. It is well established that carbohydrate (CHO) ingestion during moderate to high-intensity exercise can prolong endurance capacity (7). Evidence has since emerged to suggest that the ingestion of multiple transportable carbohydrates can increase exogenous carbohydrate oxidation and improve endurance performance, over and above the effect of a single carbohydrate (8, 14). Recently, the addition of protein to a carbohydrate containing beverage (CHO+Pro) during exercise has been reported to prolong endurance time-to-exhaustion (TTE) in the range of 13-36% (13, 30, 31). In contrast, other well controlled studies did not report such an ergogenic effect of CHO+Pro on time-trial performance (25, 35). Recently, Saunders and co-workers (32) suggested the addition of protein to carbohydrate specifically improved late-exercise time-trial performance. Thus, the efficacy of adding protein to carbohydrates for endurance performance remains unclear.

A number of methodological differences between studies make it difficult to discern whether any benefits observed for CHO+Pro are the result of a protein-mediated effect. First, there are discrepancies in the way exercise performance is assessed for CHO and CHO+Pro treatments. The ecological validity of TTE protocols is limited, because endurance athletes do not compete in events that require sustaining a fixed power output for as long as possible. Moreover, claims that CHO+Pro improves late-exercise performance were based on a time-trial protocol that was not specifically designed to record power output and measure late-stage exercise performance (32). Secondly, additional energy consumed when protein is added to a carbohydrate containing beverage may explain the performance benefits observed by others. If carbohydrate is consumed at a rate considered optimal for exogenous

carbohydrate oxidation (16), the role of protein for improving endurance performance may be negligible (35). Finally, the control of external variables (or in some cases lack thereof) has varied greatly between previous studies. Investigations attempting to implement a strictly controlled exercise environment reported no effect of protein co-ingestion for time-trial performance (25, 35).

The majority of studies examining the effect of carbohydrate-protein co-ingestion have focused on the impact on endurance performance. Several authors suggest that consuming CHO+Pro during and following endurance exercise improves recovery, defined as the recovery of endurance capacity (30, 37) and muscle function (33, 34). Reductions in plasma creatine kinase (CK) concentrations (20, 21, 28, 30, 31, 33, 34) and muscle soreness (20, 21, 28, 33), are thought to play a key role in aiding the recovery process. However, evidence to support an effect of additional protein for improving recovery is inconsistent. Others have found no difference in the post-exercise rise in plasma CK (5, 10), muscle soreness (5, 21) or the recovery of endurance capacity (4) for CHO+Pro. Thus, the effectiveness of additional protein for enhancing recovery is unclear, perhaps due to methodological and design differences among studies, including variable amounts of control. These issues require further investigation.

The primary aim of the present study was to investigate the efficacy of adding protein to carbohydrate for improvement of late-exercise performance. Unlike previous studies, we aimed to supply a recommended dose of carbohydrate during cycle exercise to determine whether the addition of protein improved cycle time-trial performance in a controlled exercise environment, when preceded by a standardized steady-state ride. Second, we aimed to determine the effect carbohydrate-protein beverages on indices of recovery, 24 h following

time-trial cycle exercise by measuring plasma CK concentration, muscle soreness and muscle function. Participant diet and physical activity were stringently controlled for the 48 h trial duration to ensure participants were in a similar state of energy balance and fuel repletion.

2.3 Methods

2.3.1 Participants

Twelve trained male cyclists were recruited through advertisements, from local clubs (subject characteristics are shown in Table 2.1). Only individuals who undertook 2 or more training sessions per week of 1 – 5 h duration were eligible to participate. Data was collected over a 4-month period, such that all participants were all in a similar phase of their training cycle. All trials were completed within a 3-week period with no more than 14-days between trials and the purpose and methodology of the study were clearly explained to participants. All participants signed an informed consent and a completed a general health questionnaire prior to taking part in the study. The experimental protocol was approved by the School of Sport and Exercise Sciences Safety and Ethics Subcommittee.

2.3.2 Experimental Design

The study design was counter-balanced, cross-over and double-blind. Maximal oxygen uptake and muscle function were assessed at baseline. Following baseline testing participants performed two trials, during which they completed a 120 min steady-state cycle, consuming either a carbohydrate (CHO) or carbohydrate-protein (CHO+Pro) treatment beverage. Following the 120 min steady-state ride at 50%Wmax, participants were then asked to complete a time-trial lasting ~1 h (Figure 2.1). Participants returned after a 7-14 days and repeated the protocol whilst consuming the alternate treatment beverage. For example if the participant consumed CHO+Pro during trial 1, they consumed the CHO treatment during trial

2. Markers of sarcolemmal disruption were obtained immediately prior to and 24 h post-exercise and the recovery of muscle function was assessed 24 h post-exercise. All tests were conducted in a fasted state at the same time-of-day to minimize any circadian variance.

Table 2.1 Participant characteristics at baseline.

	Age (Yrs)	Weight (kg)	Experience (y)	BMI ($kg \cdot m^{-2}$)	VO_2max ($mL \cdot kg^{-1} \cdot min^{-1}$)	Wmax (W)
Mean	27	70.5	7.1	22.4	62.7	350
SD	8	5	1.5	2.6	6.3	34

Data are presented as means ± SD

2.3.3   Baseline Testing

Body Mass: A digital scale was used to determine body weight to the nearest 0.1kg. Each participant was weighed in their cycling clothing without shoes. This was repeated prior to each of the two testing visits to ensure body weight remained similar throughout.

Maximal Power Output: Prior to completing the treatment trials, maximal oxygen uptake ($\dot{V}O_2max$) and maximal power output (Wmax) were determined using an incremental cycle test to exhaustion on an electrically braked cycle ergometer (LODE Excalibur Sport V 2.0 Groningen, Netherlands). The test consisted of a 3 min warm-up at 95 W, followed by an increase of 35 W every 3 min until exhaustion, at a self-selected cadence. The seat position, handlebar height and orientation used during testing were recorded and replicated on subsequent visits to the laboratory. All cycle tests were conducted in thermo-neutral conditions (23°C, 40% relative humidity). Wmax values were used to determine the workload for the pre-loaded time-trial. Heart rate (HR) was measured continuously by using

telemetry using HR monitor (Polar S625X; Polar Electro Oy, Kempele, Finland). $\dot{V}O_2$ was considered maximal if 3 of the 4 following conditions were met: 1) a plateau in $\dot{V}O_2$ with further increasing workloads (an increase of < 2 mL·kg^{-1}·min^{-1}); 2) a HR within 10 beats/min of the age predicted maximum (220 bpm - age); and 3) a respiratory exchange ratio (RER) of >1.05; 4) a rate of perceived exertion (RPE) greater than 17.

Metabolic & Physiological Measures: Expired O_2, CO_2 and RER, were measured during the last 60 sec of each stage and ~60 sec prior to the cessation of exercise to determine $\dot{V}O_2$ and $\dot{V}CO_2$. Participants indicated to the investigator the point at which they felt they were only able to exercise for one more minute. These data were obtained using Douglas gas collection bags (Plysu, Milton Keynes, UK), with air expired through a 2-way breathing valve connected to the bag. Bags were analyzed using a gas analyzer (Servomex 1440, East Sussex, UK), which was calibrated before each analyses using medical-grade gasses of known concentrations (British Oxygen Company, UK). The RPE was obtained using the 6-20 Borg scale.

Assessment of Maximal Isometric Strength: Twenty-four hours after each time–trial, a Tornvall chair (22) was used to determine maximal isometric strength (MIST). These values were compared with a single rested MIST test conducted at baseline, to determine the post-exercise loss of isometric strength. Both voluntary and stimulated isometric contractions of the *quadriceps femoris* were determined, in the non-dominant leg. A series of known weights was applied to a strain gauge and the resulting voltage output was recorded daily for conversion into force. Participants were seated with the knee at 90°, which was fixed in place by a cord fastened round the ankle. The cord was attached to a strain gauge interfaced with a computer, on which the resultant force was displayed and recorded. Two arm straps held the participant in place in the chair and minimize any extraneous movement. Damp electrodes (13 x 8 cm) were secured proximally and distally to the anterolateral thigh. At baseline,

maximal electrical stimulation was determined using a twitch overlay programme; progressively larger voltages (mA) were applied until two produced the same force. Once this was determined participants received a percutaneous electrical stimulation three times before being encouraged to extend their knee as hard as possible. Three electrical stimulations were superimposed while contraction was occurring to ensure that each effort was maximal. This procedure occurred three times during each trial with participants resting for 90 seconds between efforts. The highest value was recorded as the participant's maximal voluntary contraction.

2.3.4   Blinded Trials

Two-hour Pre-load Cycle: No less than 7 days after baseline testing, participants underwent familiarization of the time-trial whilst consuming water only. For each trial, participants reported to the Human Performance Laboratory in a fasted state (06.00 – 07.00) having undergone 24 h of dietary control prior to this visit. Participants completed 120 min of constant pre-load ergometer cycling at a work-load 50% of their previously determined Wmax (175 ± 5 W). Participants were instructed to maintain a comfortable cadence above 60 revolutions per minute (rpm).

One-hour Time-trial: Upon completion of the 120 min pre-load cycle the ergometer was set in the linear mode and participants were asked to complete an energy based target amount of work at 70% of their pre-determined Wmax. The total amount of work for a 1 h time-trial (880 ± 27 kJ) was calculated according to the formula derived from Jeukendrup et al. (15). A linear factor, calculated as work-rate at 70% Wmax divided by ($90rpm^2$), was entered into the ergometer controller. During the time-trial, participants received no verbal or visual feedback regarding performance time or physiological measures. Participants had access to a computer screen indicating the amount of the total energy target they had

completed. Trials were performed in a small testing laboratory with a screen placed behind each participant to minimize distraction. Air conditioning and a fan were used when requested by participants, for which the settings were recorded and replicated during the second trial.

Metabolic & Physiological Measures: Oxygen uptake, CO_2 production and RER data were collected for 2 min intervals every 30 min of the 120 min pre-load cycle to determine the intensity of the workload. Heart-rate and RPE were obtained also to confirm a steady state was maintained prior to the time-trial. During the time-trial HR, RPE and average power output were recorded at 25, 50 and 75% of the total energy target (kJ). These same measures were also obtained ~10 seconds prior to completion of the trial.

Assessments of Muscle Function and Force Production: Twenty-four hours after the start of the pre-loaded cycle trial (06.00 – 07.00), participants were asked to return to the laboratory in a fasted state, to undergo a MIST as described previously.

Visual Analogue Scale of Muscle Soreness: Using a 50mm line with word descriptors at each end reading 'no soreness' and 'extreme soreness', participants marked on the line, the point that they felt represented their current degree of general muscle soreness in their non-dominant leg. The distance to the mark was then measured in millimeters. VAS soreness was also rated when the knee was both flexed and extended. Average VAS soreness is reported here as it closely represents general, flexed and extended soreness values. A VAS was completed in the morning prior to each time-trial and 24 h following the onset of the pre-load cycle upon return to the laboratory prior to the MIST. The VAS scale has previously been shown to be a reliable device in measuring acute pain (6).

Blood Analysis: Upon arrival for each time-trial, a cannula (IV Venflon 20GX32mm) was inserted into the antecubital vein of the forearm. A two-way stopcock (Medex, Monsey, NY) was applied and opened to collect a 10mL sample at rest. A further blood sample was

taken upon each participants return to the laboratory for the MIST on day 2 (24 h following the onset of the pre-load cycle). Samples were centrifuged at 3000rpm at 4°C to allow the extraction of blood plasma for further analysis. All plasma samples were processed and stored frozen at -80°C. The exact time-points of all sample collections were recorded and repeated during the second blinded treatment. Enzymatic analysis of CK concentration (CK NAC CP, ABX diagnostics, UK) was performed in duplicate at each time-point using a semi-automated analyzer (COBAS MIRA S-plus, ABX, UK).

2.3.5   Treatment Beverages

The two experimental trials were a 6% maltodextrin carbohydrate beverage (65 g·h^{-1}; CHO) and a carbohydrate + protein (CHO+Pro) beverage containing 6% maltodextrin (65 g·h^{-1}) and 1.8% protein hydrolysate (19 g·h^{-1}). Treatment beverages were counter-balanced throughout the study to minimize any influence of order, with 6 participants receiving CHO first and 6 receiving CHO+Pro first. Beverages were served in non-see-through bottles, with a different flavor used in each trial to minimize the risk of taste comparisons between beverages. During pilot testing, a group of cyclists were able to successfully identify the beverage composition when flavors were the same. Flavors were added using 3g of non-energetic sweetened drink mix. Participants consumed 270mL every 15 min of the 120 min steady state pre-load, beginning at the onset of the ride (1080mL per/hour). An investigator handed the participant one container at a time and verbally encouraged them to drink at an appropriate rate. Water was consumed *ad libitum* during the time-trial with the amount recorded (~430 ± 26mL) and administered in the same volume during the second trial. Beverages were prepared by an independent investigator who had no part in the data collection of the study.
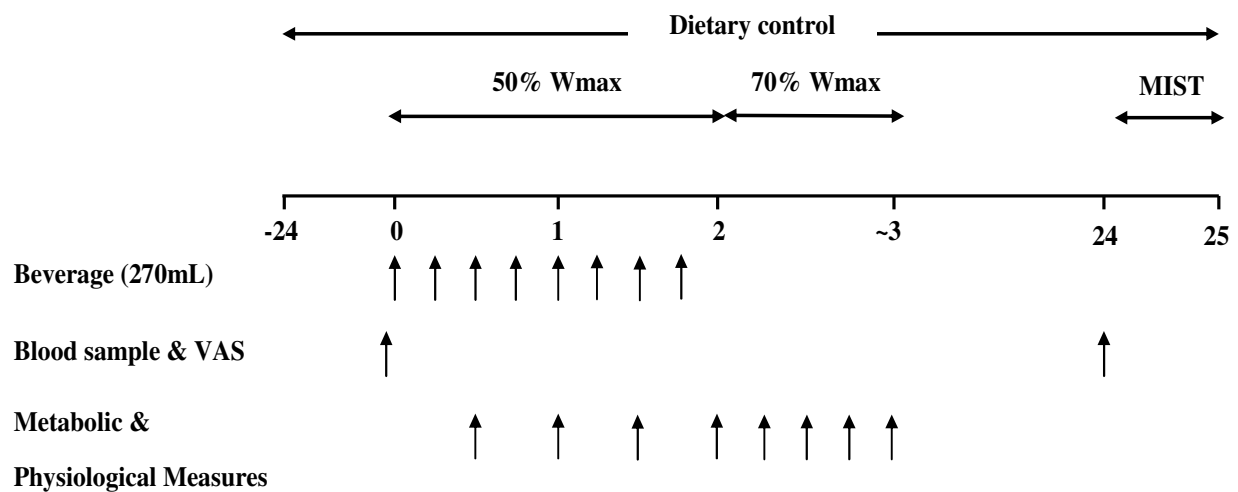
Figure 2.1 Schematic diagram of the experimental protocol. Metabolic measures include $\dot{V}O_2$, $\dot{V}CO_2$ and RER. Physiological measures include HR, RPE and average power output. Time values are in hours.

2.3.6   Diet & Exercise Control

Participant diet was standardized for the 48 h duration of each treatment. This ensured that nutrient intake was controlled for 24 h prior to each time-trial and over the 24 h course of each time-trial day, prior to the assessment of muscle function the following morning. In-between baseline testing and each blinded trial, participants completed a 3-day food diary, representative of their average week. A questionnaire of food preferences was also completed by participants. Using an on-line diet planner, each of the 3-days was logged and the energy and nutrient intake estimated. An average of the 3-days was combined with the participant's weight to calculate the total energy content of the diet. The standardized diet was matched to each participants habitual daily energy intake and contained $8g \cdot kg^{-1}$ BM carbohydrate and $1.6g \cdot kg^{-1}$ BM protein with the remainder of energy derived from fat. The macronutrient composition of the standardized diet was not significantly different from participants' habitual diet (Table 2.2). Participants were instructed to consume only the food provided for

them over the two-day testing period. The same two-day food parcel was provided to each

participant prior to the second trial. Participants were instructed to maintain normal volume

and intensity of training throughout the course of the study but to refrain from training for 48

h prior to each testing phase.

2.3.7   Statistical Analysis

Data are reported as mean ± SEM, unless otherwise stated. All data were analyzed by

two-way analysis of variance for repeated measures (Group x Time). Significant differences

between means were determined using SPSS (Version 15.)   Bonferroni adjustment was

applied to post hoc means-comparison tests. Differences were considered significant at $P <$

0.05.   In keeping with recent trends in inferential statistics, we made magnitude based

inferences about the 'true' values of the effect of treatment on outcomes by expressing the

uncertainty as 90% confidence limits (CL) and by calculating and interpreting chances that

the true effect was beneficial or detrimental. For time-trial performance data a substantial

effect was derived from 0.5 times (11) the average of estimates for the typical error of

measurement for time-trial performance and power output. For all other biochemical and

psychometric variables, the smallest standardized (Cohen) difference in the mean (0.20 times

the between-subjects standard deviation for the control group) was used to identify the

magnitude of the smallest substantive effect. Based on an analysis of the confidence limits

and P values using a published spreadsheet (2), the likelihood of a substantial benefit or

detriment, increase or decrease, for an outcome was qualified as follows: <1%, almost

certainly not; 1–5%, very unlikely; 5–25%, unlikely; 25–75%, possible; 75–95%, likely; 95–

99%, very likely; and >99%, almost certain (9); otherwise, an effect was deemed unclear or

inconclusive if the confidence interval overlapped the thresholds for positive and negative

substantial effects by >5%. When the mean and >50% of the confidence interval lie within

the threshold for a substantial effect, the effect is qualified as trivial. *P* values were provided for an inferential comparison for the main experimental outcomes only.

2.4 Results

2.4.1 Time-trial performance

Mean power output during the time-trial was not significantly different between treatments (247 ± 11 W for CHO and 247 ± 13 W for CHO+Pro, respectively). Power output was reduced at 75%, compared with 25 and 50% of time-trial completion (Figure 2.2); however, average power outputs at these time-points were not different between treatments. As a result there was also no significant difference in time-to-complete the time-trial for CHO or CHO+Pro (Figure 2.3). Inference based statistics determined the effect of CHO+Pro compared with CHO on time-to-complete the trial was unclear (*P* = 0.81). Heart rate and RPE were significantly elevated at completion of time-trial target compared with 25, 50 and 75% completion (*P* < 0.001) with no between-group differences at any time-point between CHO and CHO+Pro (Table 2.3).

2.4.2 Steady-state exercise

There was no between-group difference in heart rate and $\dot{V}O_2$ at 0, 30, 60, 90 and 120 min of the 120 min pre-load cycle. Average HR for CHO and CHO+Pro was 141 ± 12 and 142 ± 12 bpm respectively (Table 2.3). Average $\dot{V}O_2$ (34.9 ± 3.7 mL·kg^{-1}·min^{-1} for CHO and 34.3 ± 4.2 mL·kg^{-1}·min^{-1} for CHO+Pro) and RER (0.88 ± 0.01 for both) were not different between treatments at any time-point. Metabolic data indicated participants were cycling at ~55% $\dot{V}O_2$max. Throughout the steady state ride RPE was not significantly different between treatments (Between-subject range 10 – 15).

Table 2.2 Comparison of habitual and standardized diet

		Carbohydrate	Protein	Fat
Habitual diet	**(g)**	570.3 ± 41.9	104.8 ± 12	74.8 ± 41.2
	(g·kg⁻¹ BM)	8.1 ± 0.5	1.5 ± 0.1	1.1 ± 0.6
Standardized diet	**(g)**	565.2 ± 39.4	113.1 ± 7.8	73.4 ± 35.6
	(g·kg⁻¹ BM)	8.0 ± 0	1.6 ± 0	1.0 ± 0.5

Participant daily habitual energy intake was 3373.3 ± 349.1 kcal. Habitual energy intake was maintained during standardized dietary control. Macronutrient intake ($g·kg^{-1}$ BM) was not significantly different between habitual and standardized diets ($P < 0.05$). Values are presented as means ± SD.

Table 2.3 Physiological data at 25, 50, 75, and 100% of time-trial completion.

	Heart-rate (bpm)		Rate of perceived exertion (6-20)	
Trial Completion	**CHO**	**CHO+Pro**	**CHO**	**CHO+Pro**
25%	165 ± 3	166.8 ± 2.6	16.3 ± 0.5	15.8 ± 0.8
50%	167± 4	168.1 ± 2.8	17.5 ± 0.7	16.6 ± 0.7
75%	165 ± 3	169.2 ± 2.3	18.0 ± 0.7	17.2 ± 0.7
100%	[a] 180 ± 3	[a] 180.6 ± 2.1	[a] 19.4 ± 0.6	[a] 19.2 ± 0.8

*: Significant increase compared with 25, 50 and 75% completion time-points ($P < 0.001$) indicated by *a*. Data are presented as means ± SEM.

2.4.3   *Isometric knee extensor strength*

A significant time effect revealed that knee extensor MIST declined 24 h after the onset of each trial compared with baseline MIST (15 ± 3% for CHO and 11 ± 3% for CHO+Pro; *P*

< 0.002); with no significant between-group difference (Figure 2.4).   Inference based
statistics revealed the effect of CHO+Pro over CHO on the post-exercise recovery of knee
extensor MIST was most likely trivial ($P = 0.28$).

2.4.4   Visual analogue scale of muscle soreness

A significant time effect ($P < 0.05$) revealed VAS soreness increased from 4.8 ± 1.2 to
14.3 ± 4.3mm for CHO and 5.9 ± 1.9 to 14.3 ± 4.8mm for CHO+Pro, 24 h after the onset of
each trial (Figure 2.5). No between-group difference in VAS soreness was evident at 24 h
post-exercise. Inference based statistics indicated no clear difference in the extent of VAS
muscle soreness between-groups ($P = 0.91$).

2.4.5   Plasma creatine kinase concentration

A significant time interaction ($P < 0.002$) revealed plasma CK was significantly
increased for CHO and CHO+Pro at 24 h post-exercise (Figure 2.6).   No between-group
difference in plasma CK concentrations was evident at 24 h post-exercise ($P > 0.05$).
Inference based statistics revealed the effect of CHO+Pro compared with CHO on post-
exercise CK concentrations was most likely trivial ($P = 0.53$).

2.4.6   Treatment Blinding

Seven of the 12 participants completed the time-trial more quickly during their first
treatment visit, suggesting there was no learning effect between trials. Five of the 12
participants correctly identified the beverage order during testing. Of the 5 participants who
identified the beverages correctly, only 2 performed faster with CHO+Pro suggesting our
attempts to minimize treatment bias were successful. Seven of the 12 participants, performed

better when consuming a CHO beverage, with the remaining 5 completing the task more quickly with CHO+Pro.
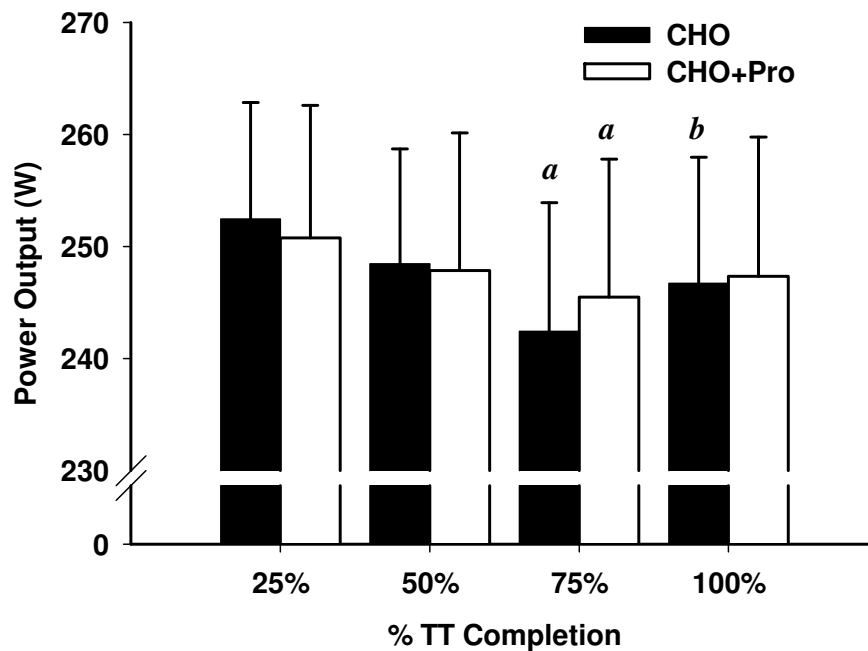
Figure 2.2 Average power output measured at quarterly completion of time-trial target. *a,* indicates significant reduction in power output vs. all prior time-points. *b,* indicates significant increase in power output vs. 75% completion ($P < 0.05$). Data are presented as means ± SEM.
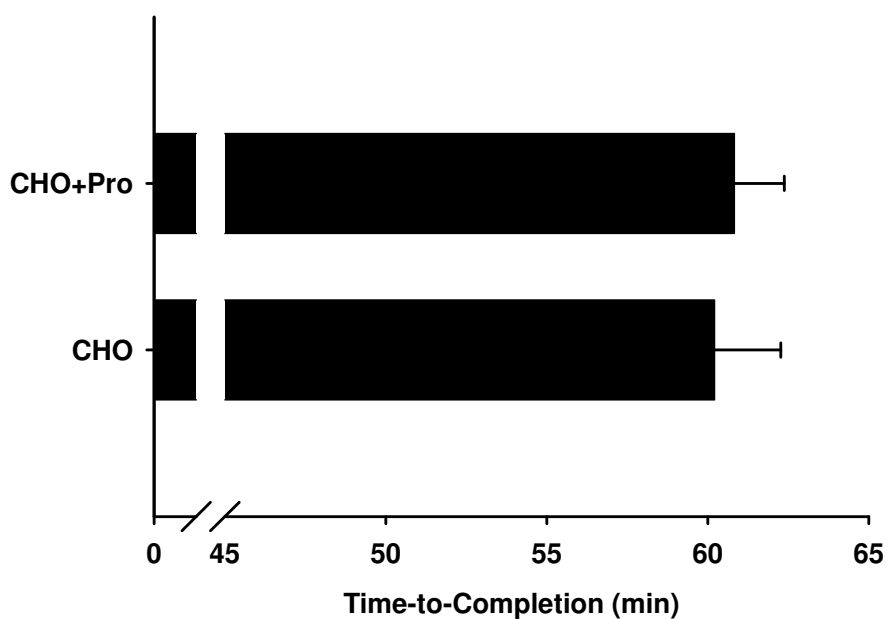
Figure 2.3 Time-trial time-to-completion. Data are presented as means ± SEM.
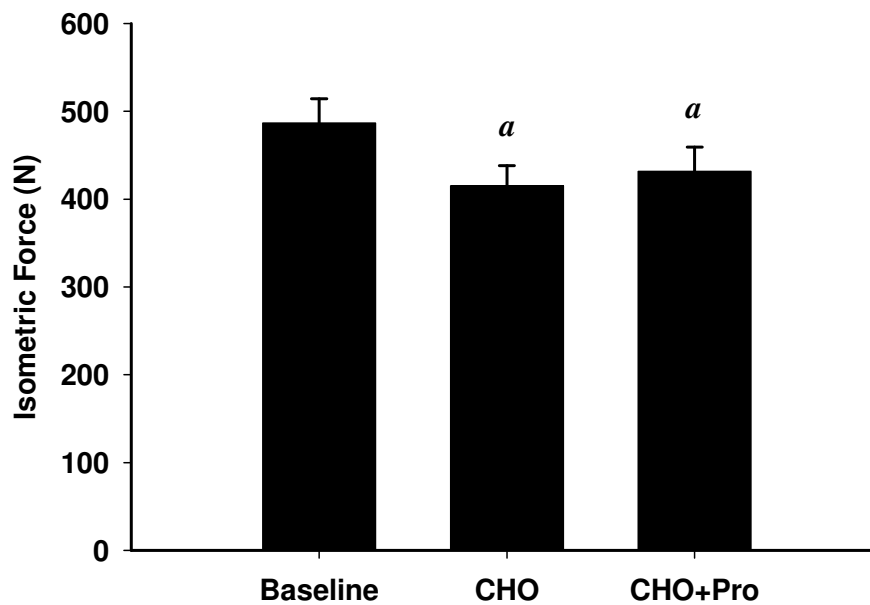
Figure 2.4 Maximum isometric force production of the knee extensors at baseline and 24 h post-exercise. *a,* indicates significant reduction from baseline (*P* < 0.05). Data are presented as means ± SEM.
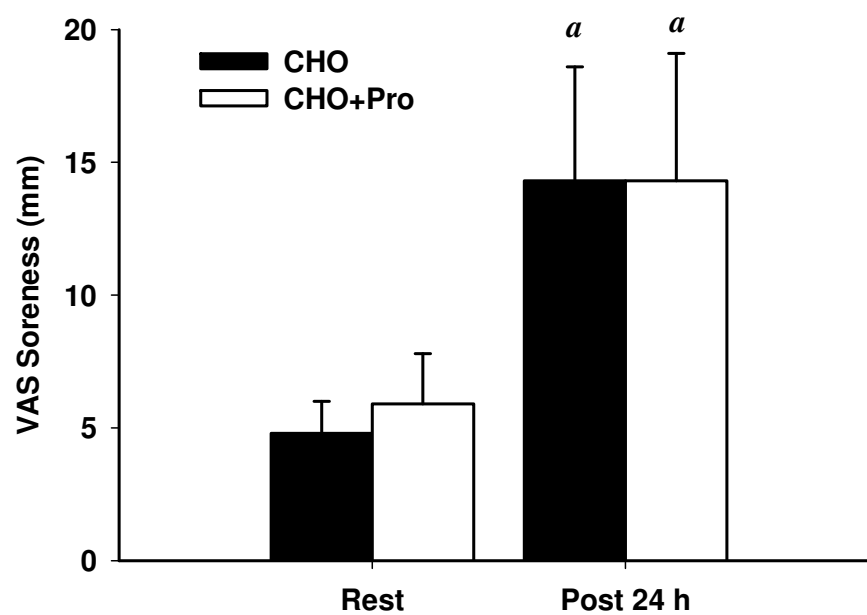
Figure 2.5 Average visual analogue scale of perceived muscle soreness at rest and 24 h post exercise. Muscle soreness scored on a scale of 0 (no soreness) to 50 (extreme soreness). *a,* indicates significant increase from rest (*P* < 0.05). Data are presented as means ± SEM.
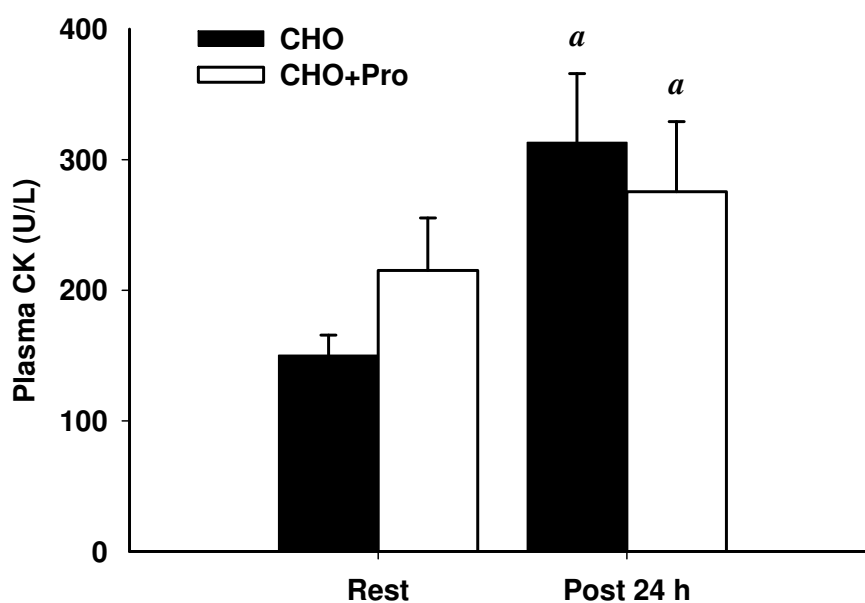
Figure 2.6 Plasma creatine kinase (CK) concentration at rest and 24 h post-exercise. *a,* indicates significant increase from rest ($P < 0.05$). Data are presented as means ± SE.

2.5 Discussion

The present study is the first to our knowledge, to determine the effect of a CHO+Pro beverage on late-stage cycle time-trial performance and post-exercise indices of recovery. Here we show that the addition of protein to a moderate carbohydrate dose did not improve late-exercise time-trial performance when beverages were consumed during a steady-state pre-load cycle. Furthermore, there were no differences in power output between treatments at any stage of the time-trial. Finally, the addition of protein to a carbohydrate containing beverage did not improve any of the measured indices of post-exercise recovery. Thus, our results do not support the notion that carbohydrate-protein co-ingestion during exercise enhances late-exercise performance or post-exercise recovery over to carbohydrate alone.

In contrast to the present findings, several investigations suggest that the addition of protein to carbohydrate improves endurance performance (13, 30-32). In many cases the

discrepancies between studies may be due to differences in the exercise test used to assess performance. Studies suggesting improved performance actually measured time-to-exhaustion (13, 30, 31), not performance *per se*. Currell and Jeukendrup (9) highlighted the fact that the ecological validity of exhaustive exercise protocols is limited, because endurance athletes do not compete in events that require sustaining a fixed power output for as long as possible. Furthermore, the precision of time-to-exhaustion is reportedly in the range of 26% (15). On the contrary, time-trial cycle tests have been found to be highly reproducible and sensitive to small changes in exercise performance (15, 18, 26). Our observation of no improvement in time-trial performance for CHO+Pro is consistent with other studies measuring overall time-trial performance (25, 35). It should be noted that the final measurement of performance is influenced not only by the exercise test, but by control measures employed and subject feedback during exercise. Knowledge of parameters such as time elapsed; distanced travelled and heart rate during exercise, may compromise the blinding of treatments, creating a placebo effect (23), particularly when additional protein is consumed which is often difficult to mask. Early studies did not report the control of these conditions (13, 28, 30-32, 34). Further to the well controlled study protocol we used, participant diet was assessed and standardized, whilst physical activity was minimized to ensure participants were in a similar state of energy balance and fuel repletion prior to and during each treatment trial. Thus, when performance, rather than endurance capacity is measured in a controlled environment, additional protein does not seem to be advantageous for performance.

Recently, the importance of additional protein on more specific aspects of endurance performance has been touted. In particular, the notion that additional protein is important for enhancement of performance late in exercise has been put forward (32). Despite no difference in 60km time-trial performance between CHO and CHO+Pro treatments, ingestion of

additional protein resulted in improved performance during the final stages of the time-trial (32). Whereas this result can be interpreted as important for endurance athletes, it should be emphasized that overall performance was not improved, thus minimizing the importance of any late exercise improvement. Given that overall performance was not different, the shorter time taken to complete late-exercise stages with CHO+Pro may have been due to a slower completion of earlier exercise stages, or greater variability in the earlier section of the time-trial relative to more reliable late-exercise performance. Whereas, these results contrast with our findings, the differences may be due to the fact that Saunders and colleagues (32) did not design their study to specifically examine the impact of additional protein on late-stage performance. The pre-loaded cycle time-trial used in the present study was designed to record power output and determine late-exercise time-trial performance and has been validated in previous studies from our laboratory (9, 15). In support of our findings, a recent study by Osterberg and colleagues (25) found no enhancement of late-exercise time-trial performance preceded by a 120 min steady-state ride, when protein was added to carbohydrate. Accurate determination of late-exercise performance requires that the exercise intensity of the prior bout should be standardized and power output reported. Thus, it seems that when properly controlled, late-stage exercise performance is not improved by the addition or protein to carbohydrate during exercise.

Furthermore, lack of a definitive physiological mechanism also contributes to doubt of the importance of adding protein to carbohydrate during exercise. Several mechanisms have been theorized (29), but only one has any support in studies. Koopman and colleagues (17) showed that ingesting CHO+Pro during ultra-endurance exercise resulted in two-fold greater protein oxidation compared with CHO. Thus, studies in which extended time-to-exhaustion was found, suggest that the addition of protein may alter substrate utilization, potentially sparing muscle glycogen (13, 30, 31). However, carbohydrate intake in these studies was too

low ($<$50 g·h^{-1}) to attain peak exogenous oxidation rates (16) and the protein plus carbohydrate provided more energy than the carbohydrate alone. Investigations in which the total energy content of CHO and CHO+Pro beverages was matched, have found no difference in time-to-exhaustion (28, 34). Thus, the benefit of adding protein to carbohydrate likely is due to the additional energy delivered by protein as opposed to a protein *per se*. We show the addition of protein (19 g·h^{-1}) to a carbohydrate dose (65 g·h^{-1}) that meets the recommended upper-limit to attain peak exogenous carbohydrate oxidation rates, did not improve late-exercise time-trial performance despite increasing the total beverage energy load by 29%. To our knowledge, no other purported mechanism for the enhancement of performance by additional protein has been demonstrated at this time.

In addition to the enhancement of endurance performance, adding protein to carbohydrate has been promoted to enhance recovery. Previous studies have shown CHO+Pro beverages prolong subsequent time-to-exhaustion in the range of 40 and 55% (30, 33, 37). Others have found the addition of protein to carbohydrate results in small increases in the recovery of muscle function, on the order of 1-2 knee extension lifts (34) and 1-2cm in vertical jump height (34). Whereas these data suggest the addition of protein to carbohydrate improves the recovery of performance and function, there are many factors that differ between studies, thus the picture is less than clear. It is difficult to determine whether these benefits were influenced by the timing of beverage ingestion (prior to, during, or post-exercise), the way in which recovery was measured, or the time given between exercise and the assessment of recovery. We chose to measure maximal isometric strength at 24 h post-exercise because of its practical relevance (1). Athletes often repeat their training or compete 24 hours after an exercise bout and therefore recovery of muscle function and exercise performance would be important in this time frame. In concert with two recent studies (5, 10), we showed that the co-ingestion of carbohydrate and protein, does not improve the

recovery of isometric strength. Others have failed to show improvements in recovery as based on improved subsequent performance assessed in various methods (4, 10, 20, 21, 28) . Thus, the impact of adding protein to carbohydrate on recovery from intense exercise must still be considered to be somewhat equivocal.

Studies that report improvements in the recovery of endurance capacity typically measured these changes several hours following protein ingestion (30, 37). The ingestion of protein is known to stimulate muscle protein synthesis (12) resulting in positive net whole-body protein balance following endurance exercise (19), which has been suggested to enhance recovery through the repair and remodeling of damaged proteins (20, 30, 31). However, the turnover of myofibrillar proteins is relatively slow, on order of days to weeks (27). Thus, it is difficult to comprehend how this process may influence changes noted in just a few hours (30, 37). So, if recovery is enhanced by adding protein to carbohydrate, it is unlikely to be due to the improved net muscle protein balance from the protein.

Whereas recovery may be defined by the ability to reproduce an optimal level of performance, several investigations have sought to measure the effect of CHO+Pro on the other markers thought to be important to the recovery process. Studies have shown that CHO+Pro beverages reduce plasma CK, a putative marker of sarcolemmal disruption and muscle soreness, (20, 21, 28, 31, 32). Others have found that lower CK concentrations and lower ratings of muscle soreness accompany improvements in the recovery of time-to exhaustion (30, 33) and vertical jump height (33). Consequently, previous studies suggest that protein co-ingestion may play an important role in reducing plasma CK and muscle soreness, thus improving recovery. However, the changes in plasma CK when protein is added to carbohydrate are often minimal (20, 28, 31, 32, 34) and the inter-individual variation is very large, making it difficult to determine the physiological significance. Moreover, the plasma CK and muscle soreness values found in our study are in a similar range to those

reported previously, yet we show that CHO+Pro did not ameliorate post-exercise markers of sarcolemmal disruption. A recent study from Betts et al. (5) used a strenuous bout of intermittent shuttle running to induce a greater degree of sarcolemmal disruption than the cycle time-trial used in our study. In support of our findings, the authors showed that the addition of protein to a carbohydrate beverage did not ameliorate post-exercise plasma CK and muscle soreness. Thus, it is not clear what impact, if any, ingestion of protein has on recovery by ameliorating CK and soreness.

It is likely that the importance of plasma CK and muscle soreness for enhancement of recovery has been over emphasized by studies that suggest the addition of protein to carbohydrate attenuates these responses (20, 21, 28, 31, 32). At best, plasma CK and muscle soreness should be considered putative markers of muscle damage. Studies have shown that these indirect markers have a poor relationship with the loss of muscle function (24) and direct markers of sarcolemmal disruption (3, 36). Moreover, 'true' treatment effects may be masked by the inherent variability of the CK response (5, 24, 36). Further to this suggestion, three participants in our study exhibited three-fold greater resting plasma CK response compared with the group average when protein was co-ingested. Resting CK concentrations for these same individuals were vastly reduced during the carbohydrate-only trial. Thus, our results suggest a clear need to further investigate the impact of protein ingestion on recovery from endurance exercise. It would seem that studying direct markers of sarcolemmal disruption (biopsy or MRI techniques) in concert with post-exercise tests of muscle function may provide clearer answers regarding the efficacy of CHO+Pro for improving recovery.

In conclusion, when energy intake is controlled and carbohydrate is ingested at rates considered optimal for peak exogenous carbohydrate oxidation, the addition of protein does not improve late-exercise cycle time-trial performance or recovery of muscle function at 24 h post-exercise. The metabolic and physiological response to CHO+Pro may be dependent on

the mode, duration and intensity of the exercise bout and warrants further study. Based on our findings and other carefully controlled studies (25, 35) there is currently no basis to recommend CHO+Pro beverages to endurance athletes for performance enhancement or improved recovery.

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Conflict of Interest

There are no conflicts of interest for any of the authors. The results of the present study do not constitute endorsement by ACSM.

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CHAPTER 3

THE INFLUENCE OF PROTEIN INGESTION FOLLOWING ENDURANCE EXERCISE ON MYOFIBRILLAR AND MITOCHONDRIAL PROTEIN SYNTHESIS.

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3.1 Abstract

Purpose: The aim of the present study was to determine mitochondrial and myofibrillar muscle protein synthesis (MPS) when carbohydrate (CHO) or carbohydrate plus protein (C+P) beverages were ingested following prolonged cycling exercise. The intracellular mechanisms thought to regulate MPS were also determined. **Method:** In a single-blind, cross-over study, 10 trained cyclists (age 29 ± 6 yr, $\dot{V}O_2max$ 66.5 ± 5 mL·kg^{-1}·min^{-1}) completed two trials in a randomized order. Subjects cycled for 90 min at ~77 ± 1% $\dot{V}O_2max$ before ingesting a CHO (25g of carbohydrate) or C+P (25g carbohydrate + 10g whey protein) beverage immediately and 30 min post-exercise. A primed constant infusion of L-[$ring$-$^{13}C_6$] phenylalanine began 1.5 h prior to exercise and continued until 4 h post-exercise. Muscle biopsy samples were obtained to determine myofibrillar and mitochondrial MPS and the phosphorylation of intracellular signalling proteins. Arterialized blood samples were obtained throughout the protocol. **Results:** Plasma amino acid, plasma urea and serum insulin concentrations increased following ingestion of C+P only. Myofibrillar protein synthesis was greater for C+P compared with CHO (0.087 ± 0.007 and 0.057 ± 0.006 %·h^{-1}, respectively; $P = 0.025$). Mitochondrial protein synthesis rates were similar for C+P and CHO. mTOR^{Ser2448} phosphorylation was greater for C+P compared with CHO at 4 h post-exercise ($P < 0.05$). p70S6K^{Thr389} phosphorylation increased at 4 h post-exercise for C+P ($P < 0.05$), whilst eEF2^{Thr56} phosphorylation increased by ~40% at 4 h post-exercise for CHO only ($P < 0.01$). **Conclusions:** The present study demonstrates that the ingestion of C+P following prolonged cycling stimulates an increase in myofibrillar MPS. These data indicate that the increase in myofibrillar MPS for C+P may be mediated through the phosphorylation of p70S6K, downstream of mTOR, which in turn may release the inhibition of eEF2 on translation elongation.

Key words: cycling, skeletal muscle, protein turnover, training adaptation.

Abbreviations: EE, endurance exercise; RE, resistance exercise; MPS, muscle protein synthesis; C+P, carbohydrate plus protein; CHO, carbohydrate-only; $\dot{V}O_2$, oxygen uptake; mTOR, mammalian target of rapamycin; p70S6K, 70kDa S6 protein kinase; Akt, protein kinase B; 4E-BP1, eukaryotic initiation factor 4E binding protein 1; eEF2, eukaryotic elongation factor 2; PRAS40, proline-rich Akt substrate 40 kDa; p38 MAPK, p38 mitogen-activated protein kinase; AMPK, AMP-activated protein kinase.

3.2 Introduction

Endurance (EE) and resistance exercise (RE) training regimens result in divergent phenotypic adaptations. Whereas RE promotes muscle hypertrophy and an increase in contractile force output (20, 29), EE training is characterized by an expansion of oxidative capacity, brought about through an increase in the size and density of mitochondria (25, 47). At the metabolic level, the adaptation to exercise is determined by summing the acute (22, 35, 48) transcriptional (24, 42) and translational responses (54) to each exercise stimulus and the subsequent increase in synthesis of muscle proteins. Recently, Wilkinson and colleagues (54) showed the response of myofibrillar and mitochondrial muscle protein synthesis (MPS) were dependant on the type of exercise performed. Furthermore, the interaction of exercise and protein nutrition varies between different protein fractions (38). Thus, it is important to examine the response of different protein pools to exercise in order to determine whether a particular nutritional intervention is having the desired effect.

Whereas the response of mixed MPS to different types of exercise has been investigated (19, 41, 50), there is less information on the response of various proteins to exercise and nutrition. It is widely recognized that ingesting protein potentiates the anabolic effect of RE (45), seemingly due to the essential amino acid content (49). Recently, Moore *et al.* (38) showed a differential response of myofibrillar and sarcoplasmic proteins to RE and protein nutrition. However, very few studies have sought to determine the effect of protein ingestion on MPS following EE. Levenhagen *et al.* (34) showed that adding protein to a carbohydrate treatment (C+P) increased post-EE leg and whole-body protein synthesis and this increase in synthesis was associated with net protein gain. However, it was unclear whether the benefits observed were due to the protein *per se*, or an increase in total energy intake. To remedy this, Howarth and colleagues (27) showed that C+P ingestion increased

mixed MPS compared with CHO treatments matched for total energy and carbohydrate content. On the other hand, another recent study showed no increase in mixed MPS when C+P was ingested following EE (19). Importantly, none of these studies (19, 27, 34) have attempted to resolve the specific protein fractions contributing to changes in mixed MPS, or the mechanism accounting for the adaptations. Therefore, specifically investigating the acute response of mitochondrial or myofibrillar protein synthesis to C+P ingestion following EE may resolve this issue.

Acute changes in transcription and translation that occur following exercise regulate skeletal muscle protein turnover through a number of intracellular signalling proteins. The mammalian target of rapamycin (mTOR) is a key regulator of translational control, integrating environmental signals from nutrients and exercise to control cell growth (13). The activation of mTOR signalling leads to the phosphorylation of downstream targets involved in the mRNA translation initiation and elongation (6), e.g. p70 ribosomal protein S6 kinase-1 (p70S6K). In concert with an increase in mitochondrial MPS following EE, Wilkinson *et al.* (54) showed an increase in the phosphorylation of signalling proteins in the mTOR–p70S6K pathway. Furthermore, human (28) and rat studies (39) indicate that post-EE C+P ingestion increases the phosphorylation of intermediates in the mTOR-p70S6K pathway. To date, no study has characterized the response of signalling proteins to changes in myofibrillar and mitochondrial MPS following post-EE protein ingestion.

Thus, the primary aim of the present study was to determine whether ingesting additional protein with carbohydrate following prolonged cycling exercise modified the synthetic response of mitochondrial and myofibrillar muscle proteins. The second aim of the study was to elucidate the potential intracellular signalling mechanisms (in the mTOR-

p70S6K pathway) regulating the differential response of myofibrillar and mitochondrial MPS following post-EE protein ingestion.

3.3 Methods

3.3.1 Participants

Ten well-trained, male cyclists were recruited from local clubs through advertisements. The mean (±SD) age of the cohort was 29 ± 6 yr, body mass was 77 ± 6.5 kg, maximal oxygen consumption was 66.5 ± 5 mL·kg^{-1}·min^{-1} and maximal power output was 383 ± 25 W. Only cyclists who undertook 2 or more training sessions per week of 1–5 h duration were eligible to participate. Participants had 6.5 ± 3 yr of competitive cycling experience. All tests were completed within a 4-week period with both treatment trials separated by 14 to 21-days, with the exception of one participant who completed the second trial 32-days after the first. The purpose and methodology of the study were clearly explained to the participants. All participants gave their informed consent prior to taking part in the study and were deemed healthy based on their response to a general health questionnaire. The experimental protocol was approved by the Black Country Research Ethics Committee (Rec No: 08/H1202/130).

3.3.2 Study Design

Participants reported to the laboratory on 3 separate occasions. During the first visit maximal aerobic fitness was determined. Approximately 2-weeks later participants performed the first blinded trial in which they consumed a carbohydrate (CHO) or carbohydrate-protein beverage (C+P; Lucozade Sport Recovery Powder, GlaxoSmithKline, Brentford, UK). Briefly, during each trial participants performed a 90 min high intensity, steady-state cycle before consuming CHO or C+P, immediately and 30 min following the exercise bout. Mitochondrial and myofibrillar MPS were measured by incorporating isotopic

tracer infusion and muscle biopsy techniques. Participants returned to the laboratory for the second blinded trial 14 to 21-days after the first trial, thereby serving as their own control. Trial order was randomized and counter-balanced.

3.3.3 *Preliminary Testing*

Body Mass: A digital scale was used to determine body mass to the nearest 0.1kg. Each participant was weighed in their cycling clothing without shoes on. Measurement of body mass was repeated prior to each of the two testing visits to ensure body mass remained constant throughout the study.

Maximal Power Output: Maximum oxygen uptake ($\dot{V}O_{2max}$) and maximal power output (Wmax) were determined using an incremental cycle test-to-exhaustion on an electrically braked cycle ergometer (LODE Excalibur Sport V 2.0 Groningen, Netherlands). The test consisted of a 3 min warm-up at 95 W, followed by an increase of 35 W every 3 min until exhaustion, at a self-selected cadence. Breath-by-breath measurements were taken throughout exercise, using an OxyCon Pro automated gas analysis system (Jaeger, Wuerzeburg, Germany). The gas analyzers were calibrated using a 4.95% CO_2 - 95.05% N_2 gas mixture (BOC Gases, Surrey, UK) and the volume transducer was calibrated with a 3L calibration syringe. Heart rate (HR) was measured continuously via telemetry using a HR monitor (Polar S625X; Polar Electro Oy, Kempele, Finland). $\dot{V}O_2$ was considered maximal if 3 of the 4 following conditions were met: 1) a plateau in $\dot{V}O_2$ with further increasing workloads (an increase of < 2 $mL \cdot kg^{-1} \cdot min^{-1}$); 2) a HR within 10 beats/min of the age predicted maximum (220 bpm - age); 3) a respiratory exchange ratio (RER) of >1.05; and 4) a rate of perceived exertion (RPE) greater than 17. The seat position, handlebar height and orientation used during baseline testing were recorded and replicated on subsequent visits to the laboratory.

All exercise bouts were conducted in thermo-neutral conditions (21°C, 40% relative humidity).

Dietary Analysis & Control: Participant diet was standardized for 48 h prior to each treatment. During the preliminary testing phase, participants completed a 3-day food diary, representative of their average week (2 weekdays and 1 weekend day). A questionnaire of food preferences was also completed by participants. Using an on-line diet planner (Weight Loss Resources), each of the 3-days was logged and energy and macronutrient intake was estimated. In our study cohort the average total daily energy intake was ~2787 ± 164 kcal (5.3 ± 0.4 g·kg^{-1} BM carbohydrate; 1.2 ± 0.1 g·kg^{-1} BM fat; 1.5 ± 0.2 g·kg^{-1} BM protein). The food parcels given to each participant matched their habitual energy and macronutrient intake. Participants were instructed to refrain from caffeine and alcohol and to consume only the food provided for them over the two-days prior to arriving for each trial. Participants were also asked to consume their final meal no later than 2200 h to ensure a 10 h fast prior to measuring myofibrillar and mitochondrial protein synthesis. An identical two-day food parcel was provided to each participant prior to the second trial.

Physical activity control: Participants were instructed to maintain their normal training volume and intensity throughout the course of the study but to refrain from training for 48 h prior to each treatment trial. To monitor physical activity between trials, participants were asked to record all training 7-days prior to each trial. No differences were noted in training volume in the 7-days prior to each trial.

3.3.4   *Experimental trial protocol*

Each participant was instructed to arrive at the Human Performance Laboratory at 0630 h after an overnight fast, where standard measures of height and weight were taken. A cannula was placed into the forearm vein of one arm and a hand vein of the other. The forearm cannula was used to infuse a stable isotopic tracer whilst the hand vein was heated for frequent arterialized blood sampling. After a resting blood sample had been obtained, participants then received a primed constant infusion of L-[*ring*-$^{13}C_6$] phenylalanine (prime: 2 $\mu mol \cdot kg^{-1}$; infusion: 0.05 $\mu mol \cdot kg^{-1} \cdot min^{-1}$; Cambridge Isotope Laboratories, MA, USA) to determine skeletal muscle protein kinetics (described below). Approximately 90 min after the start of the infusion, having rested in a supine position, participants were asked to complete a 90 min cycling exercise bout on a Lode Cycle Ergometer at a self selected cadence ≥ 60 revolutions per minute (rpm). The exercise bout consisted of a warm-up cycle at 50% Wmax (189 ± 4 W) for 10 min followed by 80 min, initially at 75% Wmax (283 ± 6 W). $\dot{V}O_2$, RER, HR and RPE were recorded over 25-30 min, 55-60 min and 85-90 min of the exercise bout (as described above). If participants indicated during exercise that the workload was too difficult and they were unlikely to complete the full 80 min, workload was lowered in 5% decrements to no less than 65% Wmax. Air conditioning and a fan were used when requested by participants. The exact settings of the air conditioning/fan and the time of any change in workload were recorded and replicated during the second trial. Throughout each trial participants were allowed to drink water *ad libitum*. The amount of water consumed throughout the course of the each trial was found to be similar (1556 ± 249mL for CHO and 1674 ± 233mL for C+P).

Muscle biopsy and blood sampling Using a 5-mm Bergstrom biopsy needle, two muscle biopsies (~100-150 mg of muscle tissue per biopsy) were obtained from the same leg during each trial. The order of biopsied leg was randomized and counterbalanced for each

trial. Prior to the exercise bout (~15min) under local anaesthetic (1% Lidocaine), the lateral portion of one thigh was prepared for the extraction of a needle biopsy sample from the *vastus lateralis* muscle. Biopsy incisions were made prior to exercise to allow the sample to be obtained as quickly as possible after exercise (5 ± 1 min post-exercise). Immediately after the post-exercise muscle biopsy was obtained, participants were asked to consume one of two treatment beverages described below. Four hours after consuming the treatment beverage, the second muscle biopsy was obtained (Figure 3.1). The second biopsy was taken ~1 cm proximal to the first biopsy. Biopsy samples were quickly rinsed, blotted and divided into two to three pieces, before being frozen in liquid nitrogen and stored at -80°C until later analysis. Arterialized blood samples from a heated hand vein were collected at rest, immediately post-exercise and every 15 min following beverage consumption for 2 h. Thereafter, blood samples were obtained at regular intervals for the remainder of the infusion. Blood was collected in EDTA-containing, lithium heparin-containing and serum separator tubes and spun at 3500rpm for 15 min at 4°C. Aliquots of plasma and serum were the frozen at -80°C until later analysis.
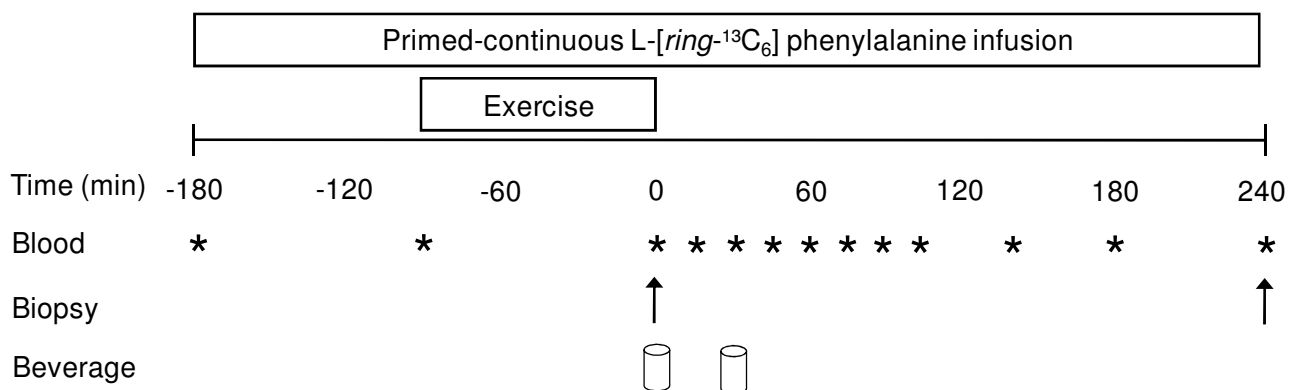
Figure 3.1 Schematic diagram of the experimental protocol.

Treatment beverages Immediately after the first muscle biopsy sample was obtained, subjects ingested either 25.2g of carbohydrate (CHO) or 25.4g of carbohydrate plus 10.2g of whey protein (C+P) dissolved in 250 mL of cold water (~11 ± 0 min post-exercise). A second identical beverage was consumed 30 min after the first beverage was finished. This dose regime provided a total carbohydrate and protein intake of 50.8g and 20.4g, respectively in C+P and a total carbohydrate intake of 50.3g in CHO. Participants were encouraged to consume the beverages within 2 min. Both CHO and C+P treatment beverages were matched for flavour (orange and passion fruit) and appearance. Beverages were administered to participants in a single-blinded manner, the order of which was randomized. Eight out of the ten participants correctly identified the order of the treatments. The amino acid content of the whey protein was (in percent content, wt:wt): Ala, 5.2; Arg, 2.2; Asp, 11.4; Cys, 2.3; Gln, 18.8; Gly, 1.5; His, 1.8; Ile, 6.7; Leu, 11; Lys, 10; Met, 2.3; Phe, 3.1; Pro, 5.7; Ser, 4.8; Thr, 7; Trp, 1.5; Tyr, 2.7; and Val, 6.1. A small amount of L-[*ring*-^{13}C$_6$] phenylalanine tracer was added to the C+P drink (6% of phenylalanine content) in order to minimize changes in blood phenylalanine enrichment after drink ingestion.

3.3.5   Analyses

Blood analyses: Plasma glucose, plasma lactate and plasma urea concentrations were analyzed using an ILAB automated analyzer (Instrumentation Laboratory, Cheshire, UK). Serum insulin was analyzed using a commercially available ELISA kit (IBL International, Hamburg, Germany), following the manufacturer's instructions. Concentrations of phenylalanine, leucine, threonine and ^{13}C$_6$ phenylalanine tracer-to-tracee (t/T) enrichment were determined by Gas Chromatography Mass Spectrometry (GCMS) (model 5973; Hewlett Packard, Palo Alto, CA). On thawing, plasma samples were diluted 1:1 in acetic acid and purified on cation-exchange columns (Dowex 50W-X8-200, Sigma-Aldrich Poole, UK). The

amino acids were then converted to their N-tert-butyldimethyl-silyl-N-methyltrifluoracetamide (MTBSTFA) derivative. Plasma $^{13}C_6$ phenylalanine enrichment was determined by monitoring at ions 234/240. Appropriate corrections were made for any spectra that overlapped, contributing to the t/T ratio. Amino acid concentrations were determined using an internal standard method (49, 51), based on the known volume of blood and internal standard added. The internal standards used were U-[$^{13}C_9$-^{15}N] phenylalanine (50µmol/l), L-[$^{2}H_3$] leucine (120µmol/l) and L-[$^{2}H_4$] threonine (182µmol/l) added in a ratio of 100 µl/ml of blood. Leucine, threonine and phenylalanine concentrations were determined by monitoring at ions 302/308, 404/409 and 336/346, respectively.

Muscle tissue analyses: Muscle samples were analyzed for enrichment of L-[*ring*-$^{13}C_6$] phenylalanine in the intracellular pool and bound myofibrillar and mitochondrial protein fractions. Intracellular amino acids were liberated from ~10-20 mg of muscle. The tissue was powdered under liquid nitrogen using a mortar and pestle and 500 µl of 0.2M perchloric acid was added. The mixture was centrifuged at 10,000g for 10 min. The pH of the supernatant was then adjusted to 5–7 with 2M KOH and treated with 20µL of urease for removal of urea. The free amino acids from the intracellular pool were purified on cation-exchange columns (described above). Intracellular amino acids were converted to their MTBSTFA derivative and $^{13}C_6$ phenylalanine enrichment determined by monitoring at ions 234/240 (as described above).

Mitochondrial and myofibrillar protein isolation was achieved using a protocol adapted from Wilkinson *et al.* (54). Approximately 70-100mg of muscle tissue was homogenized in a 2mL Eppendorf with a Teflon pestle in 10µL·mg^{-1} of ice-cold homogenizing buffer (0.1 mM KCl, 50 mM Tris, 5 mM MgCl, 1 mM EDTA, 10 mM β-glycerophosphate, 50 mM NaF, 1.5% BSA, pH 7.5). The homogenate was spun at 1,000g for 10 min at 4ºC. The supernatant

was transferred to another Eppendorf tube and spun at 10,000g for 10 min at 4°C to pellet the sarcoplasmic mitochondria (SM). The supernatant was then removed and discarded. The pellet that remained from the original 1,000g spin was washed twice with homogenization buffer. A glass Dounce homogenizer and tight fitting glass pestle were used to forcefully homogenize the pellet in homogenization buffer to liberate intermyofibrillar mitochondria (IM). The resulting mixture of myofibrillar proteins (MYO) and IM was spun at 1,000g for 10 min at 4°C to pellet out the MYO. The supernatant was removed and spun at 10,000g for 10 min at 4° C to pellet the IM. The MYO, SM and IM pellets were washed twice with homogenizing buffer containing no BSA. The MYO fraction was separated from any collagen by dissolving in 0.3 M NaCl, removing the supernatant and precipitating the proteins with 1.0 M PCA. All samples were washed once with 95% ethanol. In order to determine $^{13}C_6$ phenylalanine enrichment in the mitochondrial protein fraction, IM and SM fractions were combined as per Wilkinson *et al.* (54). Mitochondrial and myofibrillar fractions were then hydrolysed overnight at 110 °C in 0.05 M HCl/Dowex 50WX8-200 (Sigma Ltd, Poole, UK) and the constituent amino acids purified on cation-exchange columns (Dowex 50W-X8-200, Sigma-Aldrich Poole, UK). The amino acids were then converted to their N-acetyl-n-propyl ester derivative. Phenylalanine labelling was determined by gas-chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS, Delta-plus XL, Thermofinnigan, Hemel Hempstead, UK) by monitoring at ions 336/342 for labelled and unlabelled CO_2. Unfortunately during processing 2 samples were lost for the mitochondrial fractions, therefore the data represent $n = 8$. No myofibrillar fractions were lost during processing ($n = 10$).

We assessed the purity of our protein fractions on muscle tissue collected during a pilot study. Western blots revealed a greater abundance of myosin heavy chain protein content in the myofibrillar fraction and a greater abundance of cytochrome c oxidase protein content in

the mitochondrial fraction. In addition, we measured citrate synthase (CS) activity in a mixed muscle homogenate and each of the IM and SM mitochondrial fractions during method development. We found that the CS activity was ~4-fold higher in the mitochondrial fractions than the mixed homogenate. Data for these experiments is presented in **Chapter 6** (sub-section 6.1) of this thesis.

Western Blots: The remaining muscle tissue (25-40mg) was powdered on dry ice under liquid nitrogen using a mortar and pestle. Approximately 20mg of powdered muscle was homegenized in lysis buffer (50mM Tris pH 7.5; 250mM Sucrose; 1mM EDTA; 1mM EGTA; 1% Triton X-100; 1mM NaVO4; 50mM NaF; 0.50% PIC), using a hand-held homogenizer (PRO200, UK). Samples were shaken at 4ºC for 30 min (12,000rpm), centrifuged for 5 min at 6,000g and the supernatant removed for protein determination. Protein concentration was determined using the DC protein assay (Bio Rad, Hertfordshire, UK). Equal aliquots of protein were boiled in Laemmli sample buffer (250mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 0.01% bromophenol blue; 5% β-mercaptoethanol) and separated on SDS polyacrylamide gels (10 – 12.5%) for 1 h at 58mA. Following electrophoresis; proteins were transferred to a Protran nitrocellulose membrane (Whatman, Dassel, Germany) at 100 V for 1 h. The membranes were incubated overnight at 4°C with the appropriate primary antibody. The primary antibodies used were AMPK^{Thr172} (Millipore 15-115), mTOR^{Ser2448} (Cell signalling 2976), S6K^{Thr389} (Cell signalling 9234), Akt^{Thr308} (Cell signalling 4056), 4E-BP1^{Thr37} (Santa Cruz SC6025), eEF2^{Thr56} (Cell signalling 2332), PRAS40^{Thr246} (Cell Signalling 2610) and p38 MAPK^{Thr180} (Cell Signalling 9212). The following morning the membrane was rinsed in wash buffer (TBS with 0.1% Tween-20) three times for 5 min. The membrane was then incubated for 1 h at room temperature within wash buffer containing the appropriate secondary antibody, either horseradish (HRP)-linked

anti-mouse IgG (New England Biolabs, 7072; 1:1,000) or anti-rabbit IgG (New England Biolabs, 7074; 1:1,000). The membrane was then cleared in wash buffer three times for 5 min. Antibody binding was detected using enhanced chemiluminescence (Millipore, Billerica, MA). Imaging and band quantification were carried out using a Chemi Genius Bioimaging Gel Doc System (Syngene, Cambridge, UK). Intracellular signalling targets were determined with $n = 8$ for CHO and C+P trials.

3.3.6   Calculations

The fractional synthetic rate (FSR) of mitochondrial and myofibrillar proteins were calculated using the standard precursor-product method:

$$FSR\ (\%\cdot h^{-1}) = \Delta E_b/E_p \times 1/t \times 100 \tag{1}$$

Where ΔE_b is the change in bound $^{13}C_6$ phenylalanine enrichment between two biopsy samples, E_p is the precursor enrichment and t is the time between muscle biopsies.

The true precursor enrichment would be the labelled phenylalanine-tRNA (1). However, measurement of this enrichment requires large amounts of tissue, which cannot typically be obtained from human volunteers. Thus, the intracellular (IC) free phenylalanine enrichment is commonly used in studies (27, 38, 49), primarily because it is considered a superior precursor surrogate. Unfortunately, due to technical difficulties, the IC enrichment was available for only 5 participants. Thus, we chose to use the plasma precursor to estimate the IC enrichment. A comparison of plasma and IC enrichments from the samples available ($n =5$) revealed the IC enrichment to be $70 \pm 2\%$ of the plasma (range 67-77%). Furthermore, a comprehensive examination of studies (for example; (2, 30, 46)) in which phenylalanine

tracers were used to determine FSR revealed the IC phenylalanine enrichment to be ~70% of the plasma enrichment. We chose not to use the plasma enrichment, *per se*, as the precursor for ease of comparison of the results to other previously published studies.

3.3.7   Statistical analysis

A within-subject repeated measures design was utilized for the current study. Exercise variables, blood analytes and Western blot data were analyzed using a two-way ANOVA with repeated measures (treatment x time) to determine differences between each treatment beverage across time. Myofibrillar and mitochondrial FSR data were analyzed using one-factor (treatment) repeated measures ANOVA. When a significant main effect or interaction was identified, data were subsequently analyzed using a Bonferroni post hoc test. All statistical tests were analyzed using statistical package for social sciences (SPSS) version 18.0 (Illinois, Chicago, U.S). Significance for all analyses was set at $P < 0.05$. All values are presented as means ± standard error of the mean (SEM).

3.4   Results

3.4.1   Exercise variables

There was no between-trial difference in heart rate, cadence, $\dot{V}O_2$ and RER measured at 25-30, 55-60 and 85-90 min of the steady state cycle (Table 3.1). Average HR over the 90 min cycle was 172 ± 3 and 172 ± 4 bpm for CHO and C+P, respectively. Average $\dot{V}O_2$ over 90 min (51.3 ± 1.6 mL·kg^{-1}·min^{-1} for CHO and 51.6 ± 1.2 mL·kg^{-1}·min^{-1} for C+P) and RER over 90 min (0.88 ± 0.01 for CHO and C+P) were not different between treatments. Metabolic data indicated participants were cycling at 77 ± 1% of $\dot{V}O_{2max}$ during CHO and C+P trials. Average RPE over 90 min was similar for CHO and C+P (16 ± 4 for CHO and C+P).

3.4.2   Blood analytes

Fasted blood glucose was 5.1 ± 0.3 mmol·L and 5.3 ± 0.3 mmol·L for CHO and C+P, respectively and remained similar immediately post-exercise. Approximately 30 min after consuming the first treatment beverage, plasma glucose concentration increased by ~32% and ~20% for CHO and C+P, respectively, with no significant difference between treatments. Plasma glucose concentration returned to basal values by 1.5 h post-exercise for CHO and C+P and was constant for the remainder of the infusion. Fasted serum insulin concentration was 6.0 ± 0.8 and 5.5 ± 0.5 μU·ml^{-1} for CHO and C+P, respectively (Figure 3.2A). Following drink ingestion, serum insulin concentration increased for both CHO and C+P, peaking at 30 min post-exercise ($P < 0.001$). Serum insulin increased to a greater extent for C+P (~285%) compared with CHO (~60%; $P < 0.001$). Serum insulin returned to basal values by 1.5 h post-exercise for CHO and C+P and remained constant until the end of the infusion. Following exercise, plasma lactate concentration increased 150% and 175% compared with resting values for CHO and C+P, respectively ($P < 0.001$), with no difference between treatments. Lactate concentration returned to basal values by 3 h post-exercise for CHO and C+P. Resting plasma urea concentration was similar for CHO and C+P and was stable immediately post-exercise (Figure 3.2B). Following the ingestion of C+P, plasma urea concentration increased by ~20% compared with resting values and remained elevated at 4 h post-exercise ($P < 0.05$). Plasma urea concentration remained unchanged for CHO compared with resting values. From 15 min to 4 h post-exercise, plasma urea concentration for C+P was significantly greater than CHO ($P < 0.05$).

3.4.3   Plasma amino acid concentrations

Prior to and immediately post-exercise, plasma amino acid concentrations of phenylalanine, leucine and threonine were similar for CHO and C+P (Figure 3.3). Following

the ingestion of C+P, plasma concentrations of phenylalanine, leucine and threonine increased by 37, 130 and 58%, respectively ($P < 0.001$) and peaked at 1 h post-exercise (30 min after the second drink was ingested), after which, amino acid concentrations returned to basal levels such that there were no differences between CHO and C+P at 4 h post-exercise. Following the ingestion of CHO, plasma phenylalanine and threonine concentrations did not change. Plasma leucine concentration was reduced following CHO ingestion ($P = 0.04$) returning to pre-exercise levels by 2 h post-exercise.

3.4.4   Plasma and intracellular $^{13}C_6$ phenylalanine enrichment

Plasma $^{13}C_6$ phenylalanine enrichment increased from immediately pre- to post-exercise but was stable across the time of tracer incorporation, immediately post-exercise to 4 h post-exercise for CHO and C+P ($7.2 \pm 0.03\%$ t/T for both; Figure 4). These data demonstrate that the additional tracer added to the C+P treatment beverage did not appear in the circulation more rapidly than the amino acids from the protein and that all measurements were made at isotopic equilibrium. Intracellular $^{13}C_6$ phenylalanine enrichment for an available sub-set of participants ($n = 5$) was stable across the time of tracer incorporation for CHO and C+P (4.8 ± 0.5 and $4.5 \pm 0.7\%$ t/T, respectively). Based on the relationship between plasma and intracellular enrichments, the mean predicted intracellular enrichment for the complete study cohort ($n = 10$) was 5.0 ± 0.1 and $5.1 \pm 0.2\%$ t/T for C+P and CHO, respectively. Trial order did not influence the tracer enrichment in plasma (7.0 ± 0.3 and $7.4 \pm 0.2\%$ t/T for trials 1 and 2, respectively; $P = 0.2$) or in the available intracellular samples (4.5 ± 0.4 and $4.8 \pm 0.3\%$ t/T for trials 1 and 2, respectively; $P = 0.2$).

3.4.5   Post-exercise protein phosphorylation

Immediately post-exercise, mTOR^{Ser2448} phosphorylation was similar for CHO and C+P. At 4 h post-exercise mTOR phosphorylation showed a tendency to increase for C+P (P = 0.08), whereas mTOR phosphorylation tended to decrease for CHO (P = 0.07). At 4 h post-exercise mTOR phosphorylation was greater for C+P compared with CHO (P = 0.02; Figure 3.5A). Immediately post-exercise, eEF2^{Thr56} phosphorylation was similar for CHO and C+P. At 4 h post-exercise phosphorylation increased 1.4-fold for CHO compared with immediately post-exercise (P = 0.02). Furthermore, eEF2 phosphorylation for CHO was greater than C+P at 4 h post-exercise (P = 0.04; Figure 3.5B). Immediately post-exercise, p38 MAPK^{Thr180} phosphorylation was similar for CHO and C+P. At 4 h post-exercise p38 MAPK phosphorylation was unchanged for CHO and tended to increase for C+P (P = 0.08) with no significant difference between treatments (Table 3.2). Immediately post-exercise, p70S6K^{Thr389} phosphorylation was similar for CHO and C+P. The phosphorylation of p70S6K increased at 4 h for C+P compared with immediately post-exercise (P = 0.05) but was not significantly different from CHO (Figure 3.5C). Immediately post-exercise, 4E-BP1^{Thr37} phosphorylation was similar for CHO and C+P. At 4 h post-exercise 4E-BP1 phosphorylation showed a tendency to increase for CHO and C+P (P = 0.09) with no difference between treatments (Table 3.2). There was no difference in the phosphorylation of AMPK^{Thr172}, Akt^{Thr308} and PRAS40^{Ser246} immediately post-exercise between CHO and C+P. Furthermore, the phosphorylation of AMPK, Akt and PRAS40 remained unchanged at 4 h post-exercise for CHO and C+P (Table 3.2).

3.4.6   *Mitochondrial and myofibrillar FSR*

Mitochondrial protein synthesis rates were similar for CHO and C+P (confidence interval [CI]: 0.06 - 0.12 and 0.06 - 0.10 %·h^{-1}, respectively; Figure 3.6). Myofibrillar protein synthesis rates were ~35% higher for C+P compared with CHO (CI: 0.07 - 0.11 and 0.05 -

0.07 %·h^{-1}, respectively; P = 0.025). Rates of mitochondrial and myofibrillar protein synthesis were 30 ± 0.9% lower when the unadjusted plasma precursor was used in the calculation of FSR. However, the statistical outcome was not different (data not presented).

Table 3.1 Exercise variables

Ex time (min)	CHO			C+P		
	25-30	**55-60**	**85-90**	**25-30**	**55-60**	**85-90**
HR (bpm)	172 ± 5	172 ± 3	172 ± 1	174 ± 4	172 ± 1	171 ± 2
Cadence (rpm)	$*84 \pm 5$	79 ± 5	80 ± 3	$*84 \pm 4$	80 ± 3	79 ± 5
$\dot{V}O_2$ **(mL·kg^{-1}·min^{-1})**	53.2 ± 2.0	50.7 ± 2.0	50.9 ± 2.0	52.6 ± 1.9	50.5 ± 1.9	50.9 ± 2.6
% $\dot{V}O_{2max}$	80 ± 3	77 ± 2	76 ± 5	79 ± 2	76 ± 2	77 ± 3
RER	0.89 ± 0.01	0.87 ± 0.02	0.87 ± 0.02	0.91 ± 0.01	0.87 ± 0.02	0.87 ± 0.01
RPE	16 ± 1	17 ± 1	17 ± 1	16 ± 1	17 ± 1	18 ± 1

Values are the average recording over each 5 min phase and are presented as mean ± SEM. * indicates significant difference at 25-30 min compared with other time phases ($P < 0.05$).

A

B
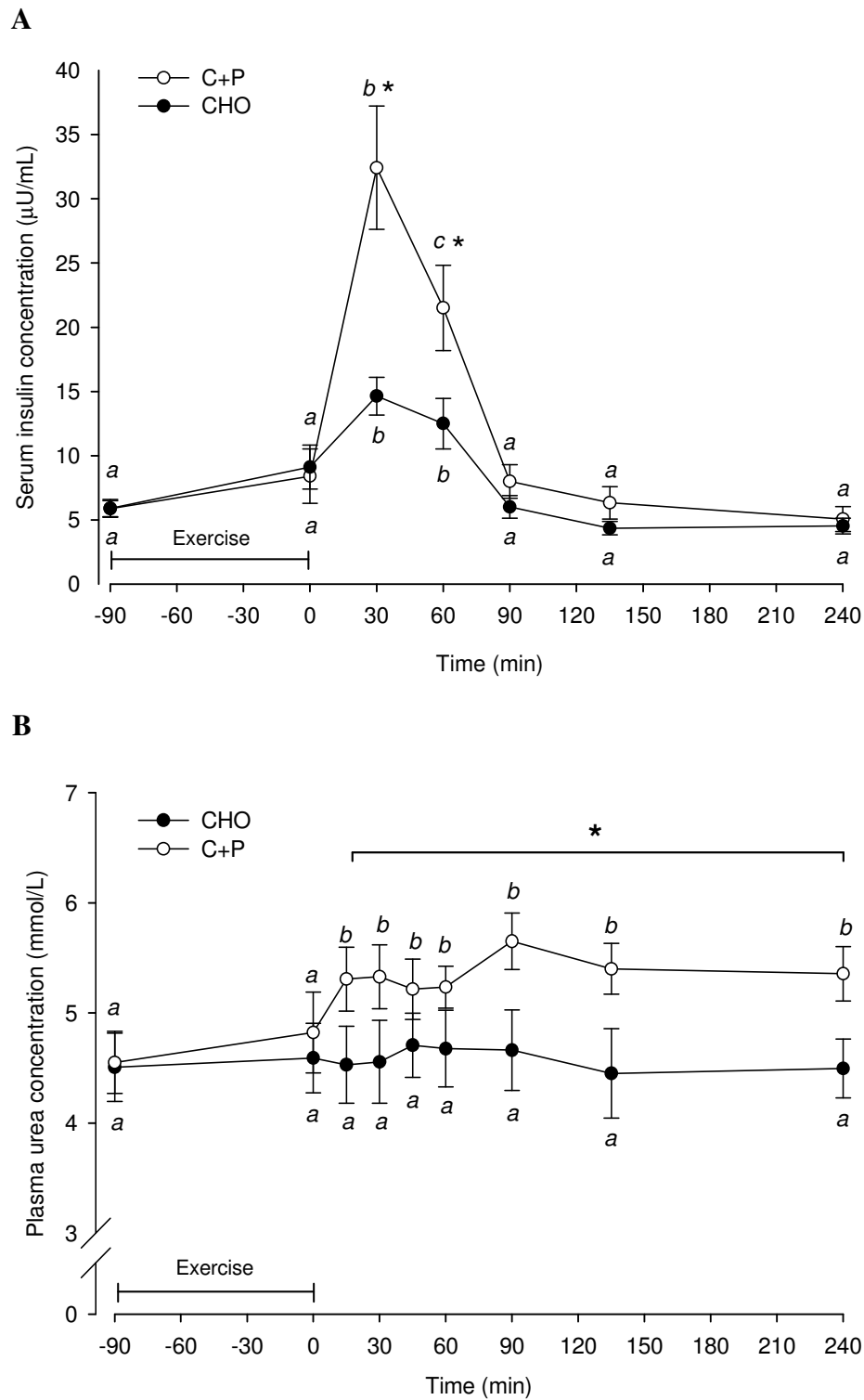
Figure 3.2 (A) Plasma urea and (B) serum insulin concentration in CHO and C+P trials. Means with different subscripts are significantly different from each other ($P < 0.05$). *: significant difference between CHO and C+P. Values are means ±SEM; $n = 10$.

A

B

C
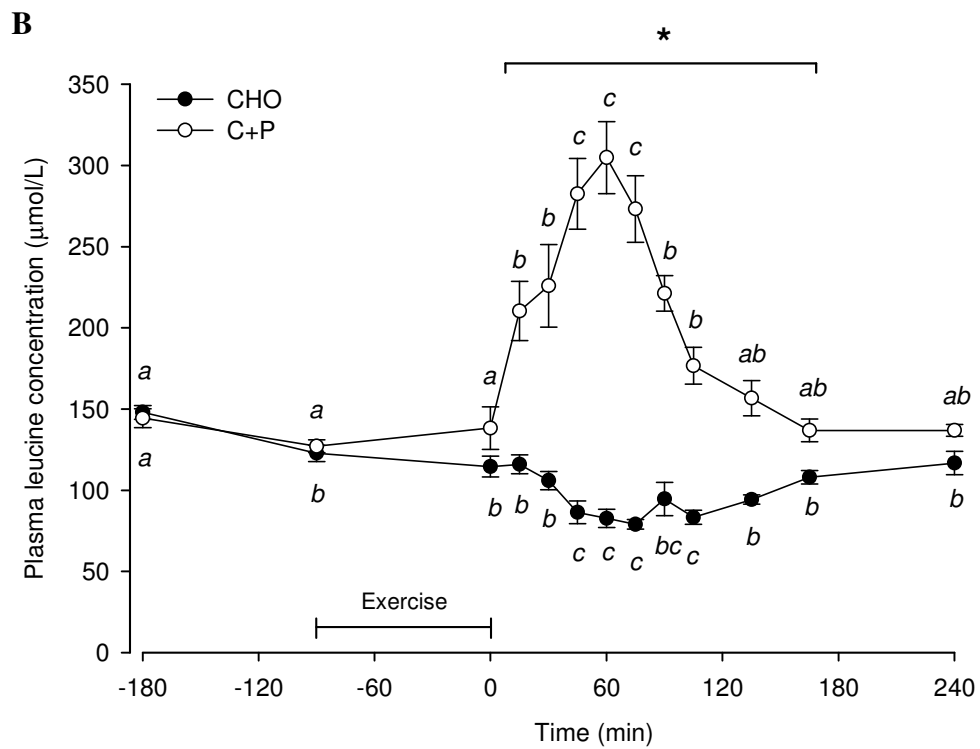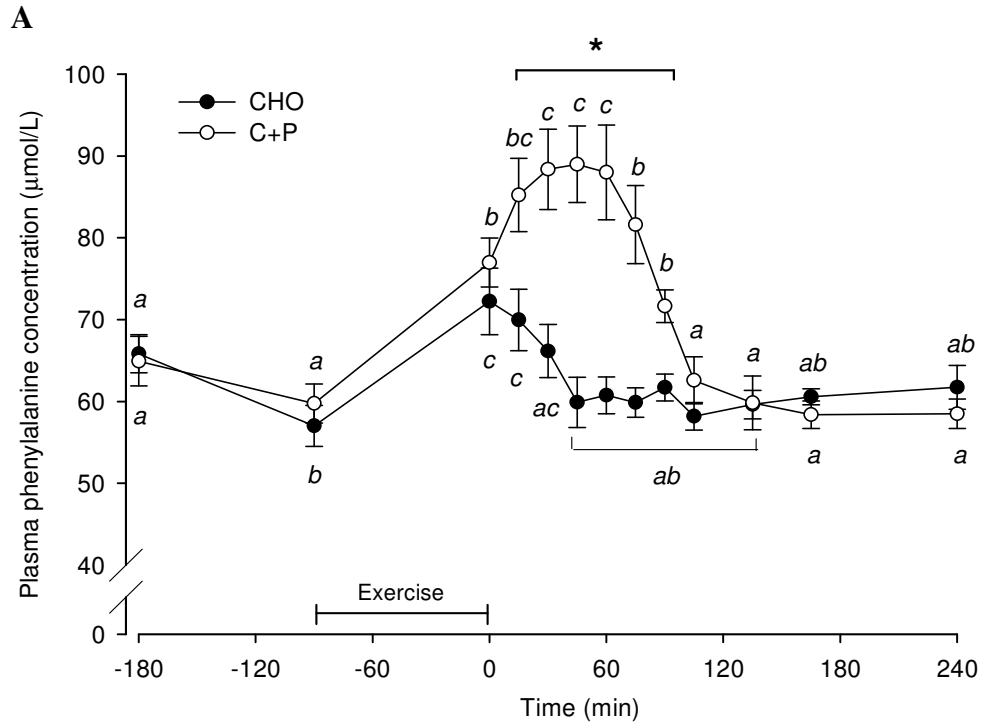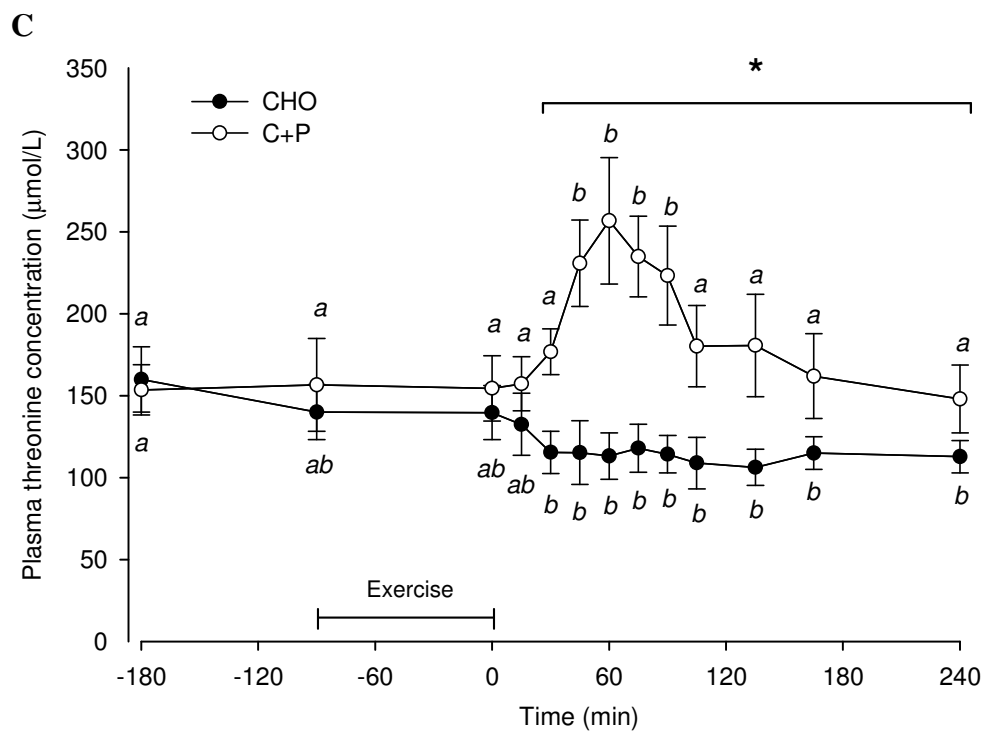
Figure 3.3 Plasma concentrations of (A) phenylalanine, (B) leucine and (C) threonine. Means with different subscripts are significantly different from each other. *: significant difference between CHO and C+P ($P < 0.05$). Values are means ±SEM; $n = 10$.
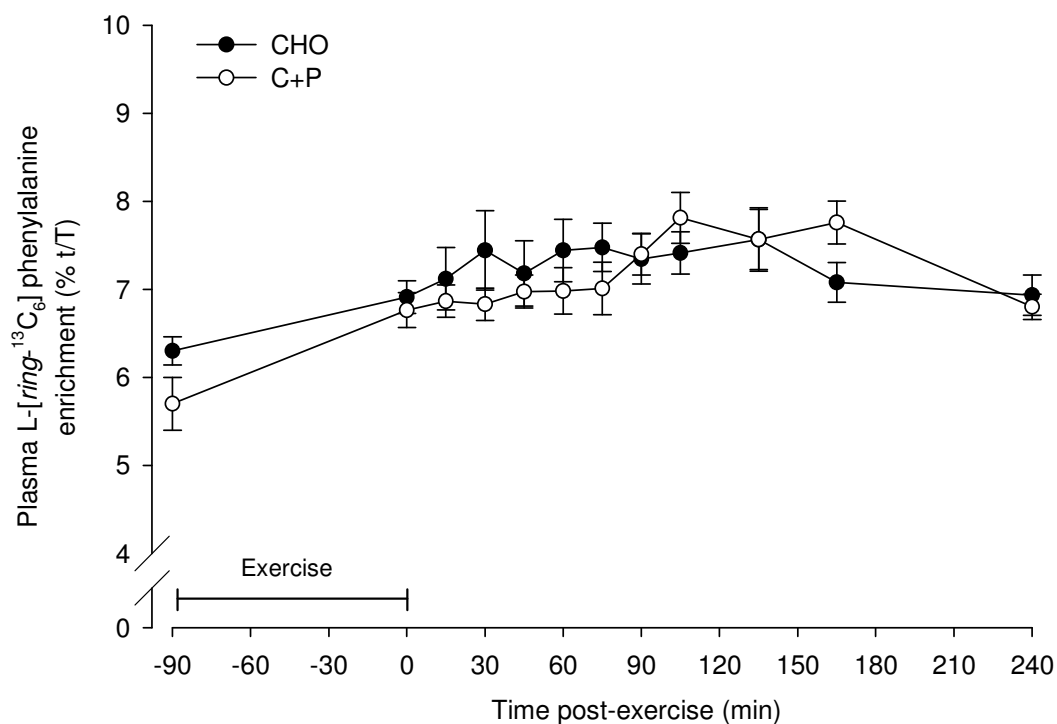
Figure 3.4 Enrichment of $^{13}C_6$ phenylalanine in plasma. % t/T: percentage of tracer-to-tracee ratio. Values are means ± SEM; $n = 10$.

Table 3.2 Post-exercise protein phosphorylation

Treatment	CHO	C+P
Time post-exercise (h)	0 - 4	0 – 4
AMPK^{Thr172}	0.95 ± 0.03	0.89 ± 0.08
Akt^{Thr308}	0.92 ± 0.04	1.04 ± 0.04
4E-BP1^{Thr37}	1.03 ± 0.02	1.04 ± 0.02
eEF2^{Thr56}	1.4 ± 0.11*†	1.05 ± 0.12
mTOR^{Ser2448}	0.91 ± 0.09	1.05 ± 0.05†
P38 MAPK^{Thr180}	1.15 ± 0.21	1.28 ± 0.08
p70S6K^{Thr389}	1.06 ± 0.04	1.13 ± 0.05*
PRAS40^{Ser246}	0.97 ± 0.08	1.03 ± 0.08

Values are mean fold-change ± SEM over 4 hours post-exercise. * indicates significant increase in phosphorylation at 4 h compared with 0 h post-exercise ($P < 0.05$). † indicates significant difference between CHO and C+P at 4 h post-exercise ($P < 0.05$); $n = 8$
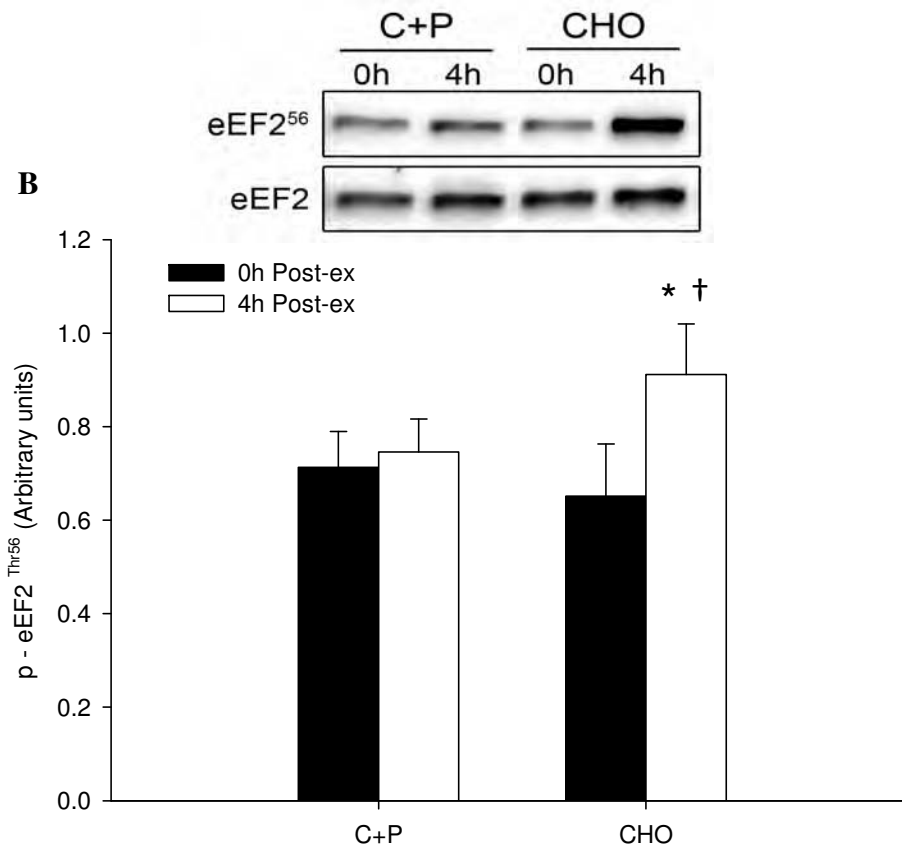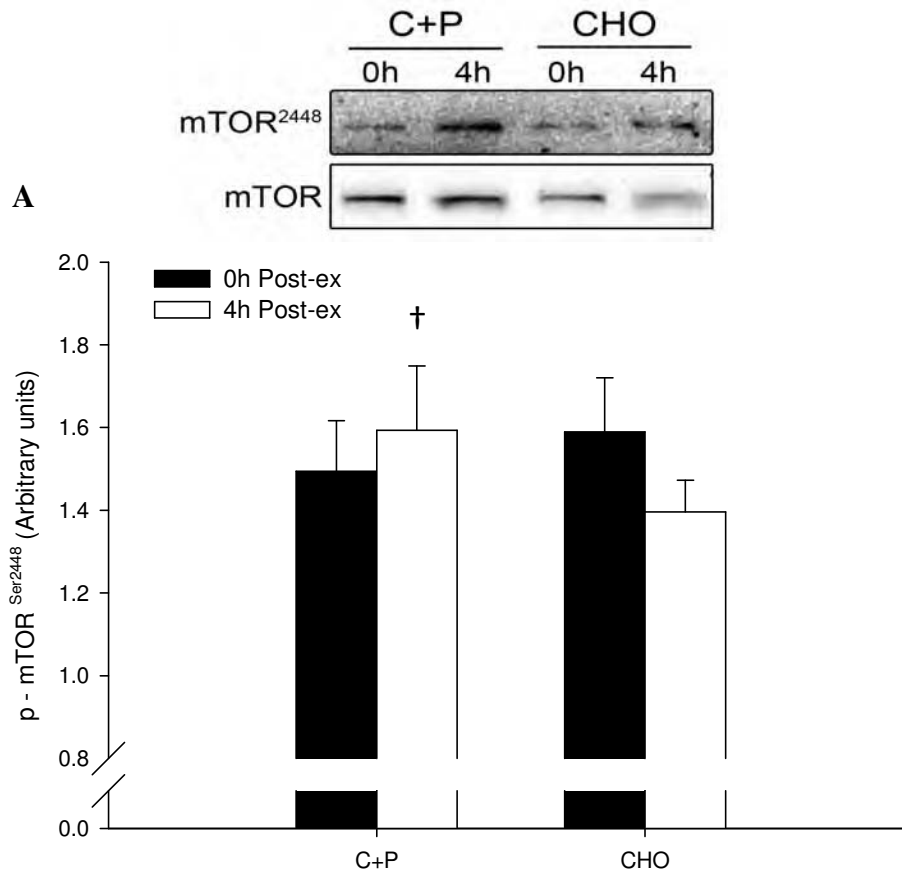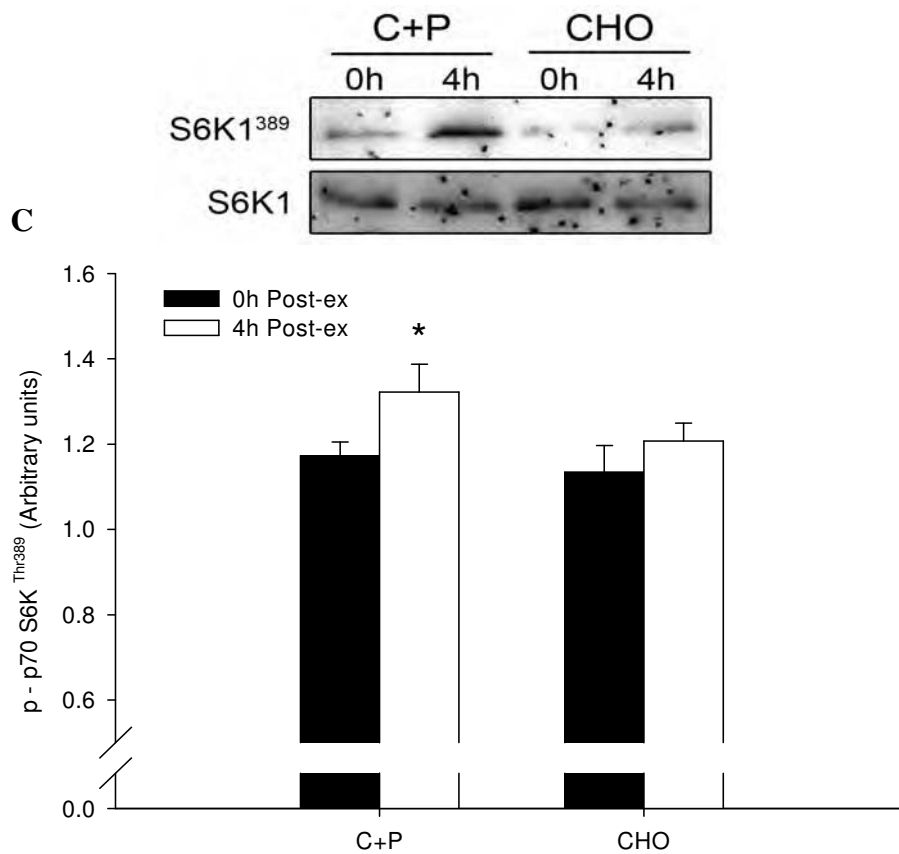
Figure 3.5 Post-exercise protein phosphorylation of (A) mTOR^{Ser2448}, (B) eEF2^{Thr56} and (C) p70S6K^{Thr389} *: significant increase in phosphorylation at 4 h compared with 0 h post-exercise ($P < 0.05$) †: significant difference between CHO and C+P at 4 h post-exercise ($P < 0.05$). Values are means ± SEM; $n = 8$.
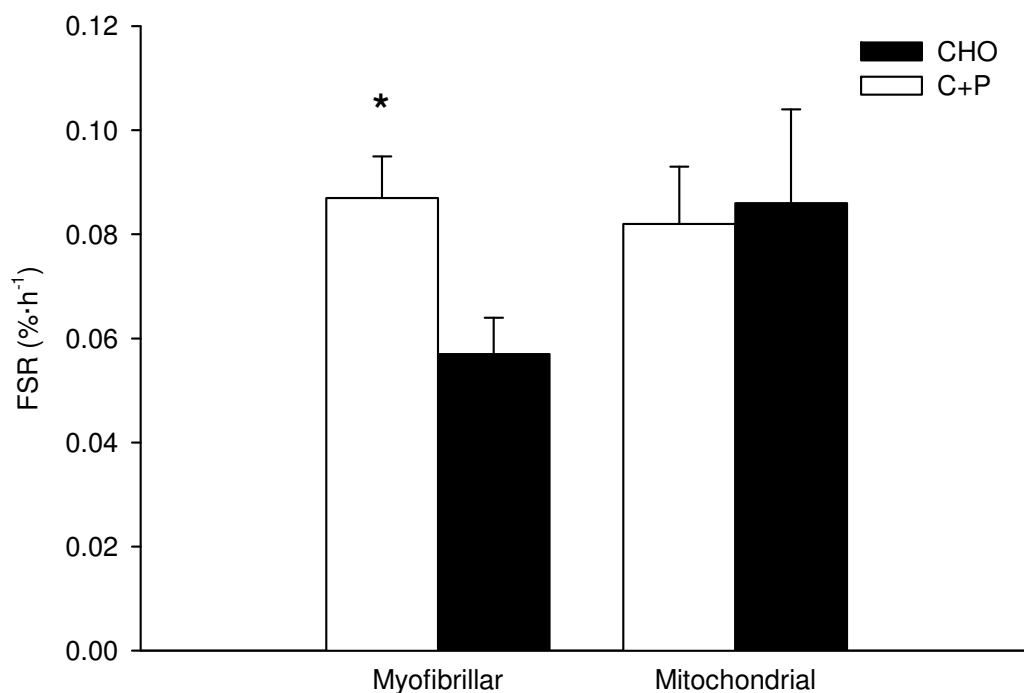
Figure 3.6 Myofibrillar (n = 10) and mitochondrial (n = 8) fractional synthetic rate. *: significant difference between CHO and C+P (P < 0.05). Values are means ±SEM.

3.5 Discussion

The present study findings expand on those of previous investigations (27, 34) to show that the ingestion of protein with carbohydrate (C+P) following 90 min of cycling by well-trained individuals, stimulates an increase in myofibrillar muscle protein synthesis (MPS) compared with carbohydrate alone (CHO). Interestingly, C+P did not increase mitochondrial MPS compared with CHO. The mechanism facilitating the adaptive response of myofibrillar MPS seems to involve the phosphorylation of mTOR and p70S6K, which in turn, may increase translation initiation and release the inhibition of eEF2 on elongation.

It has been well documented that EE increases whole-body (9) and mixed muscle protein synthesis (10, 18, 19, 50). Further, the synthesis of different protein sub-fractions

increases in response to EE (37, 54). Changes in MPS following EE are thought to be relevant for tissue repair and remodelling and not skeletal muscle hypertrophy. Indeed, it was recently shown that EE stimulates an increase in the synthesis of mitochondrial proteins, with no significant rise in myofibrillar MPS (54). The same authors demonstrate that the response of mitochondrial and myofibrillar MPS is reversed following an acute bout of resistance exercise (RE) (54). Post-RE protein ingestion also augments myofibrillar MPS (26). Given evidence that the pattern of response of mitochondrial and myofibrillar proteins to the provision of amino acids is similar under resting conditions (4, 5), it has been postulated that the increase in mixed MPS observed when protein is ingested post-EE (27, 34), could be due, in part, to an increase in the synthesis of mitochondrial proteins. However, our findings seemingly contradict this hypothesis and indicate that myofibrillar proteins are preferentially synthesized when protein is ingested following EE. Recently Harber *et al.* (19) showed no response of mixed MPS to post-EE protein ingestion following a 60 min cycle. These authors (19) measured mixed MPS at 2-6 hours post-EE, starting at 1 hour after feeding. Thus, the peak anabolic response to exercise and feeding (32, 55) may have occurred earlier than was measured, concealing any potential difference. Based on the data of Harber *et al.* (19), we suggest that the measure of mixed MPS may not be sensitive enough to reflect changes in myofibrillar and mitochondrial MPS.

The rise in myofibrillar MPS following a single bout of RE with protein ingestion (55), is predictive of gains in lean muscle mass when the training and feeding stimulus is repeated over an extended time-period (20). Thus, one interpretation of our data is that repeated post-EE protein ingestion may enhance muscle hypertrophy in endurance athletes, which, depending on the competitive goals of the athlete, may, or may not be a desirable adaptation. Gains in lean mass may result in a decreased power-to-mass ratio that accompanies an

increase in overall body weight. Indeed, evidence indicates that the primary goal of strength training for endurance populations is neuromuscular adaptations, rather than hypertrophy, to enhance performance (40). However, we hypothesize that the increase in power output that accompanies gains in lean mass may offset the gains in overall body weight, resulting in no decrement in performance. In support of this thesis, Hickson et al. (23) demonstrated that gains in thigh mass and thus, total body weight following strength training, extend short-term endurance capacity. Thus, manipulation of the power-to-mass ratio in competitive cyclists would likely have a profound impact on overall performance depending on the requirements of the particular event (11, 31). At this time, the implications of muscle hypertrophy for endurance performance are less than clear, as is the notion that post-EE protein ingestion promotes muscle hypertrophy. Clearly, these topics warrant further investigation.

Alternatively, the increase in myofibrillar MPS we observed following exercise may not lead to an increase in lean mass. Where the magnitude of myofibrillar muscle protein breakdown (MPB) equals or exceeds that of myofibrillar MPS, structural proteins would not accumulate to increase lean mass. Direct measurement of muscle protein breakdown with endurance exercise is rare and the results somewhat equivocal (12, 15, 44). Using venous blood sampling, other investigators have demonstrated that whole-body protein breakdown is transiently elevated after moderate intensity walking exercise, compared with rest (44), but others report no change following exercise (50). These studies investigated the response to moderate to low intensity exercise, rather than the high intensity exercise performed by our subjects. However, studies of endurance exercise have reported an increase in indirect markers of MPB including 3-methyl-histidine excretion (10) and net amino acid efflux from the leg (3, 52) during exercise. Thus, elevated myofibrillar MPS may also represent an increase in protein turnover in response to an increase in the degradation of myofibrillar

proteins during and following intense exercise. Combining these data, it is possible that increased myofibrillar MPS we observed when protein is ingested following intense EE, serves to counteract the fasted state losses that occurred during exercise and thus, represents an increase in myofibrillar protein turnover. Increased protein turnover may be important for the remodelling of contractile proteins. However, this hypothesis is not supported by a recent study by Harber *et al.* (19) that demonstrated that protein ingestion following an intense cycle attenuates the expression of proteolytic factors such as MuRF-1 and calpain-2. However, the relationship between the expression of these genes and protein degradation is uncertain (17). Further studies are required to determine the influence of post-EE protein ingestion on myofibrillar protein degradation and longitudinal intervention studies are needed to determine the relevance of increased protein turnover to the adaptive response to EE and performance.

The addition of protein to ingested carbohydrate did not increase the synthesis of mitochondrial proteins in the first 4 hours following exercise. Thus, the acute translational response to EE (Wilkinson *et al.*, 2008) may not be sensitive to protein ingestion. However, the long-term response of mitochondria to EE is transcriptionally mediated and occurs later than four hours post-EE (16, 21, 43), reaching a zenith between 10-24 hours post-EE (33, 37). Combining these data with recent evidence that mixed MPS remains elevated above rest at 24 h post-EE (18), it is conceivable that the major response of mitochondrial MPS to post-EE protein ingestion may have occurred later than the time frame in which we measured.

Another possibility is that the training status of the subjects and the exercise protocol utilized in the present study may explain the lack of change in mitochondrial MPS. Indeed, compared with pre-training values, Wilkinson *et al.* (54) found that the response of mitochondrial MPS to acute EE was blunted in legs that had undergone 10 weeks of either

EE or RE training. Thus, it is possible that the synthetic response of mitochondrial proteins in well-trained individuals reaches a 'ceiling'; the point beyond which, further contractile and/or nutrient stimuli fail to augment the synthetic response. Whether the pattern of myofibrillar and mitochondrial MPS we found is the same in untrained individuals remains to be elucidated.

EE can activate proteins that regulate translation initiation (7, 36, 54) (i.e. mTOR) and elongation (36) (i.e. eEF2). The transient increase in mTOR signalling following EE (7, 36), returns to basal levels by 3-4 hours post-EE (54, 56). Fujita *et al.* (14) and others have shown that ingesting protein and/or amino acids at rest increases MPS via mTOR-p70S6K signalling. To our knowledge, the only study in humans to investigate the combined effect of EE and protein ingestion on signalling protein phosphorylation was conducted by Ivy *et al.* (28). In this study, trained cyclists ingested C+P over 45 minutes after a 1 hour variable intensity cycle. The authors demonstrated that the C+P group showed higher mTOR and rpS6 phosphorylation than a non-energetic placebo. However, due to the study design, it was unclear whether these effects were due to greater amino acid availability, elevated plasma insulin concentration, or a combination of the two following C+P ingestion. The same group had earlier conducted a similar, more rigorous experiment, in which rats were fed immediately after an exhaustive swim (39). The authors demonstrated that ingesting CHO, C+P or protein-only, transiently increased the phosphorylation of mTOR, 4E-BP1, rpS6 and p70S6K, with the phosphorylation of rpS6 and 4E-BP1 sustained by C+P ingestion (39). Our data show that protein ingestion following EE sustained the phosphorylation of mTOR, such that by 4 hours post-exercise the difference between C+P and CHO was significant. Further, p70S6K phosphorylation increased by 4 hours post-exercise for C+P, compared with

immediately post-exercise. Thus, since mTOR activation is associated with increased initiation, this suggests that C+P prolongs the effect of EE on protein synthesis.

In addition to initiation, it is now recognized that translation elongation is regulated by exercise and nutrient provision. Reduced phosphorylation of eEF2 due to eEF2 kinase inhibition results in better translocation of the ribosome along the mRNA, thereby contributing to faster elongation and greater MPS (8). Mascher *et al.* (36) showed that eEF2 phosphorylation was blunted following EE, reaching a nadir at 60 min and remaining suppressed at 180 min post-EE. Concomitant with an increase in MPS, Fujita *et al.* (14) noted that essential amino acid-carbohydrate co-ingestion suppressed the phosphorylation of eEF2. Thus, eEF2 phosphorylation is sensitive to EE and protein ingestion. In this regard, we show that eEF2 phosphorylation increased by ~40% at 4 h post-EE in the CHO group, whereas there was no change with protein ingestion. Our findings are further supported by human (14) and cell culture (53) studies showing that mTOR regulation of p70S6K is inversely related to eEF2 phosphorylation. Taken together, these data suggest that the sustained phosphorylation of p70S6K, suppressed the phosphorylation of eEF2 following C+P ingestion, thereby increasing the rate of elongation. Together with the increase in initiation due to prolonged mTOR activation, this would lead to a rise in myofibrillar MPS.

In conclusion, we have shown that when protein is added to carbohydrate intervention following cycling exercise, myofibrillar proteins are preferentially synthesized. However, from our acute study it is not possible to determine whether frequent post-EE protein ingestion promotes muscle hypertrophy over time. Alternatively, the rise in myofibrillar MPS with protein ingestion, may serve to counteract a fasted-state rise in myofibrillar protein breakdown during EE. Consequently, protein ingestion may increase the rate of myofibrillar

protein turnover. The importance of an increase in myofibrillar protein turnover for endurance-trained individuals remains to be elucidated, but may be relevant for the maintenance and structural integrity of contractile proteins. Thus, we posit that post-EE protein nutrition could have important implications on the adaptive response to endurance exercise and, potentially, the recovery of muscle function. Finally, the synthetic response of different protein fractions to endurance exercise and protein ingestion may be dependent on the individual training status, intensity and duration of the exercise bout, as well as the timing and quantity of post-EE nutrient strategies. Future studies should seek to address these issues in greater depth.

Author contributions

Acknowledgements

3.6   References

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CHAPTER 4

BENEFICIAL EFFECTS OF RESISTANCE EXERCISE ON GLYCEMIC CONTROL ARE NOT FURTHER IMPROVED BY PROTEIN INGESTION

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Running title: Influence of protein ingestion on post-exercise glucose regulation.

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4.1 Abstract

Purpose: To investigate the mechanisms underpinning modifications in glucose homeostasis and insulin sensitivity at 24 h after a bout of resistance exercise (RE) with or without protein ingestion. **Methods:** Twenty-four healthy males were assigned to a control (CON; $n = 8$), exercise (EX; $n = 8$) or exercise plus protein condition (EX+PRO; $n = 8$). EX and EX+PRO completed 8 x 10 repetitions of leg press and leg extension exercise at 75% 1RM. Muscle biopsy and blood samples were obtained for all groups at rest and immediately post-RE for EX and EX+PRO. At 24 h post-RE (or post-resting biopsy for CON), a third muscle biopsy was obtained. Participants then underwent an oral glucose tolerance test (OGTT) containing 2g of [U-^{13}C] glucose. Thereafter, blood samples were obtained every 10 min for 2 h. Glucose kinetics were assessed with an infusion of 6, 6-[^{2}H$_2$] glucose during the OGTT. EX+PRO ingested an additional 25g of intact whey protein with the OGTT. A final biopsy sample was obtained at the end of the OGTT. Biopsies were analyzed for insulin-stimulated signalling phosphorylation and muscle glycogen content. **Results**: Basal, fasting plasma glucose and insulin were similar for all groups and were not different immediately post- and 24 h post-RE. Immediately post-RE, muscle glycogen was 26 ± 8 and 19 ± 6% lower in EX and EX+PRO, respectively. During OGTT, plasma glucose AUC was lower for EX and EX+PRO (75.1 ± 2.7 and 75.3 ± 2.8 mU·mL^{-1}: 120 min, respectively) compared with CON (90.6 ± 4.1 mU·mL^{-1}: 120 min; $P < 0.05$). Plasma insulin response was 13 ± 2 and 21 ± 4% lower for EX and CON, respectively, compared with EX+PRO ($P < 0.05$). Post-prandial insulin sensitivity was greater for EX and EX+PRO compared with CON ($P < 0.05$). Glucose disappearance from the circulation was ~12% greater in EX and EX+PRO compared with CON ($P < 0.05$). Basal and insulin-stimulated PAS-AS160 phosphorylation was greater for EX and EX+PRO ($P < 0.05$). **Conclusions:** Prior RE improves glycemic control and insulin sensitivity through an increase in the rate at which glucose is disposed from the circulation.

However, co-ingesting protein during a high-glucose load does not augment this response at 24 h post-exercise.

Keywords: Type II diabetes, insulin resistance, physical activity, nutrition, skeletal muscle.

Abbreviations: GLUT4; glucose transporter protein, RE; resistance exercise, AMPK; AMP-activated protein kinase, Akt; protein kinase B, AS160; Akt-substrate of 160-kDa, TBC1D1; TBC1 domain family member 1, p70S6K; ribosomal protein S6 kinase, mTOR; mammalian target of rapamycin.

4.2   Introduction

Metabolic complications, such as insulin resistance and Type II diabetes, represent a major individual and public health burden (25) and are associated with the presence of obesity and physical inactivity (13, 22, 41). Suppressed pancreatic β cell insulin production (7) and impaired glucose uptake in skeletal muscle (8, 70), are major contributors to hyperglycemia, which eventually leads to Type II diabetes. Insulin stimulates the uptake of glucose from the circulation into many body tissues, of which, skeletal muscle accounts for ~75-80% (9, 56). Insulin-stimulated glucose uptake in skeletal muscle occurs via insulin-dependent signalling that promotes glucose transporter (GLUT4) translocation to the cell membrane. Even though pharmacologic approaches are available to manage Type II diabetes, there is growing interest in lifestyle interventions, such as physical activity and dietary modification, which together can reduce the incidence of diabetes to a greater extent than pharmacological agents (61).

Skeletal muscle contraction can effectively enhance glucose uptake independent of insulin. Importantly, these properties are preserved in individuals with Type II diabetes (19, 28). A number of studies have demonstrated that aerobic exercise improves glycemic control and insulin sensitivity (10, 11, 40, 50). More recently, evidence has emerged to suggest that a single bout of resistance exercise (RE) improves whole-body insulin sensitivity (15, 30) and glycemic control (14) between 12 and 24 h post-RE. However, not all studies demonstrate a beneficial effect of RE on glucose metabolism (5, 33). The discrepant findings between studies may be due to methodological differences, including, the total exercise volume, the muscle groups exercised, the amount of time between the exercise and testing, and individual training status.

The glucose transporter GLUT4 plays an important role in skeletal muscle glucose homeostasis by regulating glucose uptake (23). Insulin regulates GLUT4 translocation from an intracellular location to the cell surface via a well-described pathway involving protein kinase B (Akt) and the recently discovered GTPase, Akt substrate of 160kDa (AS160) (21). Although enhanced glucose uptake with acute RE is thought to occur via mechanisms that bypass proximal insulin-signalling intermediates, such as Akt (3, 6, 29), AS160 appears to act as a point of convergence for insulin- and contraction-stimulated glucose transport in skeletal muscle (21, 32, 58). Recently, basal and insulin-stimulated AS160 phosphorylation was shown to increase several hours after endurance and resistance exercise in humans (12, 21) and up to 27 h post-exercise in animals (16, 60). However, the sustained response of AS160 has not been investigated in humans. Thus, the molecular and physiological mechanisms by which RE improves glucose metabolism in the 24 h after exercise have yet to be elucidated.

In addition to RE, dietary modifications that acutely raise endogenous insulin secretion, represent a clinically relevant strategy to improve blood glucose homeostasis in Type II diabetes. A number of recent studies suggest that co-ingesting protein and/or amino acids with carbohydrate induces a greater insulin release than the ingestion of either macronutrient alone (46, 49, 62). Indeed, the rise in plasma insulin with carbohydrate plus protein ingestion blunts the prevailing glucose response in Type II diabetics (34, 36), primarily due to an increase in the rate of glucose disposal from the circulation (36). Thus, in persons with Type II diabetes, co-ingesting protein with each main meal may be an effective strategy to acutely lower postprandial glucose excursions (44, 46). The combined effect of protein ingestion and RE has largely been considered in the context of muscle hypertrophy (42, 59). However, the hypothesis that post-RE protein ingestion acutely enhances glucose uptake has not been investigated in humans.

In the present study we used dual isotopic tracers during an oral glucose load to first determine the impact of an acute bout of RE on post-prandial glucose kinetics 24 h post-exercise. Second, we aimed to determine whether co-ingesting protein with the glucose load would further augment the glucose-lowering effect of the RE bout. Finally, we obtained muscle biopsy samples to examine the mechanistic basis of the effect of RE on glycemic control.

4.3 Methods

4.3.1 Participants

Twenty-four untrained, recreationally active, healthy males were recruited through advertisements to participate in the study. Individuals who were engaged in regular structured resistance or endurance training, defined as ≥ 2 training sessions per week of 60 mins or more, were ineligible to participate. All testing visits were completed within a 3-week period. The purpose and methodology of the study were clearly explained to the participants. All participants gave their informed consent prior to taking part in the study and were deemed healthy based on their response to a general health questionnaire. The experimental protocol was approved by the NHS Birmingham East, North & Solihull Research Ethics Committee (Rec No: 09/H1206/102).

4.3.2 Study design

In a randomized, parallel designed study, participants were assigned to either a non-exercise control (CON; $n = 8$), exercise only (EX; $n = 8$) or exercise plus protein (EX+PRO; $n = 8$) condition. Participant characteristics are presented in Table 4.1. Following a preliminary assessment of maximal lower-limb strength, participants reported to the laboratory in a fasted

state, on two consecutive mornings. On the first morning, a resting muscle biopsy sample was obtained, thereafter EX and EX+PRO performed an intense lower-limb workout. A second biopsy was obtained immediately post-exercise for EX and EX+PRO only. Twenty-four hours later another muscle biopsy was obtained, after which, participants completed an oral glucose tolerance test (OGTT). Participants assigned to EX+PRO co-ingested protein with the OGTT to determine whether the addition of protein augmented the impact of resistance exercise on glucose metabolism. During OGTT, dual isotopic glucose tracers were utilized and frequent blood samples obtained over 2 h to determine glucose kinetics and insulin sensitivity. A final muscle biopsy was obtained at the end of the 2 h OGTT to examine the phosphorylation of contraction- and insulin-mediated intracellular protein phosphorylation.

Table 4.1 Characteristics of participants in each group

Parameter	CON ($n = 8$)	EX ($n = 8$)	EX+PRO ($n = 8$)
Age (y)	22 ± 3	20 ± 3	22 ± 6
Weight (kg)	77.0 ± 6.3	79.7 ± 14.5	75.6 ± 13.1
BMI (kg·m^{-2})	24 ± 2.3	25.1 ± 3.9	23.5 ± 4.8
LP 1RM (kg)	200 ± 44	212 ± 50	206 ± 60
LP 1RM (kg·BM^{-1})	5.7 ± 0.8	5.9 ± 1.8	6.0 ± 1.3
LE 1RM (kg)	111 ± 22	121 ± 21	115 ± 29
LE 1RM (kg·BM^{-1})	3.5 ± 1.2	3.7 ± 0.7	3.4 ± 0.6
Exercise volume (kg)	–	18,314 ± 2154	18,277 ± 2406

CON: resting control group, EX: exercise only group, EX+PRO: exercise plus protein group BMI; body mass index, 1RM; one-repetition maximum, LP: leg press, LE; leg extension. Exercise volume is defined as number of repetitions x number of sets x weight lifted. Values are presented as means ± SD.

4.3.3   Preliminary testing

Body Mass: A digital scale was used to determine body mass to the nearest 0.1kg. Participant weight and height were recorded in exercise clothing without shoes on. This was repeated prior to each of the two testing visits to ensure body mass remained stable throughout testing.

Maximal Strength: Bilateral 1 Repetition Maximum (1RM) was determined for leg press and leg extension exercises (Cybex VR/3). After warming up at a self selected resistance, the load was set at a level designed to allow the subject to perform at least two, but less than ten repetitions before failure. This estimation protocol of 1RM was designed to minimise the number of attempts necessary to determine 1RM (39). After each successful lift the load was increased by 2.5-5 kg until failure to complete two repetitions. Between each successive attempt a 2 min rest period was allowed. A repetition was considered valid if the subject used proper form and was able to complete the entire lift in a controlled manner without assistance.

Diet and Physical Activity Control: Participant diet was standardized for the entire 48 h testing period. During preliminary testing participants completed a 3-day food diary, representative of their average week (two week days and one weekend day). A questionnaire of food preferences was also completed by participants. Using an on-line diet planner (Weight Loss Resources) the total energy and macronutrient content of each of the 3-days was estimated. Food parcels were provided to each participant with a total energy and macronutrient intake equivalent to their average habitual intake. Thus, participant diet was not manipulated during the study. Participants were instructed to consume only the food provided for them over the two-day testing period (i.e. 24 h prior to Day 1 and during Day 1). There

was no difference in total energy intake or macronutrient composition of the food parcels for CON, EX and EX+PRO (Table 4.2).

Physical activity control: Participants were instructed to maintain normal habitual activities of daily living but to refrain from any strenuous activity for 48 h prior to reporting to the laboratory on Day 1. After completion of testing on Day 1, participants were also instructed to refrain from strenuous activity prior to returning the following morning on Day 2.

4.3.4   Treatment trials

Day 1 - Exercise/Control Trial: Participants reported to the Human Performance Laboratory between 0600 and 0700 h in a fasted state, 7-14 days after preliminary strength tests. After resting in a supine position for 30 min a cannula was inserted into an antecubital forearm vein and a resting blood sample obtained for analysis of background isotopic enrichment. Thereafter, the lateral portion of one thigh was prepared under local anaesthetic (1% Lidocaine) and a 5-mm Bergstrom biopsy needle was used to extract a resting muscle biopsy sample from the *vastus lateralis* muscle. After the resting biopsy was obtained the biopsied leg was bandaged and EX and EX+PRO were instructed to complete a bout of intense lower-limb resistance exercise, whereas CON were permitted to consume the standardized breakfast and leave the laboratory. For EX and EX+PRO a second biopsy sample was obtained post-exercise (6 ± 1 min) 1 cm distal from the resting biopsy. For EX and EX+PRO both biopsy incisions were made at rest to allow the post-exercise biopsy sample to be obtained as quickly as possible. The order of biopsied leg was counterbalanced between groups. Biopsy samples were blotted and freed of any visible fat and connective

tissue, frozen in liquid nitrogen (within ~60 s of being taken from the muscle) and stored at -80°C until further analysis.

The resistance exercise bout consisted of a standardized warm-up on a leg-press machine (12 x 50% 1RM + 10 x 60% 1RM + 8 x 70% 1RM + 2 x 75% 1RM) followed by 8 sets of 10 bilateral repetitions at 75% 1RM. Participants then completed 8 sets of 10 bilateral repetitions on a leg-extension machine at 75% 1RM. In the event that a participant failed to complete 10 repetitions in a set the weight was decreased by 2.5-5kg for the following set. Failure was defined as the point at which the exercise could not be completed or technique failed. Participants were instructed on proper lifting cadence using a metronome set to 50 beats min^{-1}, which corresponded to 1 s concentric muscle action, 0 s pause and a 1 s eccentric muscle action. Strong verbal encouragement was given throughout exercise. Between-set rest intervals of 2 min were given and participants completed the exercise bout in 45 ± 3 min. Participants were permitted to consume water *ad libitum* throughout Day 1.

Day 2 - Infusion Trial: Participants returned to the laboratory the following morning between 0600 and 0700 h in a fasted state. A schematic diagram of the study protocol is presented in Figure 4.1. A cannula was inserted into the antecubital vein of one arm for the infusion of a stable isotopic tracer. A second cannula was inserted into a hand vein of the opposite arm and a resting venous blood sample obtained. At a time-point corresponding to ~23 h post-exercise for EX and EX+PRO a primed infusion of 6, 6-[^{2}H$_2$] glucose (Cambridge Isotope Laboratories, MA, USA) was initiated (prime: 13.5 μmol.kg^{-1}; infusion: 0.35 μmol.kg^{-1}.min^{-1}) and continued for ~180 min. For CON, the infusion was initiated at a time-point corresponding to ~23 h after the resting biopsy on Day 1. Approximately 60 min into the infusion (~24 h post-exercise or resting biopsy) a muscle biopsy sample was obtained from

the *vastus lateralis* of the opposite leg to that sampled the previous day. Immediately after the muscle biopsy was obtained CON and EX consumed an oral glucose load (oral glucose tolerance test; OGTT) described below. EX+PRO consumed the same glucose load plus additional protein. The time at which the beverage was completely consumed was considered *t = 0,* thereafter participants rested in the supine position and venous blood samples were obtained every 10 min until *t = 120.* A final muscle biopsy sample was obtained at *t = 120* from a separate incision (~26 h post-exercise or resting biopsy). Water intake was restricted during Day 2 to ensure participants consumed only the treatment beverage.

4.3.5   Treatment beverages

Sixty minutes into the infusion on Day 2, participants ingested 73g of glucose (80.3g dextrose monohydrate when corrected for water content) plus an additional 2g of $[U-^{13}C]$ glucose (99%, Cambridge Isotope Laboratories, MA, USA) to determine the contribution of exogenous glucose production to the total rate of appearance of glucose. Thus, participants ingested a total of 75g of glucose. Participants assigned to EX+PRO ingested an additional 25g of whey protein with the 75g glucose load. Glucose was provided in the form of dextrose monohydrate obtained from Roquette™ (Lestrem, France) and intact whey protein (Volactive ultrawhey 90) was a generous gift from Volac™ (Royston, UK). The amino acid content of the protein was (in percent content, wt:wt): Ala, 5; Arg, 2.1; Asp, 11; Cys, 2.2; Gln, 18.1; Gly, 1.4; His, 1.7; Ile, 6.4; Leu, 10.6; Lys, 9.6; Met, 2.2; Phe, 3; Pro, 5.5; Ser, 4.6; Thr, 6.7; Trp, 1.4; Tyr, 2.6; and Val, 5.9. Both treatments were consumed in water in a total volume of 300mL. Treatments were not matched for flavour or appearance due to the parallel study design. Participants were instructed to consume the treatment beverage within 2 min.
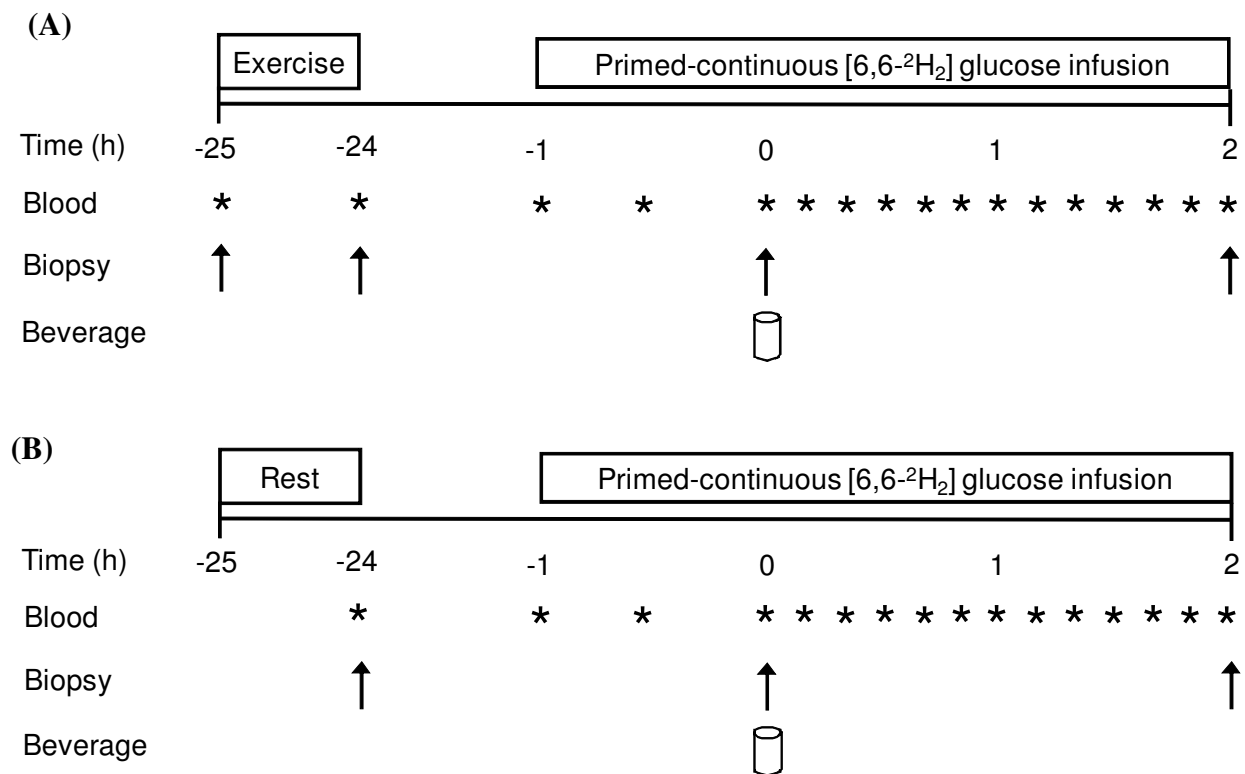
Figure 4.1 Schematic diagram of the study protocol. (A) Indicates protocol for exercise only and exercise plus protein participants. (B) Indicates protocol for non-exercised control participants.

4.3.6   Analyses

Blood analytes: Blood was collected in EDTA-containing tubes and spun at 3,500rpm for 15 min at 4°C. Aliquots of plasma were the frozen at -80°C until later analysis. Plasma glucose concentration was analyzed using an ILAB automated analyzer (Instrumentation Laboratory, Cheshire, UK). Plasma insulin concentration was analyzed using a commercially available ELISA kit (IBL International, Hamburg, Germany) following the manufacturer's instructions. The enrichment of $[^{13}C]$ and $[^2H_2]$ glucose in plasma was determined by gas chromatography-mass spectrometry (model 5973; Hewlett Packard, Palo Alto, CA). Derivatization was carried out with butane boronic acid in pyridine and acetic anhydride. The glucose derivative was quantified by selected ion monitoring at mass-to-charge ratios (*m/z*)

297, 299 and 303 for $[^{12}C]$-, $[^2H_2]$- and $[U-^{13}C]$ glucose, respectively. Two sets of enriched standards were measured containing known amounts of $[^2H_2]$- and $[U-^{13}C]$ glucose. By establishing the relationship between the enrichment of the glucose standards, the enrichment in plasma samples was determined.

Western blots: Muscle biopsy samples (~40mg) were powdered on dry ice under liquid nitrogen using a mortar and pestle. Approximately 25mg of powdered muscle was homegenized in lysis buffer (50mM Tris pH 7.5; 250mM Sucrose; 1mM EDTA; 1mM EGTA; 1% Triton X-100; 1mM NaVO4; 50mM NaF; 0.50% PIC) using a hand-held homogenizer (PRO200, UK). Samples were shaken at 4ºC for 30 min (12,000rpm), centrifuged for 5 min at 6,000g and the supernatant removed for protein determination. Protein concentration was determined using the DC protein assay (Bio Rad, Hertfordshire, UK). Equal aliquots of protein were boiled in Laemmli sample buffer (250mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 0.01% bromophenol blue; 5% β-mercaptoethanol) and separated on SDS polyacrylamide gels (10-12.5%) for 1 h at 58mA. Following electrophoresis; proteins were transferred to a Protran nitrocellulose membrane (Whatman, Dassel, Germany) at 100 V for 1 h. The membranes were incubated overnight at 4°C with the appropriate primary antibody. The primary antibodies used were total Akt, Akt^{ser473} (Cell signalling 3787), total AS160 and phospho AS160 were a generous gift from Prof. Grahame Hardie, University of Dundee, the PAS-AS160 antibody was generated by the division of signal transduction therapy (DSTT), University of Dundee. Total p70S6K and phospho p70S6K^{Thr389} was from Santa Cruz (11759/7984R). The following morning the membrane was rinsed in wash buffer (TBS with 0.1% Tween-20) three times for 5 min. The membrane was then incubated for 1 h at room temperature within wash buffer containing the appropriate secondary antibody, either horseradish (HRP)-linked anti-mouse IgG (New England Biolabs, 7072; 1:1,000) or anti-

rabbit IgG (New England Biolabs, 7074; 1:1,000). The membrane was then cleared in wash buffer three times for 5 min. Antibody binding was detected using enhanced chemiluminescence (Millipore, Billerica, MA). Imaging and band quantification were carried out using a Chemi Genius Bioimaging Gel Doc System (Syngene, Cambridge, UK). Western blots analyses were determined for 18 participants ($n = 6$ per group).

Immunoprecipitations: Endogenous AS160 and p70S6K proteins were immuno-precipitated (IP) overnight at 4°C with 0.8μg of AS160 or p70S6K antibodies in a mix of protein G-agarose beads (Millipore, Glostrup, DK) and lysate (600μg). The following day immunocomplexes were washed two times in sucrose lysis buffer and two times in TNE (10 mM Tris, pH 7.5, 150 mM NaCl, 10 mM EDTA and 0.1 mM Na_2VO_4). The immunocomplexes were re-suspended in 50 µL of 1 x Laemmli sample buffer and boiled for 5 min (96°C) upon which they were subjected to western blotting as previously described.

Muscle glycogen measurement: Powdered muscle (~20 mg) was hydrolyzed in 250 µl of 2 N HCl by heating at 95°C for 3 h. The solution was neutralized with 250 µl 2 N NaOH and the resulting free glycosyl units were assayed spectrophotometrically using a hexokinase-dependant assay kit (Glucose HK, ABX diagnostics, UK) against glucose standards of known concentrations (4).

4.3.7   Calculations

Insulin sensitivity: Plasma glucose and insulin concentrations during the 120 min OGTT were used to determine the whole-body insulin sensitivity index (ISI) according to the following equation of Matsuda (37):

Matsuda (ISI) = 1000/ √ (FPI·FPG) · (mean OGTT insulin) · (mean OGTT glucose)   *(1)*

Where FPG is the fasting plasma glucose concentration, FPI is the fasting insulin concentration and 1000 represents a constant that allows numbers between 1 and 10 to be obtained.

Post-absorptive insulin sensitivity was also estimated by the homeostasis model assessment (HOMA-IR) index which is calculated by dividing the product of FPG and FPI by 22.5 (38).

Glucose kinetics: From the [^{2}H$_2$] glucose tracer, the total R_a (*Eq. 2*) and R_d (*Eq. 3*) of glucose were calculated with the single-pool non-steady-state equations of Steele (57) as modified for use with stable isotopes (68). Total R_a represents the splanchnic R_a of glucose from ingested glucose, the liver and potentially some glycogenolysis and gluconeogenesis from the kidneys.

R_a total = F - (V· (C_2 + C_1)/2· (E_{p2} − E_{p1})/t_2 − t_1)) / (E_{p2} + E_{pl})/2)   *(2)*

R_d = R_a total – V· (C_2 + C_1/ t_2 − t_1)   *(3)*

Where E_{pl} and E_{p2} are the [^{2}H$_2$] glucose enrichments in plasma at time-points t_1 and t_2, respectively; and C_1 and C_2 are glucose concentrations at t_1 and t_2, respectively; and V is volume of distribution in 160 mL·kg^{-1}.

The [U-^{13}C] glucose tracer added to each beverage was used to calculate the R_a of glucose from the gut. The R_a of [^{13}C] glucose (R_a gut) into plasma was determined by transposition of

the Steele equation and the known ^{13}C enrichment of the ingested glucose (52) adapted for use with stable isotopes (24).

$$F_2 = R_a \cdot [(E_{p2} + E_{p1})/2 + (C_2 + C_1)/2 \cdot (E_{p2} - E_{p1})/(t_2 - t_1) \cdot V] \qquad (4)$$

Where F_2 is the R_a of [^{13}C] glucose in the blood; R_a is the previously determined total R_a of glucose (*Eq.* 2). Knowing the R_a of [^{13}C] glucose in the blood, one can determine the absorption rate of glucose from the gut from the known enrichment of the ingested glucose.

$$R_a \text{ gut} = F_2/E_{ing} \qquad (5)$$

Where R_a gut is the R_a of gut-derived glucose and E_{ing} is the ^{13}C enrichment of the ingested glucose. The rate of endogenous glucose (EGP) was calculated as the difference between R_a total and R_a gut.

$$EGP = R_a \text{ total} - R_a \text{ gut} \qquad (6)$$

R_a, R_d, R_a gut and EGP were converted to g.min^{-1} for graphical representation (= μmol·kg^{-1}·min^{-1} x kg x 180.2 / 10^{-6}).

4.3.9   *Statistical Analysis*

A between-subject repeated measures design was utilized for the current study. Exercise variables, blood analytes, plasma enrichment and Western blot data were analyzed using a two-way ANOVA with repeated measures (treatment x time) to determine differences between each condition across time. When a significant main effect or interaction was

identified, data were subsequently analyzed using a Bonferroni post hoc test. Plasma glucose

and insulin concentrations over the 120 min OGTT were calculated as area under the curve

(AUC). Within-group changes over time; glucose kinetics and blood analyte AUC data were

checked for statistical significance using one-way repeated-measures ANOVA. All statistical

tests were analyzed using statistical package for social sciences (SPSS) version 18.0 (Illinois,

Chicago, U.S). Significance for all analyses was set at $P < 0.05$. All values are presented as

means ± standard error of the mean (SEM).

4.4 Results

4.4.1 *Dietary intake*

Dietary analysis indicated that daily energy intake and macronutrient composition of the

diet was similar for CON, EX and EX+PRO (Table 4.2). Thus, the contents of the

standardized diet provided prior to and during Day 1 of the study were similar for all groups.

Table 4.2 Participant habitual dietary intake and macronutrient composition.

	CON (*n* = 8)	EX (n = 8)	EX+PRO (n = 8)
Daily energy intake (kJ)	9,353 ± 469	9,487 ± 691	10,345 ± 452
Carbohydrate (%)	71.1 ± 6.6	66.2 ± 3.9	69 ± 7.1
Protein (%)	15.8 ± 3.0	18.2 ± 3.3	17.9 ± 6.5
Fat (%)	13.1 ± 3.1	15.6 ± 0.4	13.1 ± 4.8

Groups as per Table 1. Values are presented as means ± SEM.

4.4.2 Exercise variables

Leg-press and leg-extension 1RM values determined during pre-testing, were not different between groups (Table 4.1). Based on the measured 1RM, the resistance lifted on the leg-press machine during Day 1 was 150 ± 17 and 156 ± 17 kg and for EX and EX+PRO, respectively ($P > 0.05$). Leg-extension resistance was set at 85 ± 11 and 84 ± 9 kg for EX and EX+PRO, respectively ($P > 0.05$). All participants were able to complete the leg-press exercise without reducing the weight. Six participants (four from EX and two from EX+PRO) were unable to complete the leg-extension exercise at the desired resistance, which was then lowered by 2.5-5 kg to enable participants to complete ten repetitions. However, the total exercise volume performed for EX and EX+PRO was not different (Table 4.1).

4.4.3   Plasma glucose

Fasting plasma glucose (5.1 ± 0.4, 5.3 ± 0.7 and 5.7 ± 0.2 mmol·L^{-1} for CON, EX and EX+PRO, respectively) were in the normal range. Basal plasma glucose concentrations were similar on Day 2 (24 h post-exercise) for all groups (5.6 ± 0.3, 4.9 ± 0.3 and 5.1± 0.6 mmol·L^{-1} for CON, EX and EX+PRO, respectively). During the OGTT, plasma glucose concentration increased in all groups, peaking between 30-50 min after feeding ($P < 0.05$; Figure 4.2A). Plasma glucose peaked at 92 ± 7, 88 ± 9 and 80 ± 8% above basal fasted values for CON, EX and EX+PRO, respectively. Following the peak, plasma glucose concentration decreased, such that by 120 min post-OGTT plasma glucose concentration had returned to basal fasted values. Plasma glucose AUC during OGTT was 17 ± 3% lower for EX and EX+PRO ($P = 0.02$ for both) compared with CON (Figure 4.2B).

4.4.4   Plasma insulin

Fasting plasma insulin concentrations (7.2 ± 0.6, 5.9 ± 0.7 and 7.1 ± 0.6 μU·ml^{-1} for CON, EX and EX+PRO, respectively) were in the normal range. Basal plasma insulin concentrations were similar on Day 2 for all groups (6.2 ± 0.7, 6.5 ± 0.4 and 6.9 ± 0.5 μU·ml^{-1} for CON, EX and EX+PRO, respectively). Plasma insulin concentrations during OGTT increased by 8.6-, 9.1- and 11.1-fold above basal fasted values for CON, EX and EX+PRO, respectively peaking between 30-40 min after feeding (Figure 4.3A). Plasma insulin AUC during OGTT was significantly greater for EX+PRO compared with EX ($P = 0.04$) and CON ($P = 0.01$). There was no difference in insulin AUC between EX and CON (Figure 4.3B).

Insulin sensitivity: HOMA-(IR) index on Day 1 was 1.55 ± 0.13, 1.69 ± 0.32 and 1.51 ± 0.12 for CON, EX and EX+PRO, respectively. HOMA-(IR) index was not different on Day 2 (1.46 ± 0.17, 1.5 ± 0.12 and 1.64 ± 0.17 for CON, EX and EX+PRO, respectively). Post-prandial insulin sensitivity, calculated using the Matsuda ISI, was greater for EX and EX+PRO (6.95 ± 0.5 and 6.82 ± 0.41, respectively) compared with CON (6.21 ± 0.72; $P < 0.05$). There was no difference in Matsuda ISI between EX and EX+PRO.

4.4.5   *Glucose tracer kinetics*

Plasma enrichment of infused 6, 6-[$_2$H^2] and ingested [U-^{13}C] glucose are presented in Figure 4.4A. R_a total, R_a gut, EGP and R_d over time are presented in Figure 4.4 (B, C, D, respectively). Average plasma glucose tracer kinetics are presented in Table 4.3. In all groups, plasma glucose R_a total increased over time ($P < 0.05$), peaking between 70-90 min after feeding (Figure 4.4B). There was no difference in the plasma glucose R_a total between groups. Glucose R_a gut demonstrated an increasing contribution to R_a total with time ($P < 0.05$), whereas EGP demonstrated a reduced contribution to R_a total with time ($P < 0.05$; Figure 4.4C). The increase in R_a gut peaked between 70-90 min after feeding, whereas the

decline in EGP reached a nadir between 90-100 min after feeding. Glucose R_d increased over time in all groups ($P < 0.05$; Figure 4.4D). Glucose R_d increased by 127 ± 13, 131 ± 15 and $150 \pm 18\%$ above basal values for CON, EX and EX+PRO, respectively. Glucose R_d was significantly lower for CON between 40-70 min after feeding compared with EX and EX+PRO ($P < 0.05$). Average glucose R_d and whole-body glucose disposal, (R_d expressed as % of R_a total) was significantly lower for CON compared with EX and EX+PRO ($P < 0.01$; Table 4.3). The time taken for R_d to match the R_a total was greater for CON than EX and EX+PRO ($P < 0.05$). There was no difference in average glucose tracer kinetics between EX and EX+PRO.

4.4.6   *Muscle glycogen*

Basal muscle glycogen concentration was similar for all groups (Figure 4.5). Immediately post-exercise, muscle glycogen concentration was 26 ± 8 and $19 \pm 6\%$ lower for EX and EX+PRO, with no difference between groups ($P < 0.05$). Muscle glycogen concentration at 24 h post-exercise was similar to basal values for all groups. There was no significant change in muscle glycogen concentration following OGTT (~26 h post-exercise), compared with 24 h post-exercise.

4.4.7   *Protein phosphorylation*

Western blot phospho-images are presented in Figure 4.6. Basal PAS-AS160 phosphorylation (Day 1) was similar for all groups (Figure 4.7A). Compared with basal, AS160 phosphorylation did not change immediately post-exercise but was increased at 24 h post-exercise for EX and EX+PRO only ($P < 0.05$). AS160 phosphorylation at 24 h post-exercise was significantly greater for EX and EX+PRO compared with CON ($P < 0.01$). At 26 h post-exercise, following OGTT, AS160 phosphorylation increased by 1.4-fold for CON

and 1.3-fold for EX and EX+PRO compared with 24 h post-exercise ($P < 0.05$). AS160 phosphorylation at 26 h post-exercise was greater for EX and EX+PRO compared with CON ($P < 0.05$). Basal Akt^{ser473} phosphorylation (Day 1) was similar for all groups (Figure 4.7B). Compared with basal, Akt phosphorylation did not change immediately post- and 24 h post-exercise in all groups. At 26 h post-exercise, following OGTT, Akt phosphorylation increased by 2.2-, 2.3 and 1.9-fold for EX and EX+PRO, respectively, compared with 24 h post-exercise ($P < 0.05$). Akt phosphorylation at 26 h post-exercise was greater for EX and EX+PRO compared with CON ($P < 0.05$). Basal p70S6K^{Thr389} phosphorylation (Day 1) was similar for all groups (Figure 4.7C). Compared with basal, p70S6K phosphorylation was not different immediately-, 24 h- or 26 h-post exercise. There was no between-group difference in p70S6K phosphorylation immediately-, 24 h- or 26 h-post exercise.

Table 4.3 Plasma glucose kinetics for the 3 groups during OGTT

	CON ($n = 8$)	EX (n = 8)	EX+PRO (n = 8)
R_a total (g·min^{-1})	0.34 ± 0.02	0.36 ± 0.02	0.36 ± 0.03
R_a gut (g·min^{-1})	0.24 ± 0.03	0.26 ± 0.03	0.26 ± 0.04
Exogenous contribution (R_a gut as % of R_a)	65.7 ± 6	68.5 ± 6	65.9 ± 7
EGP (g·min^{-1})	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01
Endogenous contribution (EGP as % of R_a)	34.2 ± 7	31.4 ± 7	33.9 ± 7
R_d (g·min^{-1})	0.31 ± 0.02*	0.35 ± 0.02	0.35 ± 0.02
Glucose disposal (R_d as % of R_a)	89.4 ± 1.7*	94.1 ± 0.5	95.9 ± 0.6
Time for R_d to match R_a (min)	59 ± 8*	42 ± 5	45 ± 6
Ingested glucose appearance (%)	38.4 ± 7.1	41.6 ± 7.6	42.2 ± 7.8

Data presented for [6, 6- $^2H_2$] glucose rate of appearance (R_a total) and disappearance (R_d) and R_d expressed as % of R_a. Contribution of exogenous [U-^{13}C] glucose from the gut (R_a Gut) and endogenous glucose production (EGP) to R_a total are presented. Groups as per Table 4.1. * indicates significantly lower than EX and EX+PRO ($P < 0.05$). Values are presented as means ±
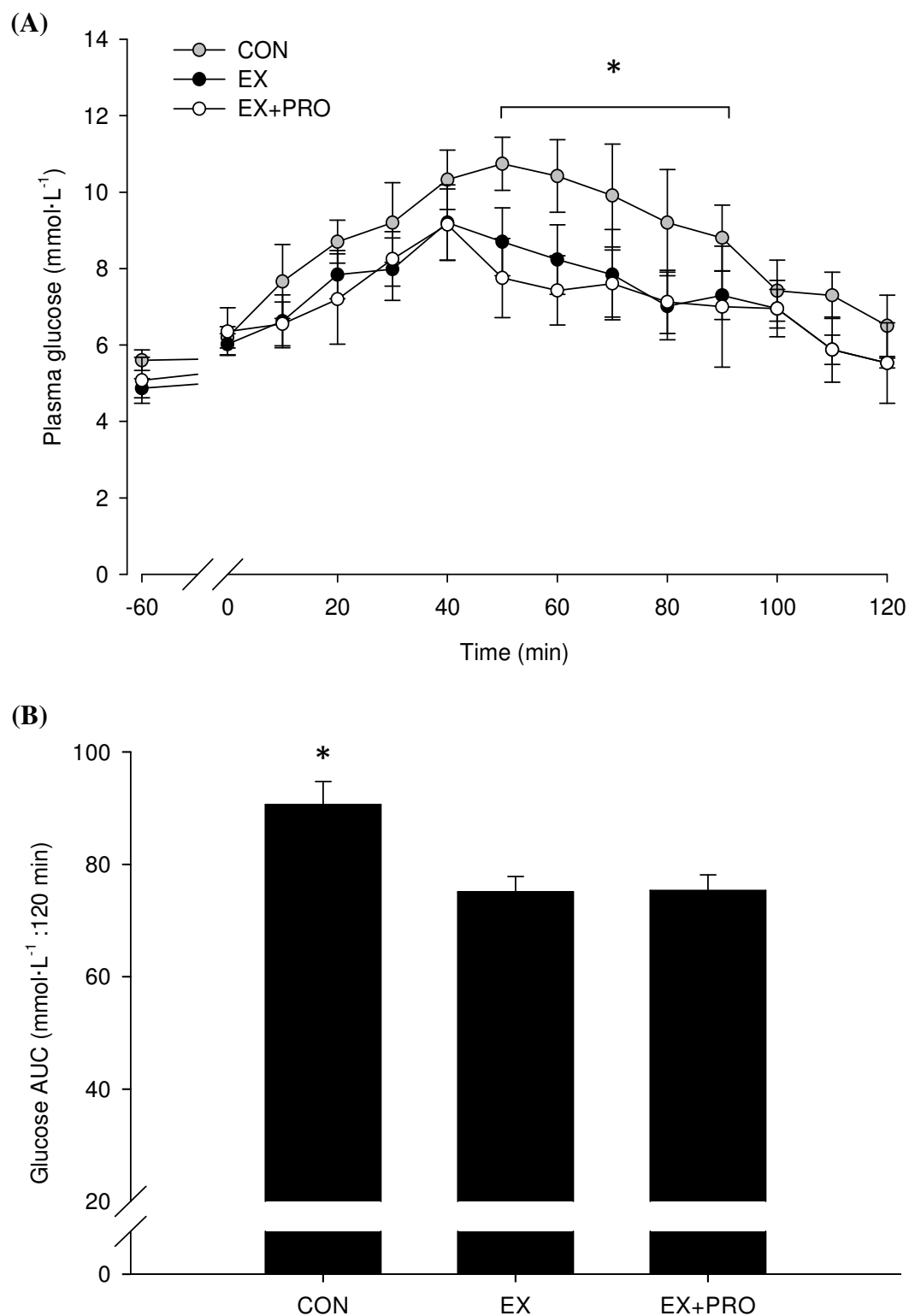
Figure 4.2 Plasma glucose concentration (A) and AUC (B) during OGTT 24 h following resistance exercise in untrained volunteers. Groups as per Table 4.1. Values are means ±SEM; $n = 8$ per group. *: significantly greater glucose concentration/AUC for CON compared with EX and EX+PRO ($P < 0.05$).
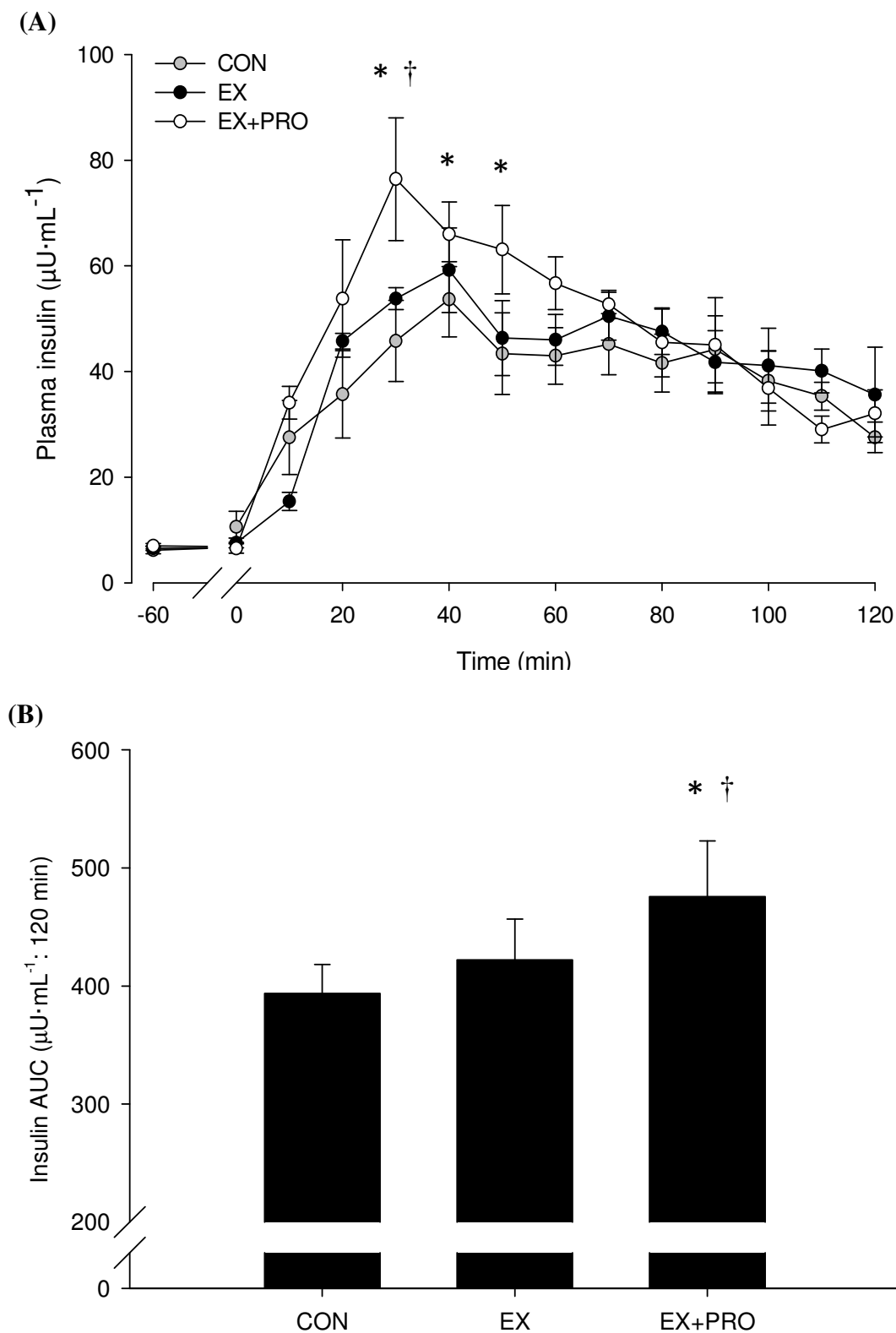
Figure 4.3 Plasma insulin concentration (A) and AUC (B) during OGTT 24 h following resistance exercise in untrained volunteers. Groups as per Table 4.1. Values are means ±SEM; $n = 8$ per group. *: significantly greater insulin concentration/AUC than CON ($P < 0.05$). †: significantly greater insulin concentration than EX. ($P < 0.05$).

(A)

(B)

(C)

(D)
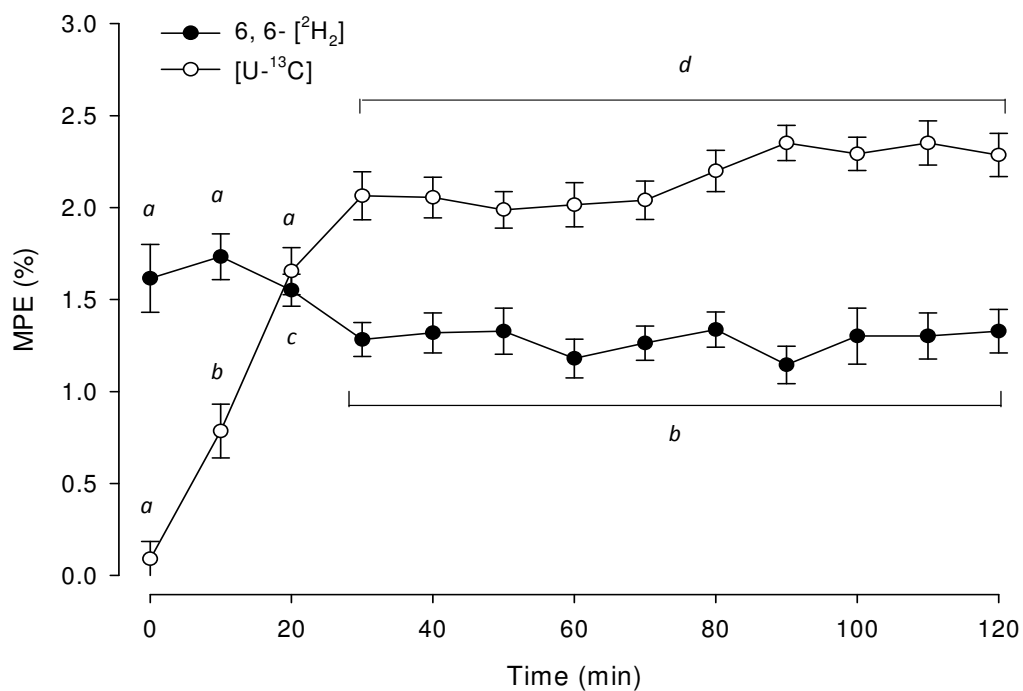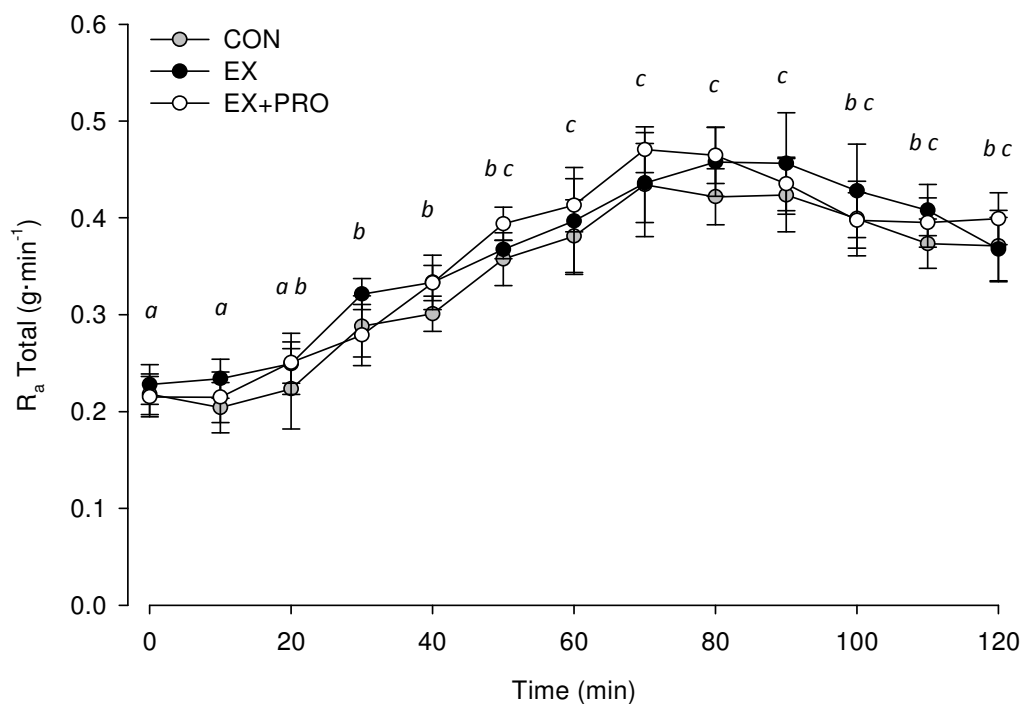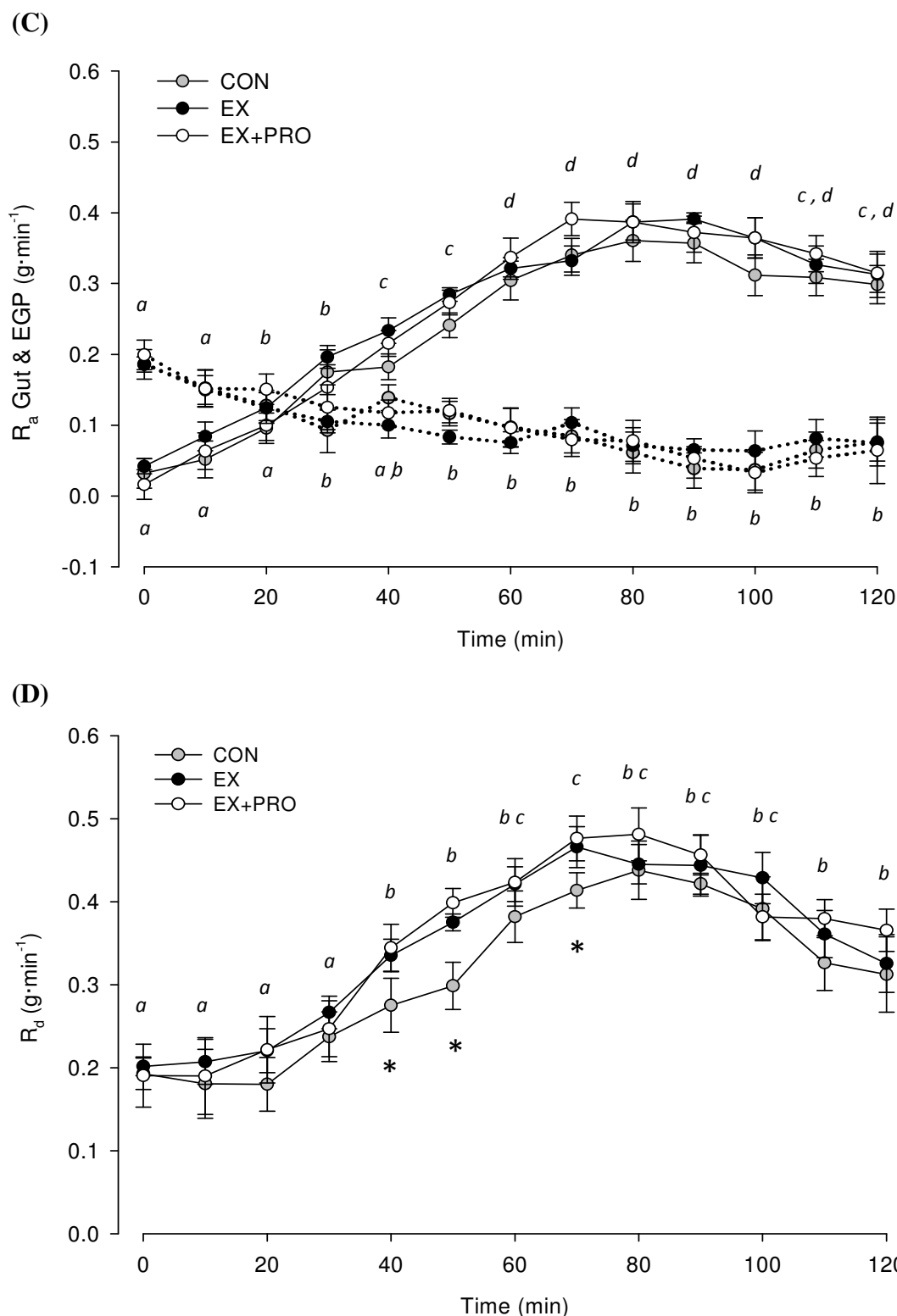
Figure 4.4 Tracer enrichments and glucose kinetics over 120 min OGTT. (A) Enrichment of 6, 6-[$^2H_2$] and [U-^{13}C] glucose in plasma. Average for all 3 groups presented, $n = 24$. MPE (%): mole percent excess. (B) Total rate of glucose appearance in plasma (R_a). (C) Contribution of exogenous glucose appearing from the gut (R_a gut) and endogenous glucose production (EGP) to the R_a Total; solid lines indicate R_a gut, dashed arrows indicate EGP. (D) Rate of glucose disappearance from plasma (R_d). Means with different subscripts are significantly different from each other. *: significantly lower R_d for CON compared with EX and EX+PRO. Values are means ±SEM; $n = 8$ per group.
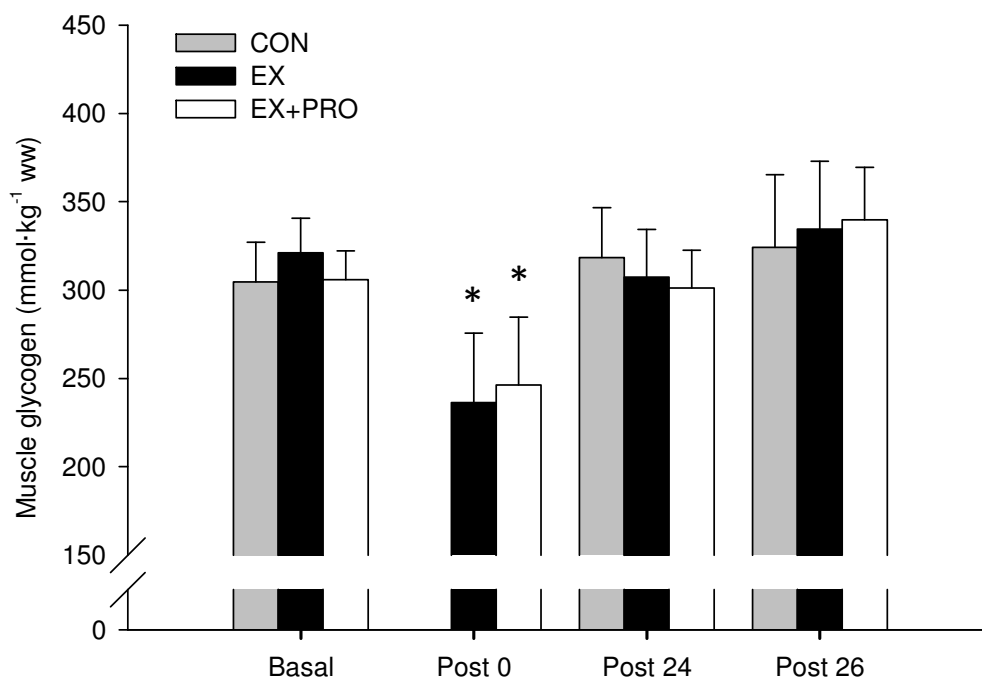
Figure 4.5 Muscle glycogen content. Values obtained at basal, immediately post-exercise (Post 0), 24 h post-exercise (Post 24) and immediately following OGTT (Post 26). Groups as per Table 4.1. Values are means ± SEM; $n = 6$ per group. *: significantly lower compared with basal ($P < 0.05$).
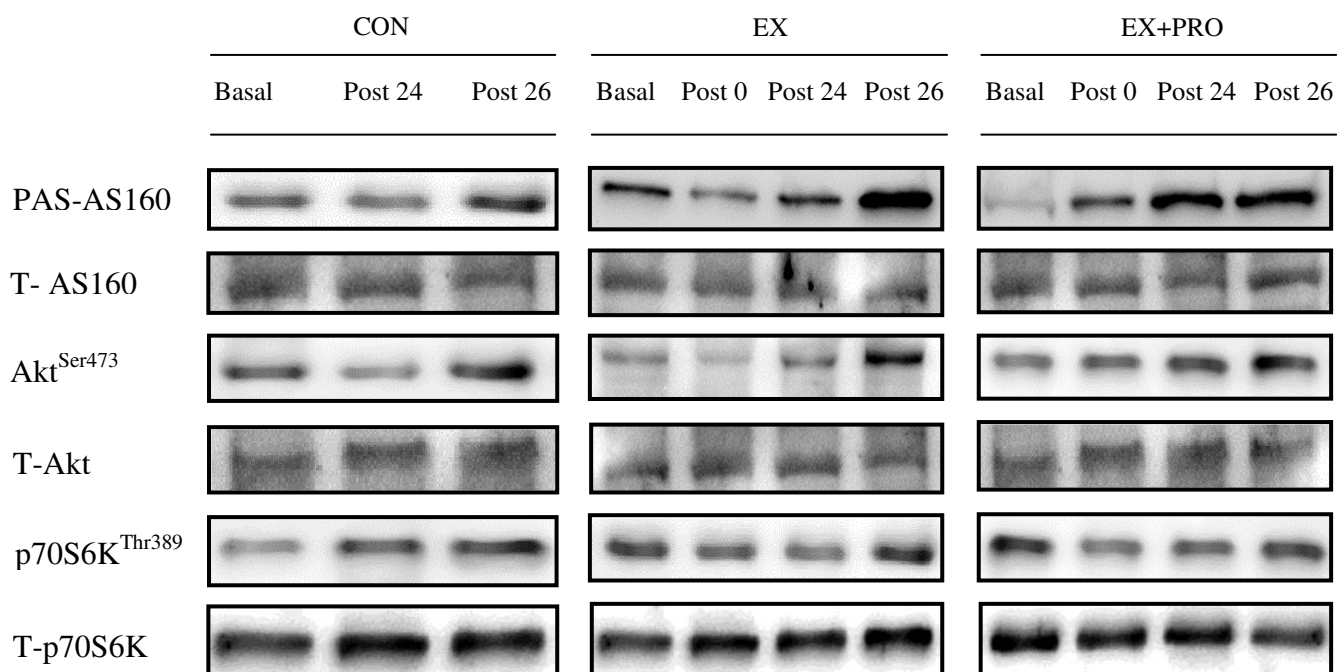
Figure 4.6 Representative protein phosphorylation blots. Proteins phosphorylation was studied in the basal, fasted state (Basal), immediately post-exercise (Post 0), 24 h post-exercise (Post 24) and 26 h post-exercise following the OGTT (Post 26) and normalized to total protein content.
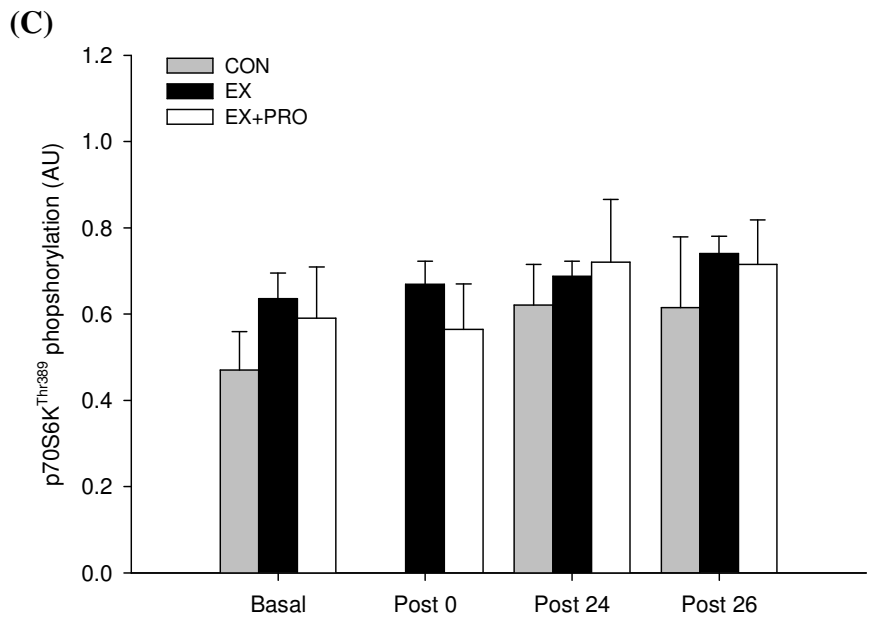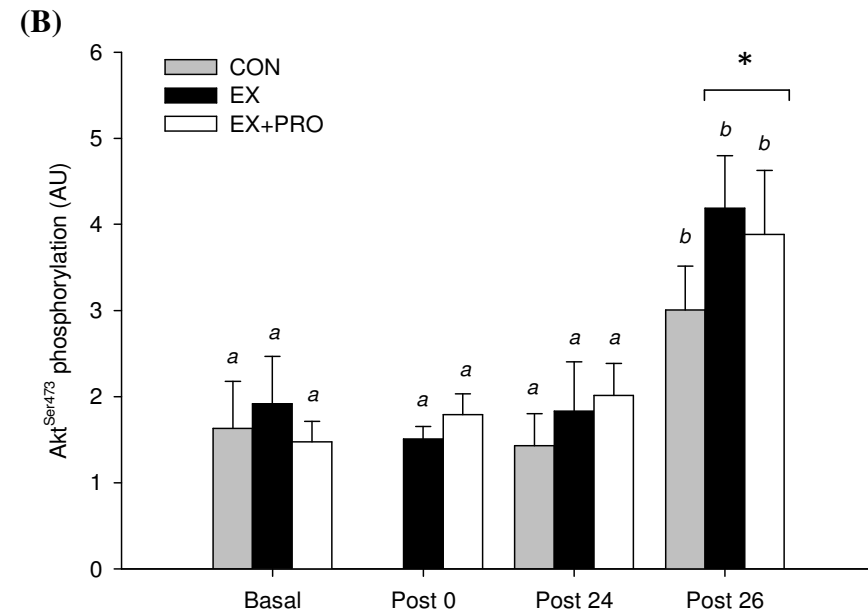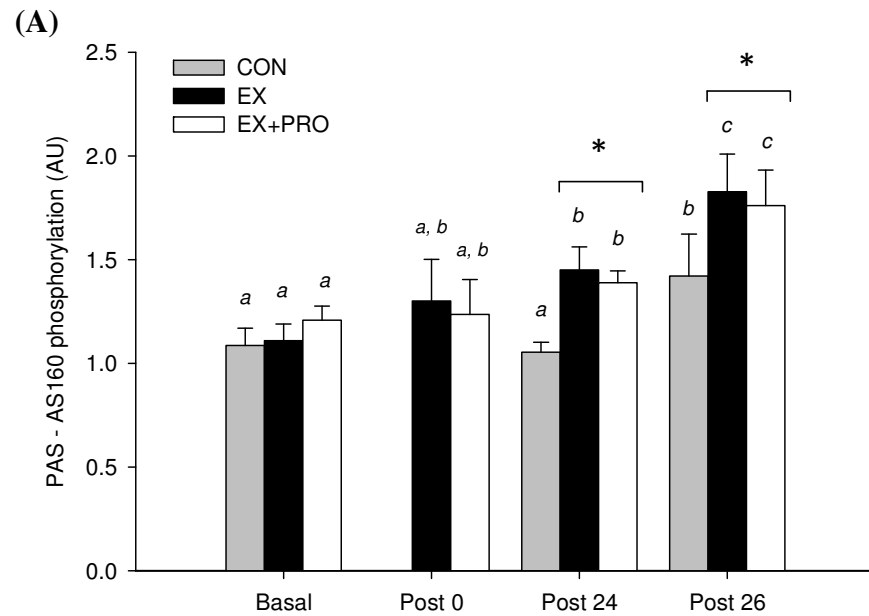
Figure 4.7 Signalling protein phosphorylation of (A) Akt^{ser473}, (B) PAS-AS160 and (C) $p70S6K^{Thr389}$ from muscle samples taken at 4 different time points. Groups as per Table 4.1. Values are means ± SEM; $n = 6$ per group. Means with different letters are significantly different from each other ($P < 0.05$). *: significantly lower phosphorylation for CON compared with EX and EX+PRO ($P < 0.05$).

4.5 Discussion

The present study expands on the findings of previous investigations (14, 30, 66) by providing a physiological mechanism to support evidence that a single bout of intense lower-body resistance exercise (RE) improves insulin sensitivity in healthy, normoglycemic adults. Using isotopic tracer techniques, we report that prior RE conducted 24 h earlier suppresses plasma glucose excursions during an oral glucose tolerance test (OGTT) by increasing the rate of glucose disappearance from the circulation (presumably taken up by skeletal muscle). Furthermore, we attribute the improvement in insulin-stimulated glucose disposal with prior RE, in part, to a greater basal and insulin-stimulated phosphorylation of AS160. However, co-ingesting protein during OGTT did not augment the response of plasma glucose, insulin sensitivity or glucose kinetics to resistance exercise despite a greater insulin response.

To date, studies investigating the acute effect of RE on insulin sensitivity and glucose homeostasis have provided conflicting results (5, 14, 15, 30, 33, 66). Whilst questions have been raised concerning the method used to assess insulin sensitivity (30), the discrepancies between prior studies may be due, in large part, to; i) the total RE volume, ii) the exercised muscle group and iii) the physical characteristics of the study cohort. Prior studies utilizing whole-body RE with a total volume of ~6500 kg (5, 15) showed no effect on plasma glucose between 12-18 h post-RE, in healthy young (15, 33) and insulin resistant populations (5, 15). On the other hand, Koopman et al. (30) demonstrated that a lower-limb RE protocol with total volume ~ 3-fold greater than the aforementioned studies (5, 15), was sufficient to improve insulin sensitivity in healthy adults. In the present study, we employed an identical RE protocol Koopman et al. (30) eliciting a similar total RE volume. Accordingly, we too demonstrated that RE improves insulin sensitivity and lowers post-prandial glucose

excursions at 24 h post-RE. However, we acknowledge that such intense RE may not be feasible for all insulin resistant/type II diabetic patients. To date, the minimum RE dose required to counteract symptoms of metabolic disease has not been determined.

In addition to the intensity of exercise, we posit that high-intensity RE confined to the leg muscles, may promote a greater increase in skeletal muscle glucose uptake than upper-body RE. Support for this notion comes from a study by Olsen et al. (48), in which, glucose uptake was found to be relatively well preserved in the arms of Type II diabetics compared with the legs, indicating that Type II diabetes may be, primarily, a disease of the lower-limbs. Taken together with the data mentioned above, these findings suggest that a substantial loading, particularly of the leg muscles, may be an important prerequisite for improved glucoregulation with RE. Thus, either intensity and/or muscles that are exercised may be important for improvement of glucose control.

Finally, in combination with RE intensity and muscle groups utilized, the physical characteristics of the study cohort should be considered when assessing the impact of RE on glycemic control. For example, Luebbers and colleagues (33) recruited young, resistance-trained males who habitually completed ≥ 3 RE sessions per week. Participants in this study completed a whole-body RE protocol at high (85% of 10RM) and low (45% of 10RM) intensities, matched for total volume (~12,600 kg). Compared with a non-exercise control trial, the authors reported no effect of low-, and more surprisingly, high-intensity RE on glucose uptake and insulin sensitivity during a hyperinsulinemic-euglycemic clamp. In contrast, others (14) have shown that normoglycemia is better maintained in Type II diabetics who completed a whole-body RE protocol (80% of 3RM) similar to that employed by Luebbers (33). Taken together, these data suggest that the physical characteristics of the study

cohort must be taken into account when prescribing an exercise volume intended to induce sufficient 'stress' across the exercised muscle to lower blood glucose.

The 17% reduction in plasma glucose response we observed with prior RE is in line with previous studies (14). However, modifications in blood analytes do not necessarily reflect adaptations in skeletal muscle glucose uptake. Therefore, we applied dual isotopic tracer methodology to determine the rate of glucose disappearance from the circulation (indicative of skeletal muscle glucose uptake (9)) and the contribution of exogenous glucose appearance and endogenous glucose production to total glucose appearance. Total glucose appearance and the contribution from endogenous and exogenous sources was not different between groups, indicating that gastric emptying, intestinal uptake and hepatic glucose output were not altered by prior RE or protein co-ingestion during OGTT. However, plasma glucose disappearance/disposal from the circulation did increase with prior RE. Further, we calculated that the time taken for glucose disappearance to match glucose appearance was reduced by ~37%. Thus, RE may be potent intervention to reverse the compromised insulin-stimulated glucose disposal that is prevalent in Type II diabetes (67). Despite the greater increase in glucose disposal in all groups during OGTT we report no increase, or between-group difference, in post-OGTT muscle glycogen content. This may not be surprising given that the amount of ingested glucose disposed from the circulation over 120 min of OGTT was not vastly different between exercised (~55%) and non-exercised groups (~50%).

Enhancement of glucose disposal with prior exercise appears to be mediated through the insulin signalling pathway. A number of studies have found that prior exercise has no effect on proximal insulin signalling steps, such as Akt phosphorylation (1, 20). Similarly, we report no effect of RE on basal Akt phosphorylation at 24 h post-exercise. In contrast, the Rab-

GTPase-activating protein AS160 is the most distal signalling protein implicated in insulin-stimulated glucose uptake in skeletal muscle. Studies in humans (12, 21) demonstrate that basal and insulin-stimulated PAS-AS160 phosphorylation is increased several hours post-RE. Until now, the sustained effect of prior exercise on PAS-AS160 phosphorylation has been demonstrated in animals only (1, 16). Here we demonstrate for the first time, in humans, that immunoprecipitated PAS-AS160 phosphorylation is greater at 24 h post-RE compared with basal and non-exercise values. Moreover, although the insulin-stimulated rise PAS-AS160 phosphorylation was similar for all groups, the final level of PAS-AS160 phosphorylation was greater for EX and EX+PRO compared with CON following the OGTT.

Important to note is that not all studies report similar adaptations in PAS-AS160 phosphorylation with prior exercise. Treebak and colleagues (60) have shown that basal and insulin-stimulated site-specific, but not PAS, phosphorylation of AS160 increased 4 h after a 60 min unilateral leg kicking protocol. Given that we and others observed a marked increase in basal PAS-AS160 phosphorylation following high-intensity RE loading, we speculate that the PAS-AS160 antibody may only be effective in detecting robust modifications in AS160 phosphorylation, whereas site-specific AS160 phosphorylation may be required to detect more subtle phospho-changes that occur with moderate-intensity exercise. Thus, regardless of the antibody used to detect AS160 phosphorylation our data expand human work (12, 21) and support animal studies (1, 16) showing the persistent increase in insulin-stimulated skeletal muscle glucose transport following acute RE is attributable, in part, to the sustained increase in basal AS160 phosphorylation. These data do not exclude the possibility that prior exercise also amplifies insulin-signalling steps other than AS160.

To our knowledge, this study is the first to attempt to determine if protein co-ingestion augments the response of RE on glycemic control at 24 h post-exercise. Under resting conditions, it has been demonstrated that the marked increase plasma insulin concentration that prevails when protein and/or amino acids are ingested with carbohydrate can effectively increase glucose disposal (36) and reduce plasma glucose excursions (62, 65). In our hands, the elevated insulin response with protein co-ingestion did not augment the RE-induced rise in glucose disposal or lower plasma glucose excursions. These data are supported by the response of the intracellular signalling indicating that although the final level of PAS-AS160 phosphorylation was greater with prior exercise, there was no difference with protein ingestion. We posit that the lack of a glycemic-lowering effect of additional protein could be attributed to the insulin response to our feeding protocol. Our data reveal that the transient rise in plasma insulin with additional protein peaked at 30 min post-feeding (21 and 13% greater than CON and EX, respectively) but was no longer evident by 60 min. In contrast, studies that report a glucose lowering effect with protein co-ingestion have favoured frequent feeding of small boluses to promote a sustained rise in plasma insulin, much greater than the present study (36, 62, 63, 65). For example, participants in the study of Manders et al. (36) ingested beverages every 15 min over 165 min supplying ~60g·h^{-1} carbohydrate alone or with ~30g·h^{-1} of additional protein. The greater plasma insulin response that prevailed with additional protein was sustained for the best part of 3 h. In contrast, the greater insulin response we observed with additional protein was relatively transient (between 30-50 min post-feeding). Thus, this relatively brief period of hyperinsulinemia may have been insufficient to further lower plasma glucose excursions. However, this approach has not been tried following resistance exercise.

A limitation of our protocol and that of other studies (36, 62, 63, 65) is that feeding high-glucose loads and/or repeated boluses does not represent a physiological feeding pattern of Type II diabetic populations. Conversely, the group of Nuttall and Gannon (17, 18, 26, 27, 45, 47) systematically investigated the insulinotropic properties of ≈ 5g of essential (arginine, phenylalanine, lysine and leucine) and non-essential amino acids (glycine, proline) co-ingested with 25g of glucose, closely mimicking the composition of a high-protein meal. By and large, these studies demonstrate that free amino acid co-ingestion initiates only a modest rise in insulin, but markedly attenuates the plasma glucose response. Combining these data with our observation that protein co-ingestion with a high glucose load did not lower blood glucose, one could suggest that co-ingesting specific free amino acids with glucose in an amount likely to be ingested during a typical meal, may lower plasma glucose excursions via subtle changes in plasma insulin or direct mechanisms acting independently of the insulin.

The lack of a glucose-lowering effect with protein co-ingestion could also be explained by the relatively modest leucine content (~2.65g) in the 25g of intact whey protein we provided. A number of studies have demonstrated that certain free amino acids can act as potent insulin secretagogues (53, 54). Indeed, a series of studies (31, 63-65) revealed that adding ~7.5g of free leucine to a protein hydrolysate plus carbohydrate beverage augmented the insulinotropic response. Subsequently, this mixture was shown to suppress post-prandial glucose excursions in Type II diabetics (35, 62). *In vitro* studies indicate this response may be due to the potency of leucine in secreting insulin release from pancreas β cells (55, 69). Furthermore, there is mounting evidence in animal and cell culture models, that leucine directly promotes glucose transport into skeletal muscle by increasing GLUT4 translocation via the mammalian target of rapamycin (mTOR) signalling pathway (2, 43, 51). Thus, given that we saw no change in p70S6K phosphorylation (downstream of mTOR) for EX+PRO, it is

possible that ingesting additional free amino acids, leucine in particular, are essential to facilitate glucose uptake in skeletal muscle and disposal from the circulation. Further work is required to determine the effectiveness of ingesting specific free amino acids, particularly leucine, in combination with RE for improving glycemic control.

Finally, it is worth mentioning that the studies discussed above, reporting a glucose-lowering effect of protein co-ingestion, were conducted under resting conditions. In contrast, we report no additive effect of protein co-ingestion, perhaps due to the fact that the glucoregulatory effects of feeding were assessed following high-intensity RE. Thus, we speculate that the robust increase in basal PAS-AS160 phosphorylation with prior exercise may have masked any additive effect of additional protein/insulin. Indeed our signalling data indicate that the insulin-stimulated rise in PAS-AS160 phosphorylation during OGTT was similar for all groups. Based on these data, it is unclear whether a synergy exists between RE and protein ingestion for promoting hypoglycemia.

In conclusion, we have shown that high-intensity resistance exercise improves insulin sensitivity and increases the rate of postprandial glucose disposal, which subsequently lowers post-prandial glucose excursions in healthy, normoglycemic adults. However, despite strong evidence that co-ingesting protein and/or amino acids with carbohydrate lowers glucose excursions at rest, we show no effect of protein co-ingestion on glycemic control, despite a marked rise in insulin secretion. Thus, we hypothesize that the co-ingestion of additional free amino acids and/or sustained hyperinsulinemia may be essential in order to increase skeletal muscle glucose uptake.

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Author contributions

L.B., A. P., C.S.S., A.E.J., K.B and K.D.T contributed to the conception and design of the experiment. L.B., A.P., C.S.S., K.B. and K.D.T. contributed to the collection of the data. L.B., A.P., C.S.S., K.B and K.D.T. contributed to the analysis and interpretation of the data. L.B., A.P., C.S.S., K.B and K.D.T. contributed to drafting or revising the content of the manuscript.

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CHAPTER 5

GENERAL DISCUSSION

Several studies have demonstrated that ingesting protein with carbohydrate during exercise can extend endurance capacity (20, 37, 38) and improve late-exercise performance (39). In the post-exercise period, protein ingestion may also impact the metabolic adaptation in skeletal muscle and potentially improve the recovery of muscle function. However, concerns regarding the way in which exercise performance and recovery are assessed, coupled with the lack of a clear physiological mechanism to explain the reported improvements, hampers our understanding of the role of protein nutrition for endurance athletes. Beyond athletic performance, there is great interest in modulating skeletal muscle metabolism to counter insulin resistance and Type II diabetes. Indeed resistance exercise has been demonstrated to improve whole-body insulin sensitivity (8, 23) and glycemic control (7), yet the mechanisms underpinning this adaptive response remain elusive. Furthermore, the notion that protein nutrition can help to maintain normoglycemia is yet to be investigated in combination with resistance exercise. Thus, the purpose of this thesis was three-fold:

i) **To determine the effectiveness of co-ingesting protein with carbohydrate during exercise on endurance performance and the recovery of muscle function.**

ii) **To determine the impact of post-endurance exercise protein ingestion on the synthesis of specific skeletal muscle proteins.**

iii) **To understand the mechanisms underpinning improved glycemic control following resistance exercise and investigate whether ingesting additional protein augments this response.**

The present chapter summarizes the key findings from the studies described in **Chapters 2-4** of this thesis. First, based on the findings described in **Chapters 2** and **3**, this section will provide recommendations for future research investigating the effectiveness of protein ingestion for endurance performance and changes in protein metabolism that, over time, may quicken the recovery of muscle function and alter the phenotypic adaptation to training. Second, based on the findings described in **Chapter 4**, this section will provide recommendations for future research studies expanding on our investigation of the effectiveness of resistance exercise, with or without protein ingestion, on glycemic control.

5.1   Protein nutrition for endurance performance

Over the last decade a number of studies have emerged to suggest that the co-ingestion of protein with carbohydrate during prolonged endurance exercise can extend endurance capacity (20, 37, 38). Furthermore, others have reported that protein co-ingestion specifically improves late-exercise time-trial performance (39). However, methodological differences in the performance test employed in previous studies makes it difficult to determine whether adding protein to carbohydrate improves endurance performance *per se or* merely time-to-exhaustion. In support of this notion, several studies report no ergogenic benefit of carbohydrate plus protein co-ingestion on time-trial performance in a well-controlled testing environment (33, 44). Furthermore, when sufficient carbohydrate is ingested the role of protein for improving endurance performance may be negligible (44). In an attempt to improve our understanding of the role of protein for endurance athletes, the logical extension of these findings was to examine the effectiveness of protein co-ingestion using a

performance model that replicates endurance competition and a carbohydrate dose that meets the recommended upper-limit to attain peak exogenous carbohydrate oxidation rates.

Thus, in our study (**Chapter 2**) we provided a recommended dose of carbohydrate (65 $g \cdot h^{-1}$) during cycle exercise to determine whether the addition of protein (19 $g \cdot h^{-1}$) improved cycle time-trial performance in a controlled exercise environment, when preceded by a standardized steady-state ride. Our data demonstrate no difference in average power output during the time-trial or overall time-to-completion between carbohydrate plus protein and carbohydrate alone. Thus, these data oppose the notion that carbohydrate-protein co-ingestion during exercise enhances late-exercise performance when sufficient carbohydrate is ingested.

5.1.1 Future studies

In the study described in **Chapter 2**, we examined the effectiveness of adding protein to a carbohydrate-containing beverage on late-exercise time-trial performance and the recovery of muscle function. Our decision not to match treatment beverages for total energy was based on the observation that others report an extended endurance capacity when protein was co-ingested with a carbohydrate dose deemed sub-optimal to attain peak rates of exogenous carbohydrate oxidation (20, 37, 38). Thus, our findings support those of van-Essen et al. (44), indicating that there is no benefit of co-ingesting additional protein in a sports beverage on time-trial performance provided sufficient carbohydrate is consumed. However, the metabolic and physiological response to carbohydrate-protein co-ingestion may be dependent on the mode, duration and intensity of the exercise bout. In this regard there is evidence that protein oxidation is increased when protein is ingested with a carbohydrate

intervention during ultra-endurance exercise (24). However, the effectiveness of protein co-ingestion for performance, in situations where carbohydrate availability is limited and muscle glycogen stores are depleted has yet to be investigated, but would prove interesting given evidence from almost 20 years ago that runners ingesting branched-chain amino acid alone took less time to complete a marathon (2).

5.2   Muscle adaptation to acute endurance exercise and protein nutrition

Endurance exercise stimulates an increase in mixed muscle protein synthesis. This response has been shown to result primarily from a rise in the synthesis of mitochondrial proteins, with little or no change reported in the synthesis of myofibrillar proteins (51). Given evidence that ingesting protein and/or amino acids potentiates resistance exercise-stimulated muscle protein synthesis, investigators have suggested that protein nutrition may enhance the synthesis of mitochondrial proteins following endurance exercise (19). Our study (**Chapter 3**) examined the response of mitochondrial and myofibrillar protein fractions to protein ingestion following endurance exercise. We report that myofibrillar protein synthesis was greater when protein was ingested with a carbohydrate beverage ($\sim0.087\%{\cdot}h^{-1}$) than carbohydrate ingested alone ($\sim0.057\%{\cdot}h^{-1}$). Conversely, protein ingestion did not stimulate an increase in mitochondrial protein synthesis. Thus, we are able to attribute the increase in mixed muscle protein synthesis demonstrated by Howarth et al. (19) to a rise in the synthesis of myofibrillar, but not mitochondrial proteins.

Although the primary aim of our study was to determine the synthesis of specific protein fractions, in collaboration with Drs Keith Baar and Andy Philp of the University of

California, Davis, we were able to measure the phosphorylation of signalling proteins implicated in the translation initiation and elongation phase of muscle protein synthesis immediately and 4 hours post-exercise for 8 participants. In spite of the fact that some of the anabolic signalling response may have occurred between 0 and 4 hours post-exercise, we demonstrate that the phosphorylation of intracellular proteins was increased (mTOR, p70S6K) or was suppressed (eEF2) following protein ingestion, thereby providing a mechanism to support our muscle protein synthesis data. Collectively, these data suggest that when protein is ingested following intense cycling exercise contractile proteins are preferentially synthesized in the initial 4 hour post-exercise period.

5.2.1   Future studies

An acute increase in myofibrillar protein synthesis, with protein ingestion and exercise, is predictive of long-term muscle hypertrophy (17, 52). Thus, one interpretation of the data presented in **Chapter 3** is that protein ingestion following prolonged high-intensity cycling over an extended period may increase lean mass. However, the rise in myofibrillar protein synthesis we observed post-exercise, may serve to counteract a fasted-state rise in protein breakdown thought to occur during and following intense endurance exercise (3, 12, 45). Thus, the rise in myofibrillar protein synthesis may represent an increase in protein turnover; important in the maintenance of a higher quality of contractile protein. However, we did not measure protein breakdown and thus, this notion is speculative at this point. Thus, it would be prudent for future studies to ascertain changes in muscle protein breakdown (and proteins implicated in proteolysis) during and following endurance exercise with protein feeding. Finally, given that resistance-exercise performed in the fed-state elevates muscle protein synthesis (54) over that performed when fasted, it is likely that the adaptive response to

protein ingestion after endurance exercise would be dramatically altered by prior feeding. This approach may also represent, more closely, common practice amongst athletes and warrants further investigation. Based on data from previous studies (3, 12, 16, 45) and our observation that myofibrillar proteins are preferentially synthesized when protein is ingested after endurance exercise, we present herein the proposed temporal response of myofibrillar net protein balance over the course of the carbohydrate plus protein trial (Figure 5.1).

In our study, muscle protein synthesis was assessed over 4 hours post-exercise. The response of mitochondria to endurance exercise is a transcriptionally mediated process and occurs later than the time-course of our investigation (11, 18, 40). Thus, a logical extension to our findings would be to investigate the response of myofibrillar and mitochondrial protein synthesis to endurance exercise and protein ingestion beyond 4 hours post-exercise. Such an approach would improve our understanding of the muscle adaptations that occur when protein is ingested after endurance exercise. Furthermore, to resolve the precise role of protein nutrition for endurance athletes we suggest that longitudinal studies must be conducted, in which, protein is ingested post-exercise over the course of a training regimen to determine the chronic phenotypic and physiological adaptations.

Other possibilities to explain the lack of change in mitochondrial muscle protein synthesis is that the training status of the subjects and the exercise protocol utilized in the present study. It has previously been demonstrated that the response of mitochondrial protein synthesis to an acute bout of cycling tends to be lower in individuals after 10-weeks of endurance training (51). Thus, our data may suggest that the synthetic response of mitochondrial proteins in well-trained individuals reaches a 'ceiling'; the point beyond

which, further contractile and/or nutrient stimuli fail to augment the synthetic response. Thus, future studies should seek to characterize the response of different protein fractions to endurance exercise in cohorts with divergent phenotypes (i.e. untrained healthy, untrained obese, resistance trained).
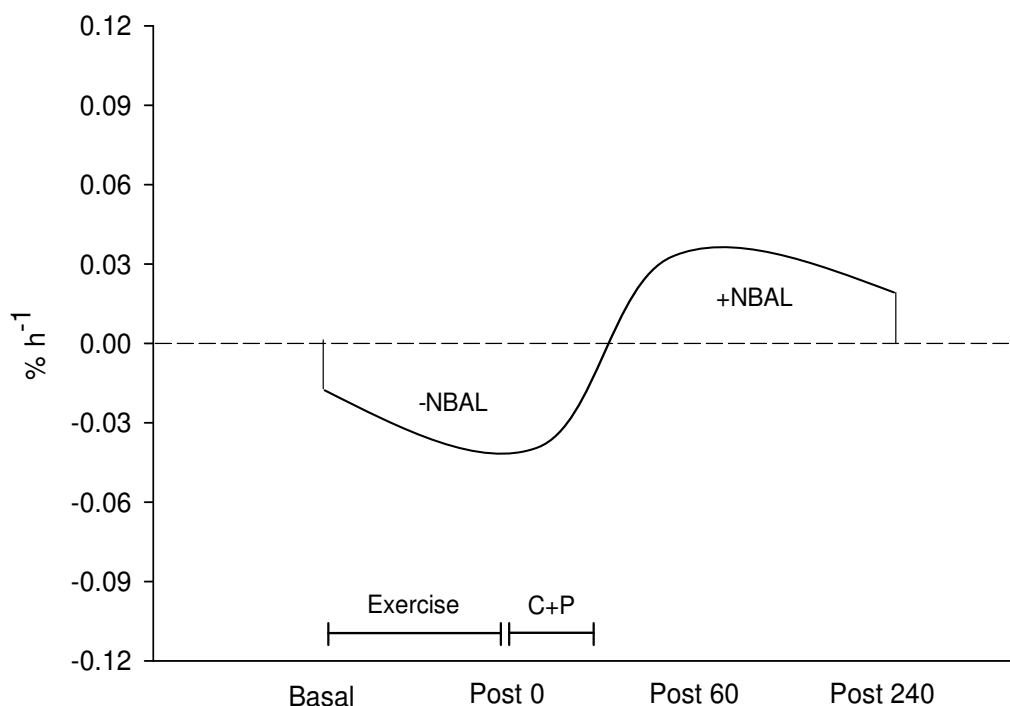
Figure 5.1 Proposed time-course of net myofibrillar protein balance following endurance exercise and protein feeding. Temporal swing of negative (-NBAL) and positive (+NBAL) net myofibrillar protein balance throughout the protocol described in Chapter 3.

5.3   Protein nutrition for recovery from endurance exercise

Although we have demonstrated that there is no effect of carbohydrate-protein co-ingestion on endurance exercise performance, the findings of **Chapter 3** indicate that protein ingestion after endurance exercise has a marked influence on the synthesis of contractile

proteins and this may play an important role in the recovery process. The increase in muscle protein synthesis we and others (19, 25) demonstrate when protein is ingested after endurance exercise has been suggested as a potential mechanism to explain how protein ingestion attenuates indirect markers of ultra-structural damage such as, for example, plasma creatine kinase concentration (26, 29, 36-38, 41, 43). Ultimately, these acute adaptations are thought to improve the subsequent rate of recovery; defined as the recovery of endurance capacity (37, 53) or muscle function (41, 43).

During the study outlined in **Chapter 2**, participants returned to the laboratory 24 hours post-exercise to determine whether the additional protein ingested during the initial exercise bout ameliorated muscle soreness, plasma creatine kinase concentration and improved the recovery of maximal isometric strength. As expected, we observed a rise in plasma creatine kinase and perceived muscle soreness and a resultant decline in isometric strength at 24 hours post-exercise. However, the additional protein did not attenuate indices of muscle damage or improve the recovery of isometric strength. Thus, despite evidence in **Chapter 3** indicating that ingesting additional protein in the post-exercise period increases the synthesis of structural proteins, we demonstrate no effect of protein ingestion during exercise on any of the indices of recovery we measured.

5.3.1 Future studies

The effectiveness of protein nutrition as a means to improve the rate of recovery is likely hampered by time lapse between exercise completion and the assessment of recovery. Reported changes in muscle damage with protein ingestion are typically demonstrated several

hours after exercise (37, 53). Despite the robust acute increase in myofibrillar protein synthesis we observed, the complete turnover of myofibrillar proteins (i.e. incorporated into the myofiber) is relatively slow; on the order of days-to-weeks (34, 35). Thus, it is difficult to comprehend how this process may influence changes noted in just a few hours. These data may explain why we and others (13) were unable to detect any influence of protein ingestion on recovery between 24 hours post-exercise. Therefore, based on our combined findings in **Chapters 2** and **3**, it would prove useful for future studies to examine recovery in the immediate hours through to several-days post-exercise.

The lack of conformity between the data presented may also be explained by the different feeding protocols employed. The absence of any improvement in recovery (Chapter 2) was demonstrated when protein was ingested during prior exercise. Conversely, the increase in myofibrillar protein synthesis we report (Chapter 3) occurred when protein was ingested following exercise. Given that no study has investigated the influence of manipulating the timing and/or frequency of carbohydrate-protein co-ingestion on subsequent rates of recovery, this avenue of investigation is important in order to shed further light on the effectiveness of protein nutrition for endurance athletes.

5.4   Metabolic adaptation to acute resistance exercise and protein nutrition

Acute resistance exercise can improve glycemic control in healthy (23) and Type II diabetic individuals (7) via insulin-independent mechanisms (6, 42). Further, co-ingesting protein and/or amino acids with carbohydrate loads potentiates the release of insulin, reducing the prevailing plasma glucose response (28, 48). Thus, resistance exercise and

protein nutrition represent safe alternatives to pharmacological interventions to alleviate or reverse the manifestations of Type II and its co-morbidities.

Our study (**Chapter 4**) examined the effectiveness of an intense lower-limb resistance exercise bout on glycemic control during a glucose challenge at 24 hours post-exercise. One group of participants co-ingested additional protein during the glucose challenge to determine whether protein feeding would augment the exercise-induced changes in glucoregulation. Using a dual stable isotopic tracer technique during the glucose challenge, we demonstrated that prior exercise increased the rate of glucose disposal from the circulation by ~12%, which ultimately, lowered post-prandial plasma glucose excursions by ~17% compared with non-exercised controls. At the skeletal muscle level, we demonstrate that prior exercise increased basal PAS-AS160 phosphorylation. These data suggest that greater basal AS160 phosphorylation with prior exercise enhances insulin-stimulated glucose uptake, as evidenced by the greater rate of glucose disposal and the final level of PAS-AS160 phosphorylation following OGTT. Interestingly, protein co-ingestion during the glucose challenge did not augment the exercise-induced improvement in glycemic control or modify signalling protein phosphorylation, despite an elevated insulin response in this sub-group.

5.4.1   Future studies

The high-intensity exercise model utilized in **Chapter 4** was adopted based on data suggesting that a substantial total exercise volume is required in order to promote a beneficial effect on glucose homeostasis (4, 8, 23, 27), particularly in non-diabetic individuals. Whilst the total exercise volume in our study and that of Koopman et al. (23) was ~3-fold greater than studies demonstrating no glucose-lowering effect of resistance exercise (4, 8, 27), the

fact that exercise was restricted to the lower-limbs may also have contributed to the reduction in blood glucose. Indeed, Olsen et al. (32) have shown that Type II diabetes may, primarily, be a disease of the lower limbs (32). Thus, investigating the chronic effects of lower-limb versus whole-body resistance training regimens on glucose homeostasis and insulin sensitivity in Type II diabetics would be an interesting route for future studies. Furthermore, although a growing body of experimental evidence suggests that long-term resistance training improves metabolic homeostasis in a manner distinct from endurance training; our understanding of how this precisely occurs is rudimentary. Thus, additional research is clearly needed to better understand how different modes of physical training (i.e., resistance, endurance or an interval exercise) change the metabolic and qualitative characteristics of skeletal muscle and improve glycemic control. Finally, given that the typical physiological characteristics of Type II diabetics are dramatically different from young, healthy, normoglycemic males we recruited, the high-intensity resistance exercise protocol in our study may not be feasible for Type II diabetic individuals to complete. Thus, future studies should seek to determine the minimum 'dose' of resistance exercise required to counteract symptoms of metabolic disease.

In **Chapter 4** we demonstrated that basal and PAS-AS160 phosphorylation was increased with prior exercise. Thus, the elevated glucose disposal with prior exercise can be attributed, in large part, to an increase skeletal muscle glucose uptake mediated by AS160. However, this does not exclude the possibility that prior exercise also amplifies insulin-signalling steps other than AS160. In addition, we and others (1, 15) report no effect of prior exercise on the basal phosphorylation of Akt^{Ser473}. Interestingly, we did observe an increase in insulin-stimulated Akt phosphorylation with prior exercise. Thus, future studies should seek to characterize, in greater detail, basal and insulin-stimulated modifications in insulin-

signalling intermediates following acute resistance exercise. Finally, it should be noted that this study presents a limitation due to the number of the participants analyzed for protein phosphorylation (6 per group). Thus, due to the small sample size and insufficient statistical power, it is possible the analyses may have contained a type 2 error. As a consequence, the lack of statistical significance in proximal insulin signalling intermediates should be taken with caution.

Under resting conditions, the insulinotropic response of co-ingesting protein with carbohydrate is thought to facilitate the reduction in plasma glucose excursions (46, 49). In **Chapter 4**, we show that, despite an elevated insulin response, protein co-ingestion during a glucose challenge did not augment the beneficial effect of resistance exercise on plasma glucose disposal or glucose excursions. We posit that this null finding may be due to; i) the relatively brief period of hyperinsulinemia with protein co-ingestion, ii) the absence of specific amino acids or iii) the fact that the robust effect of prior exercise on insulin signalling and glucose disposal may have masked an additive effect of protein/insulin. Indeed, the hypoglycaemic effect of protein co-ingestion has been demonstrated with different feeding protocols to that used in our study. In summary, these data suggest that a period of sustained hyperinsulinemia (28, 46, 47, 49) or specific free amino acids (9, 10, 21, 22, 30, 31) may be important in order for protein ingestion to lower blood glucose concentration. Given the potency of protein ingestion for maintaining glucose homeostasis, further studies should investigate the effect of different protein feeding strategies (for example, frequent feeding and branched-chain amino acid ingestion) following resistance exercise to ascertain whether protein and/or amino acids can augment the metabolic benefits we have found.

Finally, it would be remiss not to mention that ingesting protein and/or amino acids confers other benefits to metabolic health benefits besides glucoregulation. In addition to hyperinsulinemia, co-ingesting protein with carbohydrate promotes hyperaminoacidemia and has been found to stimulate protein synthesis and inhibit protein breakdown (14, 50). Thus, ingesting amino acids following resistance exercise may blunt the markedly elevated muscle protein breakdown rates commonly found in uncontrolled Type II diabetes (5). Future studies should seek to clarify this hypothesis.

5.5   Conclusions

In this thesis we have demonstrated that protein nutrition during exercise does not improve cycle time-trial performance, attenuate purported markers of muscle damage or enhance the recovery of muscle strength. Alternatively, protein ingestion following endurance exercise can affect the acute response of intracellular signalling proteins implicated in mRNA translation, thereby increasing the synthesis of contractile muscle proteins. Thus, over time this acute adaptive response may; i) promote muscle hypertrophy or ii) confer benefits for muscle recovery and regeneration. However, it is possible that an increase in mitochondrial protein synthesis may occur at a later time-point in the recovery process.

Beyond athletic performance and adaptation, data presented in this thesis demonstrate that a single bout of high-intensity resistance exercise increases insulin-stimulated glucose uptake, lowering plasma glucose concentration during feeding 24 hours post-exercise. The mechanism accounting for this action appears to involve a greater phosphorylation of distal

insulin-signalling intermediates that subsequently promote an increase in insulin-stimulated glucose transport in skeletal muscle. However, in this study the ingestion of additional protein did not impact the signalling response in skeletal muscle or improve glycemic control.

5.6   References

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CHAPTER 6

APPENDICES - METHOD DEVELOPMENT

6.1   Method of mitochondrial and myofibrillar protein isolation

Studies measuring abundant skeletal muscle protein fractions, for example, myofibrillar and sarcoplasmic proteins have been relatively forthcoming since methods to separate out these fractions were developed in the late 1990s. Yet, studies reporting the effects of interventions on mitochondrial protein synthesis have been elusive. This apparent underreporting of mitochondrial protein synthesis is likely due to technical difficulties rather than a lack of aspiration by researchers in this area. Indeed, the amount of mitochondrial protein from rat skeletal muscle yields ~1 and 2-4 mg/g muscle wet weight of sub-sarcolemmal (SS) and intermyofibrillar (IMF) mitochondria, respectively (5). Most human muscle biopsies taken from the *vastus lateralis* muscle weigh ~100-200 mg wet. Therefore, the expected yield of mitochondrial protein from muscle biopsy tissues would be < 1 mg. This amount of protein is challenging to measure using current GCMS and GC-C-IRMS methods. Therefore, it is not surprising that limited research is available on the regulation of mitochondrial protein synthesis with exercise or nutrient provision.

There are two sub-populations of mitochondria in the muscle; IMF mitochondria are located between myofibrils and SS mitochondria are located beneath the sarcolemma. SS mitochondria represent 20% of mitochondria within skeletal muscle and IMF mitochondria account for the remaining 80% (2). In humans, both populations of mitochondria have been

shown to adapt to endurance exercise training by increasing in number (3, 4). Until recently, studies in humans had only characterized the rates of protein synthesis of SS mitochondria at rest (1, 6). In one of these studies, Rooyackers et al. (6) stated that they were unable to obtain the IMF mitochondria because the proteolytic enzymes required to do so interfered with the measurements of the rate of synthesis of myofibrillar proteins. More recently, Wilkinson et al. (7) characterized the synthetic response of mitochondrial proteins to different modes of exercise by combining both IMF and SS mitochondrial fractions obtained during sample processing.

In **Chapter 3** of this thesis, we adapted the method of protein isolation from Wilkinson et al. (7). This method utilizes techniques of muscle tissue homogenization and centrifugation to separate cellular components of different densities. The principle underlying this technique is that the heaviest (or most dense) cell components pellet in less time and with less force than is required to pellet lighter organelles such as mitochondria. Therefore, once the tissue homogenate is obtained, a first slow speed spin (~1000g for 10 min) is used to pellet the myofibrillar protein. The supernatant is then removed and transferred to another tube and centrifuged at a higher speed (~10,000g for 10 min) to pellet the lighter mitochondria. Prior to study analyses, we confirmed (in human tissue from pilot studies) that this method was appropriate to obtain both mitochondria and myofibrillar proteins from a mixed muscle homogenate.

6.1.1   Method preparation and supplies

- Tissue needed: Possible with 40-50mg but better with 70-80mg.
- 4mL black screw top glass vials.
- Coated razorblade to divide muscle in N2.

- Teflon (polypropylene) pestles (Schutt Labortechnik GmbH: Product # 3.200.512).
- Steel dish to store samples in N2.
- 1.5mL Eppendorf.
- Pipette tips (blue, yellow, pastette pipettes).
- Glass culture tubes (Fisher scientific 10x75mm: Product # 14-961-25).
- Glass Dounce homogenizer with glass pestle x 2.

6.1.2   Sample preparation prior to isolation

- When weighing the sample always keep on N2 or dry ice.
- Use fresh 1.5mL eppendorf to zero the balance. Once sample (in cryotube) is out of duer and into dish of N2 (or dry ice) open cap and tip muscle out into the N2.
- Using razorblade/scalpel, chip the frozen muscle to weigh out very specific amounts then add to 1.5mL eppendorf.
- Discard old cryotube.

6.1.3   Reagent preparation

- 0.3 M Sodium hydroxide (NaOH)
- 95% Ethanol (EtOH)
- 6 M Hydrochloric acid (HCl)
- 1 M Perchloric acid (PCA)
- β-2-glycerophosphate (Sigma: Product # G9891-25G)
- MgCL (Sigma 99.0 – 102%: Product #M9272-100G)
- NaFl (Sigma: Product # 51504-100G)
- Tris base / Tris HCl (Sigma: Product #T6066-1KG / Sigma: Product # T5941-500G)
- EDTA (Siga: Product # E9884-100G)
- KCl (Sigma 99%: Product # P4505-1KG)

Buffer is made up into 20mL of 50mM Tris buffer. Tris buffer contains 6.75g/L of Tris HCl and 1.18g/L of Tris base for 0.05 M solution. The following reagents are weighed and placed into 50mL falcon tube with 20mL of Tris buffer added on top.
.

- 10mM β-2-glycerophosphate = **43.2mg**

- 5mM MgCl^2 (203.3 g/mol) = **20.33mg**

- 50mM NaFl (42 g/mol) = **42mg**

- 1mM EDTA (372.2g/mol) = **7.4mg**

- 0.1mM KCl (10mM stock) = **20uL**

Split buffer into 2 x 10mL aliquots, 1 x 10mL with 1.5% BSA and 1 x 10mL BSA free.

Therefore 10mL buffer with 1.5% BSA = **150mg** BSA added.

Note: A minimum of 70mg of muscle tissue is required to reliably and repeatedly extract myofibrillar and mitochondrial (combining IMF and SS mitochondria) protein fractions using a [^{13}C$_6$] phenylalanine tracer.

Figure 6.1 Protein isolation protocol. Procedure for separating myofibrillar, IMF and SS mitochondrial, collagen and sarcoplasmic protein fractions

Dounce homogenization of muscle on ice

1. Add 10 µl /mg cold homogenization buffer (+BSA) to sample in the 1.5mL eppendorf. Use Teflon pestle whilst sample is still frozen to homogenize until majority of tissue suspended.
2. Spin tube at 1000 g for 10 min @ 4ºC
3. Transfer supernatant with pipette (being careful not to pipette any of the pellet) to another eppendorf labelled **SS MITO**

1.5ml eppendorf tube

1. Add ~400 µl of homogenization buffer (+BSA) to the pellet
2. Homogenize with Teflon pestle. Transfer all to glass Dounce homogenizer.
3. Homogenize in Dounce tube with glass pestle leaving no large amts of tissue (TIGHT pestle is best).
4. Transfer to new eppendorf labelled **MYO**
5. Add 100µl more buffer (+BSA) to Dounce to wash extra muscle in and also transfer to MYOFIB tube.
6. Spin at 1000 g for 10 min @ 4ºC
7. Pipette off supernatant to new 1.5mL eppendorf labelled **IMF MITO**

SS and IMF MITO tubes

1. Spin tubes at 10,000 g for 10 min @ 4ºC
2. Remove supernatants and put in one 1.5mL eppendorf labelled **SARC** being careful not to disturb mitochondrial pellet

SS and IMF MITO pellets

1. Add 500 µl buffer (-BSA). Vortex to fully suspend. Spin at 10,000g for 5 min @ 4 ºC.
2. Remove and discard supernatant
3. Repeat steps 1 and 2.
4. Add 500 µl EtOH (95%). Vortex and spin @ 10,000g for 10 min @ 4 ºC.
5. Remove and discard EtOH
6. Add 500µL 0.05 M HCL to pellet and break up using pipette.
7. Add re-suspended pellet to new glass tube
8. Repeat steps 6 and 6
9. Add 1mL Dowex resin and hydrolyze overnight @ 110 ºC

SARC tube

1. Add 500 µl of 1 M PCA. Vortex to suspend. Spin at 10,000g for 5 min @ 4ºC (precipitate the AA's).
2. Remove and discard supernatant
3. Add 500 µl EtOH (95%). Vortex quickly do not re-suspend pellet. Spin at 10,000g for 5 min @ 4 ºC.
4. Repeat steps 6-9 of SS and IMF MITO pellets (see right)

MYO and COLLAGEN pellet

1. Add 500 µl homogenizing buffer (-BSA) and vortex to fully suspend. Spin at 1000g for 5 min @ 4ºC.
2. Remove and discard supernatant. Repeat (to remove BSA protein from myofibrillar and collagen pellet)
3. To the remaining pellet add 1 mL 0.3 M NaOH. Vortex thoroughly and heat @ 37 ºC for 30 mins.
4. Transfer supernatant in glass vials for IRMS.
5. Add 500 µl 0.3 M NaOH and vortex to fully suspend then heat @ 37 ºC for 10 mins
6. Remove supernatant and add to glass vial. Pellet contains **COLLAGEN**.

MYO tube (Myofibrillar protein recovery)

1. To the supernatant add 1000 µl of 1 M PCA.
2. Spin tube at 1000 g for ~10 min @ 4ºC. Place vials (without caps) in centrifuge cups using tweezers.
3. Remove and discard supernatant
4. Add 500 µl EtOH. Spin at 1000g for 10 mins @ 4ºC. Remove and discard EtOH.
5. Add 500µL 0.05 M HCL to pellet and break up using pipette
6. Repeat steps 6-9 of SS and IMF MITO pellets (see above)

6.1.4   Analysis of [$^{13}C_6$] phenylalanine enrichment by gas chromatography-combustion-isotope ratio mass spectrometry

Establish: $^{13}C_6$ phenylalanine in mitochondrial and myofibrillar fractions.

Rate (°C/min):   10°C/min   1.0°C/min   20°C/min

Temp (°C):   110°C - - - - - - ▶ 235°C - - - - - - ▶ 245°C - - - - - - ▶ 280

Time (min):   1.0'   3.0'   1.0'   10.0'

- Column: Agilent technologies DB 1701 (length 30 m, I.D. 0.25 mm, film 1µm).
- RT: 33-34 min (run standard prior to every run. RT may change as column ages).

Inlet method

Base Temp: On

Base Temp: 250°C

Mode: Splitless

Split flow (ml/min):   50

Splitless time (min):   1

Surge pressure: Off

Surge pressure (kPa):   3

Surge duration (min):   0

Constant purge: On

Stop purge at (min):   0

Autosampler method

Sample volume: 1 ul

Air volume: 0.5 ul

Injection delay: 2 s

Pullout delay: 1 s

Injection speed: 100 ul/s

Pull-ups pumps: 5

Pull-ups volume: 5 ul

Pull-ups delay: 0 s

Pre-injection washes: 1

Pre-injection volume: 5 ul

Post-injection washes: 3

Post-injection volume: 8 ul

6.1.5   Citrate synthase activity assay

A citrate synthase (CS) activity assay was used as a measure of the mitochondrial content of each fraction. In short, the different fractions obtained from the muscle homogenization were re-suspended in the homogenization buffer (described above, minus BSA), containing 0.1% Triton-X to solubilize the mitochondrial inner and outer membranes. A Bradford assay was performed in order to determine protein yield. An equal amount of protein from each fraction was tested for CS activity. Our aim was to achieve a low level of CS activity in the sarcoplasmic and mixed muscle fractions and a high level of CS activity in the mitochondrial fractions. The protocol for the CS activity assay was as follows; 1mL of 0.1 M Tris buffer, pH 8.0, heated to 37°C, was added to a cuvette containing 10 µl of 10mM DTNB in 0.1M Tris buffer and 2 µl of 30 mM acetyl-CoA in water. To this, a volume of the muscle homogenate was added to obtain 12 µg of protein, which was then tapped gently by hand to mix. The spectrophotometer (Thermo, Helios Lambda) was zeroed and 10 µl of 50 mM oxaloacetic acid in 0.1M Tris buffer were added to start the reaction. Absorbance were recorded at 412 nm every 30 s for 5 min. The premise of this assay is that the faster the absorbance changes the greater the CS activity. CS activity assay was performed on human skeletal muscle tissue (*vastus lateralis*) obtained during pilot testing.
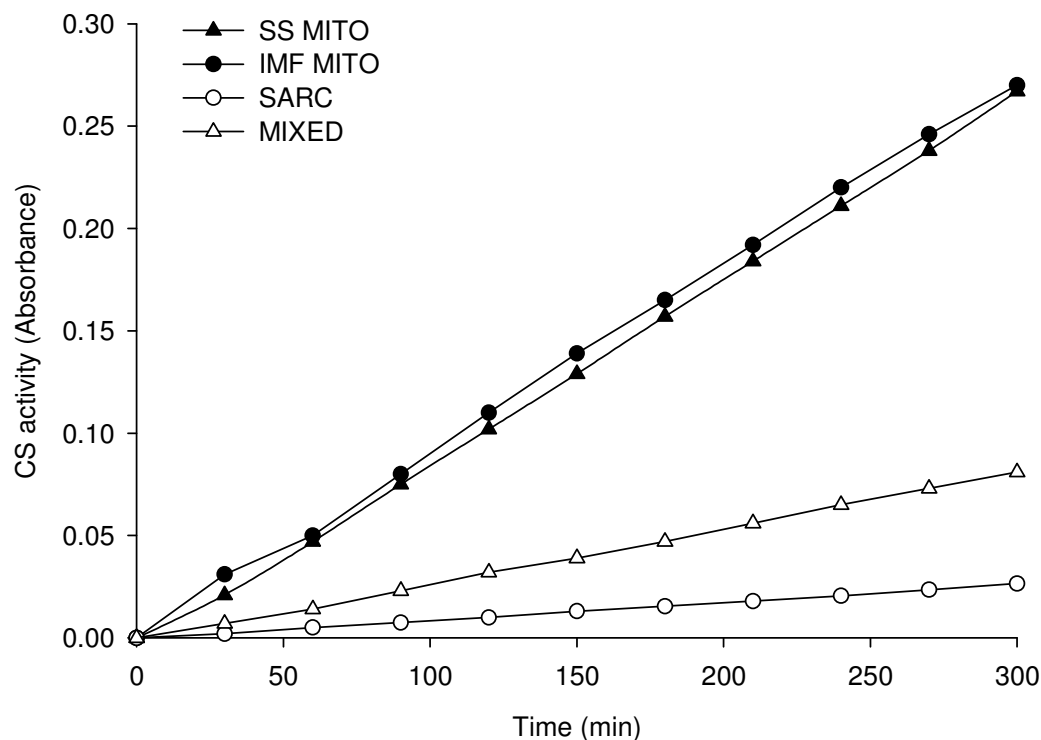
Figure 6.2 Citrate synthase activity assay. Change in absorbance of 12µg of mixed and sarcoplasmic protein (low activity) and intermyofibrillar and sub-sarcolemmal mitochondrial protein (high activity) obtained from human skeletal muscle tissue.

6.1.6   Western blots

We tested for the presence of myofibrillar and mitochondrial proteins in each fraction using gel electrophoresis. In brief, Bradford assays were used to determine the protein concentration of each fraction, after which samples were standardized to 1 mg/ml by dilution with Laemmli loading buffer in. Fifteen µg of protein/lane was loaded onto Criterion XT Bis–Tris 12% SDS-PAGE gels (Bio-Rad, Hemel Hempstead, UK) for electrophoresis at 200 V for 60 min. Gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol) for 30 min before proteins were electroblotted onto 0.2 lm PVDF membranes (Bio-Rad) at 100 V for 30 min. After blocking with 5% low-fat milk in TBS-T (Tris Buffered Saline and 0.1% Tween-20; both Sigma-Aldrich, Poole, UK) for 1 h, membranes were rotated overnight with primary antibody against the aforementioned targets at a concentration

of 1:2,000 at 4°C. Membranes were washed (3 x 5 min) with TBS-T and incubated for 1 h at room temperature with HRP-conjugated anti-rabbit secondary antibody (New England Biolabs, UK), before further washing (3 x 5 min) with TBS-T and incubation for 5 min with ECL reagents (enhanced chemiluminescence kit, Immunstar; Bio-Rad). Blots were imaged visually inspected for the presence of slow myosin heavy chain (MHCI; DSHB A4.951) and cytochrome C oxidase (COX; Cell Signalling 4280) protein using the Chemidoc XRS system (Bio-Rad, Hemel Hempstead, UK). A positive test would reveal the presence of slow MHC isoforms in the myofibrillar fraction but not in the mitochondrial fractions. Likewise, a positive test would reveal the presence of COX in the mitochondrial, but not myofibrillar fractions.
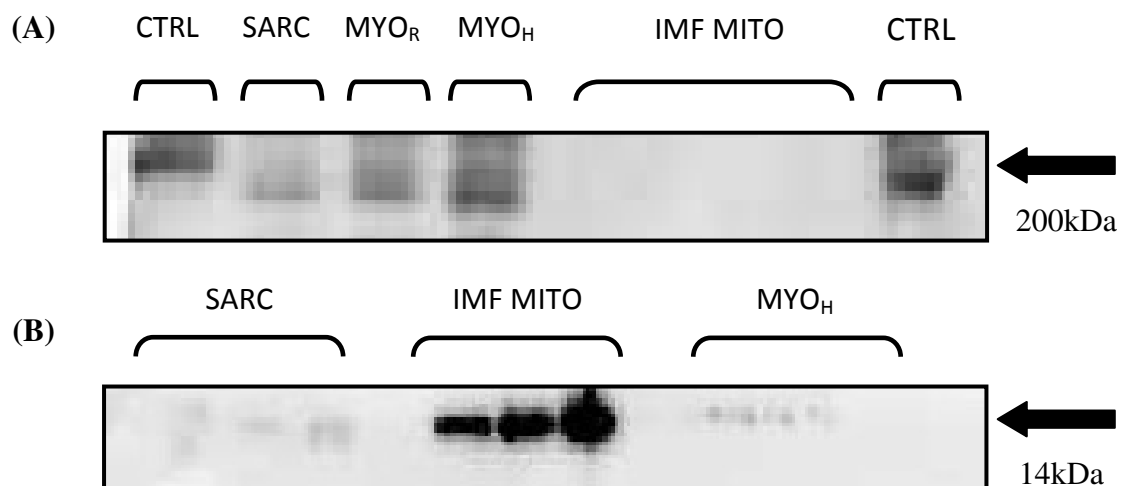
Figure 6.3 Western blot analysis of isolated protein fraction purity. (A) Total slow myosin heavy chain protein content in HeLa control (CTRL), sarcoplasmic (SARC), rat myofibrillar (MYO$_R$), human myofibrillar (MYO$_H$), intermyofibrillar mitochondria (IMF MITO) fractions; (B) Total Cytochrome C Oxidase protein content in SARC, IMF MITO and MYO$_H$ fractions. NB: For blot (A) IMF MITO was analyzed in triplicate, for blot (B) all fractions were analyzed in triplicate.

6.2   Method of plasma glucose enrichment

<u>6.1.1   Reagent preparation</u>

- Methanol
- Chloroform
- Distilled water with pH 2 (use HCL, about 4ml 1M HCL in 500ml water)
- Butaneboronic acid
- Acetic anhydride
- Ethylacetate
- Pyridine

<u>6.1.2   Protocol for deproteinisation and derivitization of samples</u>

- 150 µL plasma or standard in glass screw-cap tube.
- **+** 150 µL of distilled water PH 2.
- 1 min vortex (plate shaker).
- **+**3 mL methanol:chloroform (2.3:1) (500 mL = 348:152).
- 5 min vortex (plate shaker).
- 10 min on ice.
- 15 min centrifugation at 4ºC, 3500 rpm.

Pipette top clear layer into a new glass screw-cap tube (use glass pipette)

- **+** 2 mL chloroform.
- **+** 1 mL distilled water pH 2.
- 15 min vortex (plate shaker).
- 15 min centrifugation at 4ºC, 3500 rpm.

Pipette top clear layer into a new glass screw-cap tube (using glass pipette)

- Dry for ~ 3 hours under nitrogen at 40ºC.
- The above plasma sample or 30 µL standard **+** 150 µL Butaneboronic acid (10 mg / 1mL pyridine).
- 15 min vortex (plate shaker).
- 30 min incubate at 95ºC.

- **+**150 µL acetic anhydride.
- 90 min vortex (plate shaker).

Dry under nitrogen at 50ºC (check every 15 min).

Prepare sample for GC-MS

- **+**150 µL ethylacetate.
- 10 min vortex (plate shaker).

Transfer in glass vial to GC-MS

6.1.3   Analysis of [U-^{13}C] and 6, 6-[^{2}H$_2$] glucose enrichment by gas chromatography- mass spectrometry

Establish: U-^{13}C and ^{2}H$_2$ glucose in plasma.

Rate (°**C/min):**   4°C/min   50°C/min

Temp (°**C):**   135°C -------▶ 215°C -------▶ 250°C

Time (min):   1.0'   2.0'   2.0'

- Column: HP-5MS (30m length, 0.25mm I.D., 0.25um film)

Front inlet: Split injection, split ratio: 50:1
Flow rate: 2ml /min
Solvent delay: 1.5 min

6.3   References

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