INSULIN-MEDIATED REGULATION OF MUSCLE PROTEIN TURNOVER IN HEALTHY HUMANS

INSULIN-MEDIATED REGULATION OF MUSCLE PROTEIN TURNOVER IN HEALTHY HUMANS

By

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ABSTRACT

Only basal concentrations of insulin (i.e., 3-10 $\mu U \cdot ml^{-1}$) appear to be required for the maximal stimulation of muscle protein synthesis (MPS) during sustained hyperaminoacidemia in healthy adult humans at rest and during resistance exercise recovery. Whether plasma insulin at greater than basal concentrations can further stimulate MPS after the feeding of an MPS-saturating bolus dose of whole protein remains unexplored. We investigated the potential for insulin, via increased carbohydrate consumption, to augment mixed MPS, femoral artery blood flow, and muscle anabolic signalling at rest and after resistance exercise when co-ingested with whey protein at a dose previously shown to maximally stimulate MPS. Nine men (23.0 ± 1.9 y, 24.2 ± 2.1 $kg \cdot m^{-2}$) performed two trials consisting of a bout of unilateral knee extension exercise (4 sets x 8-12 RM to failure) followed by ingestion of a drink containing 25g of whey protein (PRO) or 25g of whey protein-plus-50g of maltodextrin (PRO+CARB). The trials were conducted in a double-blind, randomized, cross-over fashion. Muscle biopsies and stable isotope methodology were used to measure MPS. The areas under the glucose and insulin curves were 17-fold ($P < 0.05$) and 5-fold ($P < 0.05$) greater, respectively, for PRO+CARB than for PRO. Femoral artery blood flow was increased after exercise ($P < 0.05$) but was not different between PRO and PRO+CARB. Exercise increased MPS ($P < 0.05$), but there were no differences between PRO and PRO+CARB in the resting or exercised legs. Phosphorylation of protein kinase B (Akt) was greater in the PRO+CARB than the PRO trial ($P < 0.05$), and phosphorylation of Akt ($P = 0.05$) and acetyl coA carboxylase-β (ACC; $P < 0.05$) was greater after exercise than at rest. There was no

differential phosphorylation with exercise or condition of other proteins thought to be important in translation initiation, elongation, or mitogen activated kinase signalling pathways. In conclusion, the concurrent ingestion of 50g carbohydrate with 25g of protein did not further stimulate mixed MPS than the ingestion of the protein alone, at rest or following intense resistance exercise, suggesting that the insulinemia after protein ingestion is sufficient to fully stimulate MPS.

PREFACE

Skeletal muscle is the body's largest site of postprandial glucose disposal and lipid oxidation, and it is also the most modifiable contributor to basal metabolic rate and insulin sensitivity. The potential to up-regulate muscle's metabolic roles by increasing the muscle's size suggests that the preservation of a large skeletal muscle mass is instrumental in maintaining health and/or preventing and treating many metabolic diseases. A recent study demonstrated that resistance training and muscle hypertrophy decreased insulin resistance, reduced the glycation of hemoglobin, reduced blood glucose, and improved lipid profiles to a similar or greater degree of magnitude as endurance training (17); improvement in these metabolic disease risk factors appears to be beneficial in the prevention and treatment of type II diabetes, cardiovascular disease, and other insulin-resistant states. We, the laboratory of Dr. Stuart Phillips, seek to identify the stimuli for hypertrophy in healthy human muscle, in order to generate an understanding of the kinetics of muscle protein turnover (i.e., synthesis and breakdown) in the healthy state, which can subsequently be used to decipher the perturbations incurred by the inactivity, disease, and aging of human skeletal muscle. Ultimately, our research will be instrumental in the design of simple and applicable yet maximally effective nutrition and exercise programs, which will assist in the prevention and treatment of the fatal diseases associated with metabolic disorder, as well as other muscle wasting conditions such as inactivity, ageing, cancer, AIDS, and muscular dystrophy.

"Those who think they have not time for bodily exercise will sooner or later have to find time for illness."
Edward Stanley, Earl of Derby (1799-1869)

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My blue-eyed girl Sara (and my brown-eyed girl Molly) has kept me grounded over these past years; always willing to get out and hit the trail/province to clear my head, or just relax with a good show to break up the studying. Thanks for always being there. Thank-you to my family, with whom the learning process began and will never end. I cannot imagine a better way to grow up than on the hillside-view farm with my three brothers, two sisters, and with the extended family and countless GSA friends nearby. Mom and Dad are the most loving and hard-working people, and they introduced and encouraged each of my many passions and hobbies at any cost to themselves. Thanks to the boys for always pushing me to (try to) be better at everything than they were, and to the girls for assuring me that I didn't always have to be better at everything (or for at least trying). I love all you guys, and look forward to all the future memories as much as I cherish those of the past.

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CHAPTER I: Literature Review

1.1 INTRODUCTION

Animal tissues were once thought to be structurally and metabolically inert (80). The compounds required to comprise animal tissues were proposed to be acquired via the consumption of plants, because plants synthesized the compounds from elements occurring in the environment (111). In the 1930s, Rudolph Schoenheimer labelled the amino acid tyrosine with a stable isotope of nitrogen and used the tyrosine tracer to explore the metabolism of amino acids (80, 111). Schoenheimer demonstrated that the amount of labelled nitrogen excreted by a rat was only 50-60% of the total that had been consumed by the rat during a 10 day period (85). The majority of the labelled nitrogen that remained in the body had been incorporated into the proteins of the liver and muscle tissues. Further, the labelled nitrogen had been removed from the original tyrosine carbon chain and was incorporated into the nitrogenous groups of other amino acids (85). Contrary to popular theory at the time, Schoenheimer's early work demonstrated that animal tissues modify dietary compounds to direct their own synthesis (80, 85, 111).

Skeletal muscle tissue is now understood to be in a perpetual state of protein remodelling or protein turnover. It is the net balance between the synthesis and breakdown of skeletal muscle proteins that determines whether skeletal muscle tissue is accruing or losing proteins (14, 51, 72, 81). Not to be forgotten is the potential loss of amino acids to oxidation and intermediary metabolism (Figure 1). Net anabolism only occurs when protein synthesis exceeds the processes of protein loss (breakdown). Muscle hypertrophy, or a gain in skeletal muscle mass, would occur when the accumulation of muscle proteins during successive periods of net anabolism (ie., after resistance exercise

with feeding) exceeds the loss of muscle proteins during the intervening periods of net catabolism (ie., in the absence of feeding) (14, 51, 72, 81).

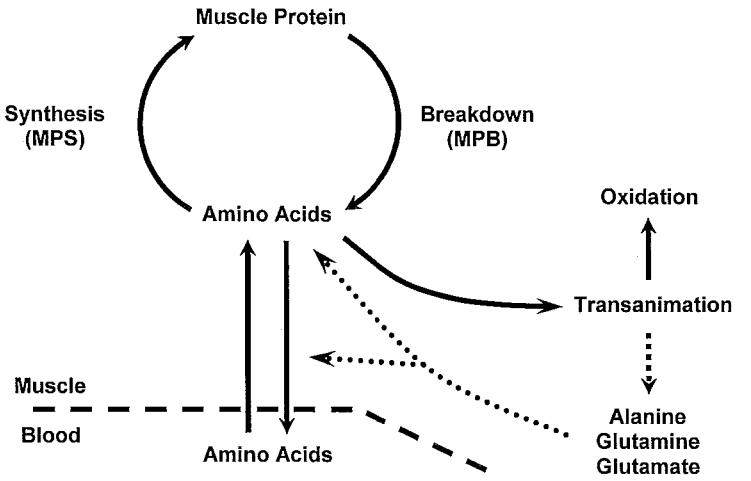

Figure 1. Overview of muscle protein turnover and the many metabolic fates of amino acids in skeletal muscle. Schematic adapted from Phillips (72).

Protein turnover has generally been measured during a brief period (1-4 h) after an experimental perturbation. More recently, the measurement of solely muscle protein synthesis (MPS) has become an increasingly common practice. The acute measurement of MPS may appear to provide insufficient information to explain changes in muscle mass over a period of adaptation to a stimulus, because changes in MPS have temporal variation dependant on feeding (64, 73, 92) and can persist for up to 48 h after exposure to an exercise stimulus (18, 57, 73). Moreover, stimuli that regulate MPS also tend to regulate muscle protein breakdown (MPB) and can, in some cases, stimulate oxidation (64, 73, 92). However, emerging research has provided validation of the acute MPS

response as a qualitatively valid predictor of long-term skeletal muscle mass gain (38, 102, 105) and loss (68, 69). Although the importance of MPB and oxidation cannot be discounted, MPS is the variable in the turnover equation that changes with the largest degree of magnitude in response to stimuli such as feeding and resistance exercise (73, 95), which further suggests that net protein balance has MPS as the locus of regulation in healthy humans (51, 77).

1.1.1 Muscle Protein Turnover

Human skeletal muscle is in net catabolic protein balance at rest in the post-absorptive state, because the rate of MPB exceeds the rate of MPS (4, 7, 73). The amino acids liberated from the muscle proteins may be recycled for the synthesis of new skeletal muscle proteins, may be oxidized or used in intermediary metabolism by the muscle, or may be released from the muscle (72). The function and target tissue of amino acids that are released by muscle in the fasted state remains unclear. It has been suggested that muscle-derived amino acids serve as precursors for the synthesis of more central tissue proteins, such as the proteins of the liver and the gut, because the central tissues do not accrue as large of an amino acid 'reservoir' (i.e., tissue free pool) to support the turnover of their proteins during periods of decreased amino acid availability, such as during fasting (109). Skeletal muscle may therefore be an endogenous amino acid reservoir that is exploited when the exogenous supply of amino acids is insufficient to support the turnover of central tissues (109). This hypothesis remains contentious because the feeding of amino acids does not alleviate the breakdown of skeletal muscle proteins when plasma insulin is maintained at basal concentrations (35). Any inhibition of MPB during amino

acid provision appears to be initiated by the insulin that is secreted in response to amino acid provision (35, 51). Contrarily, the overnight infusion of amino acids can produce a sustained decrease of MPB without sustained elevation of plasma insulin (55), suggesting that the demand of other tissues for muscle-derived amino acids can be alleviated if the availability of amino acids is continuously elevated. Whereas amino acids may or may not have an inhibitory effect on MPB, it is clear that the primary effect of amino acid provision on muscle protein turnover is the stimulation of MPS (9, 10, 64, 79).

Amino acid feeding directly stimulates the muscle's translational machinery (21, 25, 75) and provides amino acid precursors for the synthesis of muscle proteins (9, 32). Although each of the 20 physiological amino acids is required to synthesize proteins, the stimulation and maximal rates of MPS can be achieved with the provision of only the essential (indispensable) amino acids (EAA) (96, 100); a dose of 10g of EAA is sufficient to maximally stimulate MPS in healthy humans at rest (20). The stimulation of MPS by amino acid feeding at rest is transient and returns to basal synthesis rates within hours after feeding (10, 64). At rest in a healthy human, it is proposed that the transient periods of net anabolism stimulated by amino acid feeding balance against the periods of net catabolism that occur between feedings. Therefore, a healthy, recreationally active human's skeletal muscle mass may be preserved by the near perfect balance of the net anabolic and net catabolic periods (14, 71). Based on this scheme, it is clear that amino acid feeding must interact with another stimulus, namely high intensity resistance exercise, to promote muscle hypertrophy. Research in inactive muscle has demonstrated that feeding an abundance of amino acids stimulates MPS (68) but generally cannot

prevent the loss of muscle mass induced by inactivity (69, 70, 97-99). Moreover, when plasma amino acid availability is maintained by constant infusion of amino acids at rest, MPS becomes refractory to the provision of amino acids (10). This refractory characteristic of MPS has recently been confirmed using oral feedings (P. Atherton and M. Rennie, personal communication). Any amino acids that are delivered in excess of the demand of the muscle synthetic machinery are solely oxidized by the muscle tissues (62).

Resistance exercise has been recognized as a stimulus for muscle hypertrophy for decades, yet human skeletal muscle is not stimulated to a state of net anabolism during the 48h of recovery after an isolated bout of resistance exercise in the post-absorptive state (18, 57, 73). Resistance exercise does stimulate MPS more than MPB, but the rate of MPS is unable to exceed MPB without concurrent amino acid feeding (5, 12, 73, 74). When exogenous amino acids are provided in relatively close temporal proximity to a resistance exercise bout, MPS is stimulated to a greater extent than the sum of the MPS rates after exposure to either stimulus alone (6, 13, 78, 95). Part of the synergistic stimulation of MPS by amino acids and resistance exercise may occur because the exogenous amino acids provide precursors to the synthetic machinery to permit the full expression and maximal rates of exercise-mediated stimulation of MPS (9, 32, 89). The amino acids themselves are also a stimulus for the translational machinery, and thereby increase the stimulus for MPS (21, 25, 75). MPB is not inhibited by amino acid provision during recovery from resistance exercise (6, 13, 78, 95). It is not known whether the breakdown of muscle proteins after resistance exercise can be reduced if exogenous amino acids are made continuously available, as has been suggested to occur after

overnight infusion of amino acids in rested muscle (55). Ultimately, the primary effect of amino acid provision on muscle protein turnover after resistance exercise, akin to the primary effect of amino acid provision at rest, is a stimulation of MPS.

It is clear that muscle hypertrophy in young, healthy humans requires, at minimum but perhaps not exclusively, resistance exercise and close temporal consumption of amino acids and/or protein. Current research on muscle protein turnover is focused on the identification of the characteristics of the resistance exercise bout and amino acid meal that specifically modulate the rates of MPS and MPB. For an overview of the emerging regulatory characteristics, see Burd *et al.* (14) and Kumar *et al.* (51). Although the provision of energy in the form of carbohydrates and fats does not appear to regulate muscle protein turnover, there is considerable debate regarding the potential regulation of muscle protein turnover by insulin, which is secreted in response to ingestion of carbohydrates and some amino acids.

1.2 INSULIN AND MUSCLE PROTEIN TURNOVER AT REST

Early in vivo investigations into the actions of insulin on human protein turnover demonstrated that systemic infusion of insulin inhibited whole body protein synthesis (16, 28) and whole body protein breakdown (16, 22, 28, 94), and that the magnitude of inhibition of synthesis and breakdown scaled with the magnitude of hyperinsulinemia (28). Using the two-pool (blood/non-blood) arterial-venous (A-V) balance methods, a number of studies observed that systemic hyperinsulinemia did not change the rate of disappearance of an amino acid tracer from the blood entering a limb (1, 22, 33, 94), which is a measure of amino acid uptake that is often referred to as limb protein synthesis

(LPS), when the tracer amino acid is not oxidized by muscle. The rate of appearance of the amino acid tracee in the blood exiting the limb, which is a measure of the outward flux of amino acids from the free amino acid pool into the blood that is often referred to as limb protein breakdown (LPB), did not decrease in response to systemic hyperinsulinemia (1, 94), unless the concentration of insulin reached the upper limits of physiological concentrations (22, 33). The insulin-mediated inhibition of whole body and LPB observed in vivo was congruent with previous research performed in animals and human muscle cells, but the absence of an insulin-mediated stimulation of whole body and LPS conflicted with the apparent stimulation of protein synthesis in the animal and human muscle cell models.

1.2.1 Muscle Protein Synthesis - Insulin Infusion

Plasma amino acid concentrations are observed to decrease during the systemic infusion of insulin in vivo (16, 28, 94). It was proposed that insulin induced plasma hypoaminoacidemia that prevented the expression of insulin's stimulation of whole body and LPS (16, 28, 94). When plasma amino acids were maintained at basal concentrations during systemic hyperinsulinemia, the insulin-mediated reduction in whole body protein synthesis was prevented (16, 28), but whole body synthesis was not increased (16, 28), whereas LPS was stimulated (3, 67, 107). Furthermore, when insulin was locally infused to prevent the hypoaminoacidemia induced by systemic insulin infusion, LPS (4, 7, 32, 56, 76) and MPS (2, 4, 7, 32, 76) increased. Therefore, the whole body synthesis response did not necessarily reflect the synthesis of the muscle (which comprises 25-30% of whole

Table 1. Effect of insulin on limb protein synthesis, amino acid delivery, and limb blood flow at rest

Reference	Activity	Insulin		Amino Acids/Protein	Rd	F_{in}	Blood Flow
Tessari P *et al.* (1987) (93)	Rest	hINS	S		↔	↓	↑
Arfvidsson B *et al.* (1991) (1)	Rest	hINS	S		↔	↓	↑
Denne SC *et al.* (1991) (22)	Rest	sINS	S		↔	↓	↑
Heslin MJ *et al.* (1992) (39)	Rest	hINS	S		↔	↓	↑
Moller-Loswick AC *et al.* (1994) (60)	Rest	hhINS	S		↔	↓	↑
Gelfand RA and Barrett EJ (1987) (33)	Rest	hhINS	L		↔	↑	↑
Louard RJ *et al.* (1992) (56)	Rest	$hINS_1$ $hINS_2$	L L		↔ ↔	↔ ↔	↑ ↑
Bennet WM *et al.* (1990) (3)	Rest	hINS	S	bAA	↑	↑	↑
Wolf RF *et al.* (1992) (107)	Rest	hINS	S	bAA	↑	↑	↑
Newman E *et al.* (1994) (67)	Rest	hINS	S	bAA	↑	↑	↑
Hillier TA *et al.* (1998) (42)	Rest	sINS	L		↑	↑	↑

The statistics and findings reported in the literature were used to designate a significant increase (↑), a significant decrease (↓), or no significant change (↔) from the kinetics during exposure to basal plasma insulin concentrations. All subjects were young, healthy, recreationally active but untrained humans studied in the post-absorptive state.

hINS, hyperinsulinemia (30-100 $\mu U \cdot ml^{-1}$); hhINS, very high (100-170 $\mu U \cdot ml^{-1}$) hyperinsulinemia; sINS, supraphysiological (2000+ $\mu U \cdot ml^{-1}$) hyperinsulinemia; L, local infusion; S, systemic infusion; bAA, basal amino acid concentrations; Rd and F_{in} are the three pool amino acid kinetic parameters as defined by Biolo *et al.* (4).

Table 2. Effect of insulin on muscle protein synthesis, limb protein synthesis, and amino acid delivery at rest

Reference	Activity	Insulin		Amino Acids/Protein	FSR	$F_{O,M}$	Rd	F_{in}	Blood Flow
Biolo G *et al.* (1995) (5)	Rest	hINS	L		↑	↑	↑	↑	↑
Biolo G *et al.* (1999) (7)	Rest	hINS	L		↑		↔	↑	↑
Bell JA *et al.* (2005) (2)	Rest	hINS	S		↔	↔	↔	↔	↑
		hINS	L		↑	↑	↑	↑	↑
Fujita S *et al.* (2006) (32)	Rest	mINS	L		↔	↔	↔	↔	↑
		$hINS_1$	L		↑	↑	↑	↑	↑
		$hINS_2$	L		↔	↔	↔	↔	↑
Rasmussen BB *et al.* (2006) (76)	Rest	hINS	L		↑	↑	↑	↔	↔
Greenhaff PL *et al.* (2008) (35)	Rest	mINS	S	MAA (OPT)	↔		↔		↔
		hINS	S	MAA (OPT)	↔		↔		↔
		hhINS	S	MAA (OPT)	↔		↔		↔

Studies with a measure of the direct incorporation of an amino acid tracer into mixed muscle proteins (fractional synthesis rate; FSR) were included in this table. The statistics and findings reported in the literature were used to designate a significant increase (↑) or no significant change (↔) from the kinetics during exposure to the same amino acid/protein consumption at basal plasma insulin concentrations. All subjects were young, healthy, recreationally active but untrained humans studied in the post-absorptive state.

mINS, moderate (15-30 $\mu U \cdot ml^{-1}$) hyperinsulinemia; hINS, hyperinsulinemia (30-100 $\mu U \cdot ml^{-1}$); hhINS, very high (100-170 $\mu U \cdot ml^{-1}$) hyperinsulinemia; L, local infusion; S, systemic infusion; MAA, mixed amino acids; OPT, rate of intake was sufficient to maximize the amino acid stimulation of MPS as defined by Cuthbertson *et al.* (20) and Moore *et al.* (63); $F_{O,M}$, Rd, F_{in} are the three pool amino acid kinetic parameters as defined by Biolo *et al.* (4).

body protein synthesis), and insulin stimulated MPS so long as the plasma amino acids were maintained at basal concentrations (Table 1 and 2). Amino acid delivery to the muscle was subsequently proposed to be responsible for insulin's actions on MPS at rest. When the stimulation of blood flow by hyperinsulinemia was coincident with hypoaminoacidemia, the delivery of amino acids to the muscle and MPS did not change (1, 22, 33, 94). When hypoaminoacidemia was prevented during hyperinsulinemia, the stimulation of blood flow by hyperinsulinemia increased the delivery of amino acids to the muscle, which subsequently stimulated MPS (3, 4, 7, 32, 39, 56, 76, 107). Whether insulin simply promoted the direct stimulation of MPS by amino acids, or whether the amino acids provided precursors to permit the expression of insulin's direct stimulation of MPS, remains unknown (Figure 2).

1.2.2 Muscle Protein Synthesis - Carbohydrate Ingestion

Protein synthesis is an energy consuming process (~0.7 $kcal \cdot g^{-1}$ of protein synthesized) that could potentially be regulated by the supply of energy to the synthetic machinery (101). Carbohydrate feeding, which provides energy, increases blood glucose, and stimulates insulin secretion, might therefore stimulate MPS greater than hyperinsulinemia alone. Few studies have directly examined the regulation of muscle protein turnover by nutritional energy in human, but MPS does not appear to be further increased by the co-consumption of carbohydrates with a dose of amino acids that is (47), or is not (59), sufficient to maximally stimulate MPS. However, these studies used small bolus feedings to administer the nutrients, which is arguably a non-practical method of delivering nutrition (47, 59). In addition, the use of separate intervention groups, crystalline amino

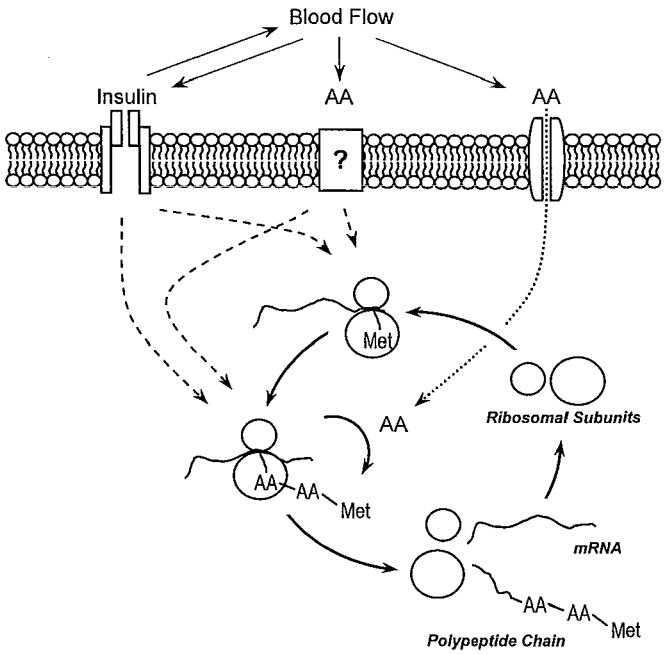

Figure 2. Potential interactions between the insulin- and amino acid-mediated stimulation of the muscle protein synthetic machinery. Insulin stimulates an increase in blood flow that increases the delivery of amino acids to the limb. The increased delivery of amino acids may increase the uptake of amino acids into the muscle (right) and provide precursors for insulin-stimulated MPS (left), and/or the amino acids may directly stimulate the synthetic machinery (centre). The dashed arrows indicate stimulation of the synthetic machinery, whereas the dotted arrow indicates movement of amino acids from the blood into the free amino acid pool.

acids with glucose, which is an inferior insulin secretagogue to the maltose used by Koopman *et al.* (47), makes the findings of Miller *et al.* (59) difficult to interpret in the context of carbohydrate and its effect on protein metabolism.

The provision of nutritional energy (carbohydrates, fatty acids, and triglycerides) in excess of the demands of MPS during systemic hyperinsulinemia produces a rate of MPS that is typically observed in healthy young humans at rest in the post-absorptive state (2). Amino acid availability, not energy provision per se, is clearly the predominant

factor in the feeding-induced stimulation of MPS (2). Whether energy becomes rate limiting for MPS during amino acid-induced hyperinsulinemia at rest, remains poorly studied in humans. The availability of energy to muscle could, for example, potentially limit the synthesis of muscle proteins during periods of negative energy balance. However, for the most part, any stimulatory effect of carbohydrate feeding on MPS appears to be due to the increase in plasma insulin that is stimulated by carbohydrate feeding and not due to provision of energy.

1.2.3 Synthesis - Dose-Response Characteristics

When plasma amino acids are maintained at basal concentrations, there is a minimum concentration of plasma insulin that is necessary for insulin-mediated stimulation of MPS. Local infusion to moderate hyperinsulinemia (20-40 $\mu U \cdot ml^{-1}$) did not increase LPS (32, 56) or MPS (32), whereas higher concentrations of insulin (40-70 $\mu U \cdot ml^{-1}$) increased LPS (32, 56) and MPS (32). At very high but physiological hyperinsulinemia (70+ $\mu U \cdot ml^{-1}$), insulin did not further increase LPS or MPS (32), suggesting that the insulin-mediated stimulation of MPS has a considerably high activation threshold (40-70 $\mu U \cdot ml^{-1}$ is a level of insulin that would require ingesting at least 40-50g of carbohydrate) and is a saturable process and/or is inhibited by an insulin-mediated mechanism that is only expressed at very high local concentrations of insulin (Figure 3). The lack of further insulin-mediated MPS stimulation at increasingly higher local hyperinsulinemia may have been due to the concurrent increase in systemic insulinemia (32, 56). Systemic hyperinsulinemia causes hypoaminoacidemia due to a suppression of proteolysis; thus, if insulin-mediated stimulation of blood flow is not sufficient to offset the

hypoaminoacidemia then delivery of amino acids to the muscle would decrease and effectively limit insulin's stimulation of MPS. Infusion of amino acids to maintain the basal plasma concentrations of amino acids may permit insulin's stimulation of MPS at higher concentrations of insulin by preventing the MPS-inhibiting hypoaminoacidemia initiated at those higher concentrations of insulin. It is clear that basal amino acid replacement could not permit infinitely greater stimulation of MPS by increasingly greater concentrations of insulin, because the stimulation of MPS by amino acid feeding at rest can be saturated whether the muscle is (35) or is not exposed (20) to hyperinsulinemia. Thus, MPS cannot be infinitely accelerated by amino acids or insulin. If, however, the mechanisms for insulin- and amino acid-mediated stimulation of MPS act synergistically, insulin might sensitize the muscle by increasing the synthetic response to a dose of amino acids that would otherwise be unable to maximally stimulate MPS. Such a dose would be less than 10g of essential amino acids according to Cuthbertson *et al.* (20) or 20g of protein according to Moore *et al.* (63). The insulin-mediated sensitization of MPS to amino acid feeding has not been well explored, but may help to elucidate the relationship between the insulin- and amino acid-mediated stimulation of MPS.

1.2.4 Breakdown - Dose-Response Characteristics

Systemic hyperinsulinemia does not inhibit LPB (1, 94), unless insulin concentrations reach very high physiological or supraphysiological levels (22, 33). When plasma amino acids are maintained at basal concentrations, insulin has (4, 32, 56, 76) and has not

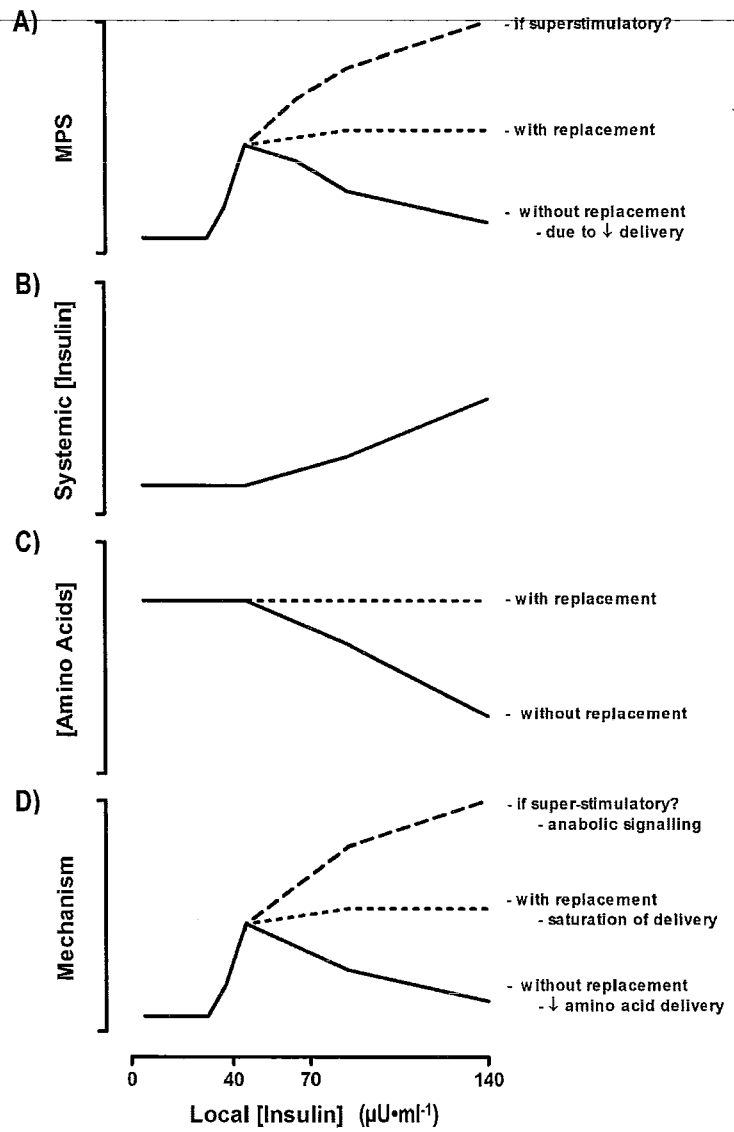

Figure 3. Dose-response curve for the effects of local hyperinsulinemia on muscle protein synthesis at rest. Dose-response curve for the local infusion of increasing plasma concentrations of insulin and muscle protein synthesis (A), the systemic plasma concentration of insulin (B), the plasma concentration of amino acids (C), and the mechanism of insulin-mediated stimulation of muscle protein synthesis (D). The solid lines represent data assembled from studies that examined the dose-response characteristics of local insulin infusion on limb (32, 56) and muscle protein synthesis (32) at rest without the replacement of amino acids. The dashed lines represent the hypothetical alterations of the dose-response characteristics if the plasma amino acid concentrations are maintained at basal levels.

(4, 7, 32, 76, 112) been observed to decrease protein breakdown at rest; however, the amino acid kinetic model used to measure breakdown appears to be responsible for the discrepant results. A decrease in protein breakdown was reported during local (4, 32, 56, 76) or systemic hyperinsulinemia (22, 33) whenever the rate of appearance (Ra) of amino acids into the blood was measured. However, Ra does not account for amino acids liberated by MPB that are lost to oxidation or recycling for protein synthesis in the muscle. Insulin-mediated alterations of oxidation and recycling would alter the release of amino acids from the limb, without necessarily altering the breakdown rate of muscle proteins. Thus, a minimal rate of absolute loss (efflux) of amino acids from tissues has been measured during local and systemic hyperinsulinemia. Whenever the kinetic movement of amino acids from the bound protein to the unbound free amino acid pool has been reported, MPB did not change in response to local hyperinsulinemia (4, 7, 32, 76, 112). Therefore, insulin may inhibit the release of amino acids from the muscle free pool into the blood, likely by stimulating synthesis, but insulin does not inhibit the breakdown of muscle proteins when amino acid delivery is maintained to resting muscle. Insulin must initiate an expansion of the muscle free pool or increase the recycling of amino acids for MPS, because a phenylalanine or leucine tracer was employed in each of the contributing studies, and phenylalanine is not appreciably oxidized by muscle and insulin does not increase the oxidation of leucine (86, 108).

Chow and colleagues (19) recently demonstrated that the movement of amino acids from the protein bound to the free amino acid pool was inhibited when amino acids were replaced to basal concentrations during systemic hyperinsulinemia. The discrepancy

between Chow's observations (19) and the response to local hyperinsulinemia (4, 7, 32, 76, 112) is not easily resolved, because the magnitude of hyperinsulinemia and the delivery of amino acids were comparable between studies. Chow examined MPB at 4h post-onset of hyperinsulinemia (19), whereas the local infusion studies examined MPB at 3h post-onset of hyperinsulinemia (4, 7, 32, 76, 112), and although the temporal response of the inhibition of MPB has not been well characterized, it is possible that there may be a latency period for the onset or for the peak inhibition of MPB after exposure to hyperinsulinemia. It is therefore only possible to suggest that insulin may initiate the retention of amino acids in the tissue free pools. Insulin-mediated retention of amino acids in the muscle free pool may potentially promote the insulin-mediated stimulation of MPS, by ensuring a continuous supply of amino acids coming from MPB, although this hypothesis is speculative but at least congruent with existing data.

1.2.5 Amino Acid Provision and Concurrent Hyperinsulinemia

Providing amino acids in excess of basal concentrations during hyperinsulinemia generated LPS and MPS rates that were greater than post-absorptive (31, 42) and hyperinsulinemic (42, 112) rates at rest, without changing LPB (42) or MPB (112). Amino acid feeding is clearly a potent stimulus for MPS, even against a background of hyperinsulinemia. When muscle protein turnover was investigated during hyperinsulinemia with the concurrent delivery of amino acids at a rate that was sufficient to maximally stimulate MPS, LPS and MPS were observed to be greater than post-absorptive rates, but the changes in LPB from post-absorptive rates conflicted between studies (31, 35). At comparable systemic concentrations of insulin, LPB and MPB

increased during hyperinsulinemia stimulated by carbohydrate feeding (31), whereas LPB decreased during hyperinsulinemia maintained by systemic infusion (35). Few studies have examined muscle protein turnover after carbohydrate-induced hyperinsulinemia at rest, but it is possible that differences in the inhibition of LPB by carbohydrate- and infusion-induced hyperinsulinemia might occur as result of differences in the temporal pattern of insulin appearance in the blood. Differences in muscle protein turnover in response to feeding- (ie., the physiological appearance of insulin in the blood) and infusion-induced (ie., the square-wave appearance of insulin in the blood) hyperinsulinemia must be further examined, in order to validate the use of insulin infusion as an appropriate model of in vivo insulin secretion.

Two studies have examined the effect of hyperinsulinemia on muscle protein turnover at rest during amino acid provision in excess of basal concentrations. When amino acids were infused at a rate that was not sufficient to maximize the amino acid contribution to MPS, net limb protein balance was greater during local hyperinsulinemia than in the absence of local hyperinsulinemia (29). Hyperinsulinemia appeared to increase LPS and decrease LPB, although these changes were not statistically significant (29). An aggregate measure of synthesis and breakdown such as a direct measurement of MPS (FSR) and the fractional breakdown rate of muscle proteins (FBR), respectively, may have demonstrated the greater stimulation of synthesis and the greater inhibition of breakdown during hyperinsulinemia, because the insulin-mediated changes in LPS and LPB indicated by the static measures were small but apparent and perhaps significant when compounded by time. When the rate of amino acid delivery was sufficient to

maximally stimulate MPS, basal concentrations of insulin were sufficient to maximally stimulate LPS and MPS (35), because systemic infusion to moderate (30 $\mu U \cdot ml^{-1}$), high (72 $\mu U \cdot ml^{-1}$), or very high (167 $\mu U \cdot ml^{-1}$) concentrations of insulin did not further increase LPS or MPS (35). Conversely, moderate hyperinsulinemia (30 $\mu U \cdot ml^{-1}$) inhibited LPB relative to basal insulinemia, with no further inhibition of breakdown at higher concentrations of insulin (35). Therefore, hyperinsulinemia may potentially enhance the synthesis of muscle proteins when amino acid provision is insufficient to maximally stimulate MPS, but hyperinsulinemia is unable to enhance synthesis when amino acid provision is sufficient to maximally stimulate MPS. Moreover, only basal concentrations of insulin are required to maximally stimulate MPS during optimal amino acid provision. Whether LPB, and perhaps MPB, can be maximally inhibited by concentrations of insulin lower than 30 $\mu U \cdot ml^{-1}$ remains to be determined.

1.2.6 Summary - Insulin and Muscle Protein Turnover at Rest

The effect of insulin on muscle protein turnover in resting muscle is largely modulated by the delivery of amino acids to the muscle, which in turn is modulated by the stimulation of blood flow by insulin and the provision of exogenous amino acids (Figure 4). If the delivery of amino acids to the muscle decreases during hyperinsulinemia (i.e., carbohydrate ingestion only with no amino acid ingestion), insulin appears to inhibit MPS and MPB (1, 22, 33, 94). If the delivery of amino acids to the muscle is maintained or increases during hyperinsulinemia, insulin stimulates MPS (3, 4, 7, 32, 39, 56, 76, 107), and inhibits LPB or the release of amino acids from the muscle free pool into the blood (4, 32, 56, 76), but has little effect on MPB (4, 7, 32, 76, 112). There appears to be

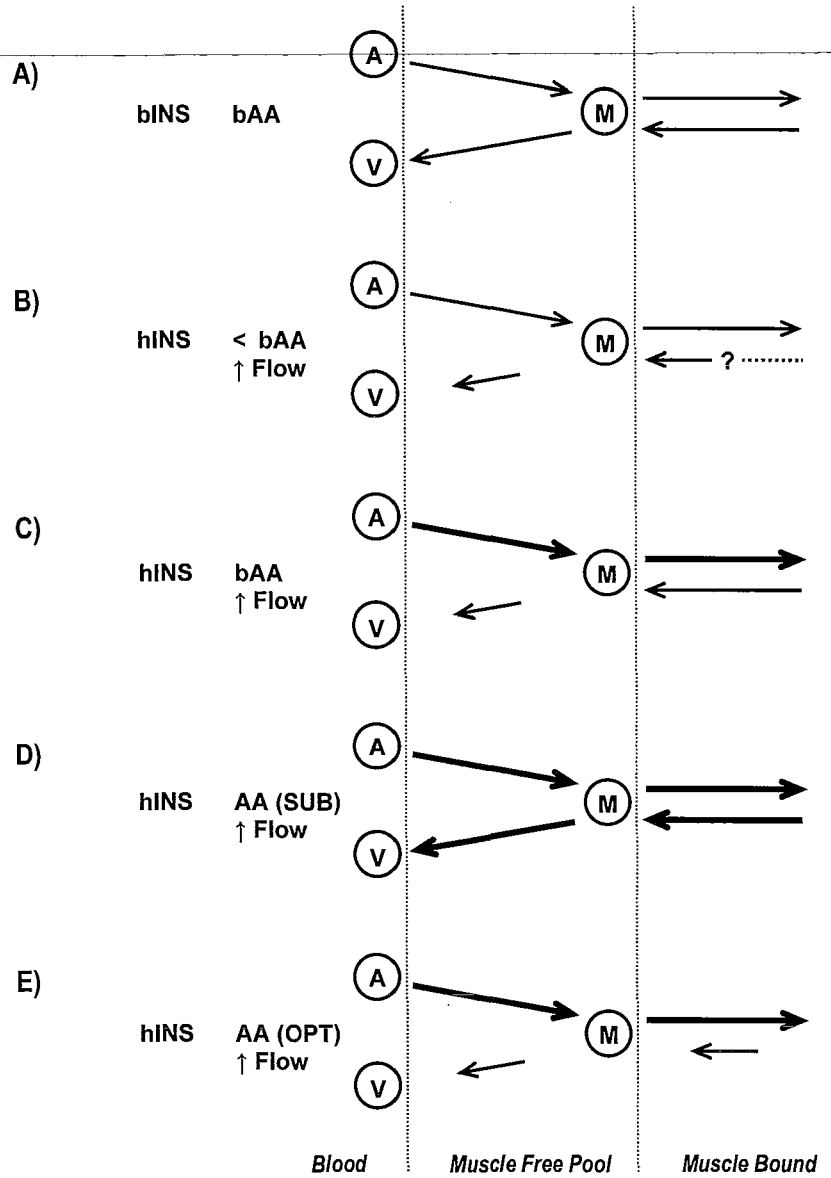

Figure 4. Hyperinsulinemia, amino acid availability, limb blood flow, and muscle protein turnover at rest. Muscle protein turnover is represented at basal post-absorptive (A), during systemic insulin infusion (B), during local insulin infusion (C), and during hyperinsulinemia plus the provision of amino acids at a rate that is not sufficient (SUB; D) and is sufficient (OPT; E) to maximally stimulate MPS. Conditions B-E are expressed relative to condition A. Larger arrows denote an increase relative to condition A, whereas smaller arrows denote a decrease relative to condition A. The literature sources used to construct the figure are highlighted in the literature summary figures of Appendix A. The pool kinetics appear to demonstrate insulin- and amino acid availability-mediated shifting of the amino acid pool that is preserved.

a threshold for amino acid delivery to the muscle, beyond which insulin appears less effective at stimulating MPS and may only 'sensitize' the muscle to the amino acid-mediated stimulation of MPS when amino acids are given at less than maximally stimulatory doses (29). The inhibition of MPB appears to emerge concurrently with the switch in insulin's stimulatory action on MPS (29). When the delivery of amino acids to the muscle is sufficient to saturate the stimulation of MPS by amino acids, plasma insulin concentrations greater than basal concentrations are unable to further stimulate MPS, whereas the inhibition of LPB and perhaps MPB is fully manifested by moderate hyperinsulinemia at most (35).

1.3 INSULIN AND PROTEIN TURNOVER AFTER RESISTANCE EXERCISE

The synergistic stimulation of MPS by amino acid feeding and resistance exercise has been well documented (6, 13, 78, 95), but amino acid feeding does not appear to alleviate the breakdown of muscle proteins after resistance exercise (35, 51). On the basis of insulin's actions on muscle protein turnover at rest, it was proposed that the infusion of insulin or the consumption of carbohydrates might potentiate MPS or inhibit MPB during resistance exercise recovery.

1.3.1 Muscle Protein Turnover and Hyperinsulinemia

In the absence of amino acid provision, systemic hyperinsulinemia does not further increase LPS (12) or MPS (12, 84) after resistance exercise (Table 3). The lack of enhanced stimulation of MPS by hyperinsulinemia is likely due to a lack of amino acid delivery due to systemic hypoaminoacidemia. When insulin was locally infused insulin to

Table 3. Effect of insulin on muscle protein synthesis, limb protein synthesis, and amino acid delivery after resistance exercise

Reference	Activity	Time of Intake	Insulin		Amino Acids/Protein	FSR	Rd	F_{in}	Blood Flow
Roy BD *et al.* (1997) (84)	RE	0 + 1 h Post-RE	hINS	C		↔			
Biolo G *et al.* (1999) (7)	RE	0 h Post-RE	hINS	L		↔	↔	↔	↔
Børsheim E *et al.* (2004) (12)	RE	1 h Post-RE	hINS	C		↔	↔		↔
Koopman R *et al.* (2007) (47)	RE	0 + ½ h + . . . + 6h Post-RE	$mINS_1$ $mINS_2$	C C	PRO (OPT) PRO (OPT)	↔ ↔			

Studies with a measure of the direct incorporation of an amino acid tracer into mixed muscle proteins (fractional synthesis rate; FSR) were included in this table. The statistics and findings reported in the literature were used to designate no significant change (↔) from the kinetics during exposure to the same activity and the same amino acid/protein consumption at basal plasma insulin concentrations. All subjects were young, healthy, recreationally active but untrained humans, except for Roy *et al.* (84), which was performed in resistance trained humans. All resistance exercise bouts were performed in the post-absorptive state, and the exercise bouts consisted of intense leg resistance exercise.

RE, resistance exercise; mINS, moderate (15-30 $\mu U \cdot ml^{-1}$) hyperinsulinemia; hINS, hyperinsulinemia (30-100 $\mu U \cdot ml^{-1}$); L, local infusion; C, carbohydrate ingestion; PRO, casein protein hydrolysate; OPT, rate of intake was sufficient to saturate the amino acid stimulation of MPS as defined by Cuthbertson *et al.* (20) and *Moore et al.* (63); Rd and F_{in} are the three pool amino acid kinetic parameters as defined by Biolo *et al.* (4).

prevent the hypoaminoacidemia associated with systemic insulin infusion, hyperinsulinemia did not increase limb blood flow or amino acid delivery to the muscle, and consequently did not increase LPS or MPS after resistance exercise (7). It not known whether insulin could stimulate MPS later in resistance exercise recovery, by stimulating blood flow and amino acid delivery when the exercise-stimulated increase in blood flow has subsided. Overall, it is clear that the potential for insulin to stimulate MPS, in resting and perhaps in exercise muscle, is highly dependent on amino acid delivery (7, 12, 84), although the relative contributions of insulin- and amino acid-mediated stimulation of the translational machinery remains unknown (Figure 2).

Hyperinsulinemia does not appear to effect whole body protein breakdown after resistance exercise (84). Contrarily, LPB was inhibited by locally-induced (7) and carbohydrate-induced hyperinsulinemia (12), although the onset of inhibition appeared to be delayed after carbohydrate ingestion, because the inhibition of LPB only began to be manifested at 3-4h post-ingestion (12). When an aggregate measure of myofibrillar protein breakdown was employed, urinary 3-methylhistidine (3-MH) excretion over a 24h period was lower, indicating inhibition of MPB, when participants consumed carbohydrates after resistance exercise compared to fasting post-exercise (84). Importantly, LPB reflects the release of amino acids from the limb and not the release of amino acids directly from muscle proteins. Biolo and colleagues measured the rate of appearance of amino acids from the bound muscle into the muscle free amino acid pool after resistance exercise with and without hyperinsulinemia, and demonstrated a hyperinsulinemia-mediated inhibition of MPB after resistance exercise (7). The inhibition

of MPB after resistance exercise contrasts with the lack of hyperinsulinemia-mediated inhibition of MPB in resting muscle (4, 7, 32, 76, 112); however, it is not known whether hyperinsulinemia inhibits MPB as effectively as LPB after resistance exercise, or consequently, whether the inhibition of insulin-mediated inhibition of LPB acts to preserve a supply of amino acid precursors for MPS. The latter has previously been suggested to occur for resting muscle. LPB appears to be inhibited by much lower concentrations of insulin after resistance exercise (12) than at rest (1, 22, 33, 94) during hypoaminoacidemia, and MPB only appears to be inhibited by insulin after resistance exercise, suggesting that resistance exercise may enhance the inhibition of LPB and MPB by insulin.

1.3.2 Protein Turnover with Hyperinsulinemia and Amino Acid Provision

Amino acid-carbohydrate co-ingestion during resistance exercise recovery stimulates LPS (78) and MPS (23) greater than resistance exercise alone. Amino acids are responsible for most of the stimulation of MPS by co-ingestion of carbohydrate and amino acid/protein because hyperinsulinemia in the post-exercise recovery period does not stimulate LPS or MPS (7, 12, 84), but amino acid provision further stimulates LPS (11, 59) and MPS (48, 49) during hyperinsulinemia after resistance exercise. It is possible that hyperinsulinemia may enhance the amino acid stimulation of LPS during resistance exercise recovery, because LPS was greater during amino acid feeding with concurrent hyperinsulinemia than without hyperinsulinemia, although this effect only began to be observed 3h after onset of hyperinsulinemia (59). The delivery of amino acids to the muscle was not increased during hyperinsulinemia, but the net uptake of amino acids into

the muscle was greater during hyperinsulinemia with increasing time post-amino acid ingestion (59). A possibility is that the stimulatory effect of insulin on LPS may be temporally delayed when increasing amounts of amino acids are provided, because the uptake of amino acids into the muscle is saturated during hyperaminoacidemia immediately following amino acid ingestion; when the plasma amino acid concentrations decline with increasing time post-ingestion, uptake in the muscle may then be stimulated by hyperinsulinemia, perhaps via persisting elevations of blood flow. This potential effect of insulin is speculative and would likely only occur when amino acid delivery is insufficient to maximally stimulate MPS. When the rate of amino acid delivery was sufficient to maximally stimulate MPS after resistance exercise, carbohydrate-induced hyperinsulinemia did not further stimulate MPS (47). The insensitivity of MPS to hyperinsulinemia during optimal amino acid provision in the post-exercise recovery period is consistent with the observation that only basal concentrations of insulin are necessary for amino acids to saturate MPS in resting muscle (35). Therefore, it appears as though insulin may stimulate MPS, in rested and perhaps exercised muscle, when the delivery of amino acids is maintained between basal delivery and delivery that is insufficient to maximally stimulate MPS; however, insulin only permits the amino acid-mediated stimulation of MPS when the delivery of amino acids is insufficient to maximally stimulate MPS. The minimum dose of insulin that is required to permit the saturation of MPS by amino acid provision after resistance exercise has not been determined, but would be unlikely to differ substantially from the basal concentration of insulin that appears to be sufficient for resting muscle (35).

stimulated LPB compared to resistance exercise alone, when the amino acid dose consumed was insufficient to maximally stimulate MPS (78). The provision of amino acids increased LPB during post-exercise hyperinsulinemia when a sub-MPS-saturating dose of amino acids was consumed (59), but decreased MPB when a MPS-saturating dose of amino acids was consumed (11). The insulin-mediated inhibition of breakdown during amino acid provision may therefore be regulated by the amount of amino acids provided, or more likely, by the magnitude of stimulation of MPS. A single study has examined LPB after amino acid provision during exercise recovery with and without concurrent hyperinsulinemia (59). Hyperinsulinemia, which decreased LPB when amino acids were not provided post-exercise (7, 12), did not decrease LPB after post-exercise provision of a dose of amino acids that was not sufficient to saturate MPS (59). Whether post-exercise hyperinsulinemia inhibits LPB when the provision of amino acids maximally stimulate MPS has yet to be examined within a single study, but is suggested to occur by the observed decrease in LPB caused by amino acid provision during hyperinsulinemia post-exercise. Therefore, the preliminary investigations suggest that insulin-mediated LPB is modulated by the availability of amino acids and/or the magnitude of MPS stimulation in the post-resistance exercise recovery period.

1.4 SUMMARY: INSULIN AND MUSCLE PROTEIN TURNOVER

The effect of insulin on muscle protein turnover in resting muscle and muscle post-resistance exercise is affected to the greatest degree by the delivery of amino acids to the muscle, whereas LPB and perhaps MPB may be further regulated by the magnitude of

stimulation of MPS. Insulin-mediated increases in resting blood flow appear to permit the stimulation of MPS by insulin, so long as plasma amino acids are maintained at or above basal concentrations (3, 4, 7, 32, 39, 56, 76, 107). This is presumed to occur simply via an increase in the delivery of amino acids to the muscle, but it remains unclear whether the amino acids directly stimulate MPS and/or serve as precursors for MPS stimulated directly by insulin. If plasma amino acid concentrations decline, such as during systemic hyperinsulinemia, which would occur during ingestion of carbohydrate alone (1, 22, 33, 94), or if hyperinsulinemia is unable to further stimulate blood flow and amino acids are maintained (7, 12, 84), insulin does not appear to increase the delivery of amino acids to the muscle or the stimulation of MPS. LPB and MPB appear to be inhibited whenever insulin cannot stimulate MPS in resting (1, 22, 33, 94) and exercised muscle (7, 12, 84). LPB but not MPB is inhibited when insulin concurrently stimulates MPS in resting muscle (4, 7, 32, 56, 76, 112), whereas LPB and MPB are both inhibited when insulin concurrently stimulate MPS in exercised muscle (7). It remains to be determined whether insulin causes an expansion of the muscle free amino acid pool and/or an accelerated rate of 'recycling' of muscle-derived amino acids, due to a stimulation of synthesis and/or suppression of amino acid oxidation or use for intermediary metabolism. When amino acids are provided to resting (29) and perhaps resistance exercised (59) muscle during hyperinsulinemia, LPS and MPS increase and LPB increases, so long as the amino acids are provided at a rate that is not sufficient to maximally stimulate MPS. When amino acid provision is sufficient to saturate MPS, hyperinsulinemia cannot further stimulate MPS in

resting (35) or exercised muscle (47), but appears to inhibit MPB in resting (35) and exercised muscle (47).

Overall, the regulation of insulin-mediated protein turnover by amino acid delivery has been explored in some detail, but particular aspects of protein turnover (i.e., MPB) have not been as well described as others (i.e., MPS). An understanding of the kinetics, regulation, and mechanisms of insulin-mediated muscle protein turnover will remain incomplete until studies incorporate a direct measure of MPB, because hyperinsulinemia appears to have independent and opposing effects on LPB and MPB under specific conditions (4, 7, 32, 56, 76, 112), although the significance and function of this modulation remains to be determined. The independent effects of insulin on muscle protein turnover promote a less catabolic net protein balance, but insulin needs to interact with amino acid provision to promote an anabolic net protein balance at rest or during resistance exercise recovery (11, 12). Insulin may sensitize the muscle to stimulation of MPS by amino acid provision when the rate of amino acid delivery is insufficient to maximally stimulate MPS, but only basal concentrations of insulin appear to be necessary to permit the saturation of MPS by a sufficient delivery of amino acids (35). When MPS is maximally stimulated by amino acids, insulin appears to inhibit LPB likely MPB, and the inhibition of breakdown by insulin is saturated by concentrations of insulin not much larger basal concentrations (35). Therefore, sufficient amino acid provision precludes hyperinsulinemia for establishing maximal net anabolic protein balance, but hyperinsulinemia may potentially enhance net anabolic protein balance, to a limited extent, by providing greater inhibition of MPB.

1.5 INSULIN-MEDIATED MECHANISMS

Insulin has direct mechanisms of action on skeletal muscle that can affect MPS and MPB, and a brief explanation of these mechanisms is provided.

1.5.1 Blood Flow-mediated Stimulation of Muscle Protein Synthesis

Regulation of the delivery of amino acids to the muscle is an important component of insulin's stimulation of MPS. Insulin affects amino acid delivery by stimulating blood flow to the muscle. Briefly, insulin signalling through its surface receptor on the vascular endothelium stimulates the synthesis of nitric oxide, and nitric oxide causes the smooth muscle at capillary junctions to relax, effectively decreasing the resistance of the capillary to blood flow (65). As a result, blood flow to the capillary bed is enhanced (65), and this increases the delivery of amino acids to the muscle tissue served by the capillary bed, so long as the plasma amino acid concentrations are maintained or are greater than basal concentrations (3, 4, 7, 24, 32, 39, 56, 76, 107). It is proposed that the insulin-mediated delivery of amino acids to the muscle is responsible for the increased uptake of amino acids into the muscle free amino acid pool during hyperinsulinemia (Figure 5 A), and that these amino acids are necessary to serve as precursors to permit the expression of insulin-stimulated MPS (3, 4, 7, 24, 32, 39, 56, 76, 107), because insulin is unable to stimulate uptake or MPS when amino acid delivery to the muscle is prevented (1, 22, 24, 33, 94) or saturated (7, 12, 84). Moreover, regression analyses demonstrated that blood flow to the limb, the delivery of amino acids to the muscle, and the availability of amino acids in the muscle free pool are responsible for the majority of the variation in MPS during hyperinsulinemia at rest (32, 76). The regulation of MPS by the delivery of amino acids

to the muscle has also been proposed by Bohé and colleagues, who observed that the concentration of essential amino acids in the blood was highly and positively correlated with the rate of MPS when increasingly large doses of essential amino acids were consumed at rest. The intramuscular availability of essential amino acids was unrelated to the synthetic response after amino acid ingestion, and Bohé proposed the existence of a sensor of essential amino acids, likely membrane bound, that regulates MPS (9). Therefore, it appears more likely that the insulin-mediated increases in blood flow simply provide the essential amino acids to the muscle, and it is the essential amino acids that directly stimulate MPS.

It is possible that insulin-mediated amino acid delivery and uptake could play a role in sustaining the MPS response when the breakdown of muscle amino acids is inhibited, because the availability of amino acid precursors may then limit the expression of MPS. The uptake of amino acids into the muscle may not only be increased by insulin-mediated blood flow, but may be further increased by an insulin-mediated enhancement of the activity of the muscle's amino acid transmembrane transport systems (4, 5, 7). Insulin potently increases the transmembrane transport of alanine at rest and after resistance exercise (4, 5, 7) via direct or indirect stimulation of the transport system A (21), which is primarily responsible for the transport of alanine and glutamine. However, alanine and glutamine are typically not rate-limiting amino acids for MPS, and insulin does not appear to directly stimulate the transport systems for the other physiological amino acids (4, 5, 7). The increased uptake of essential amino acids observed during hyperinsulinemia at rest is proposed to be caused by hyperinsulinemia-induced increases

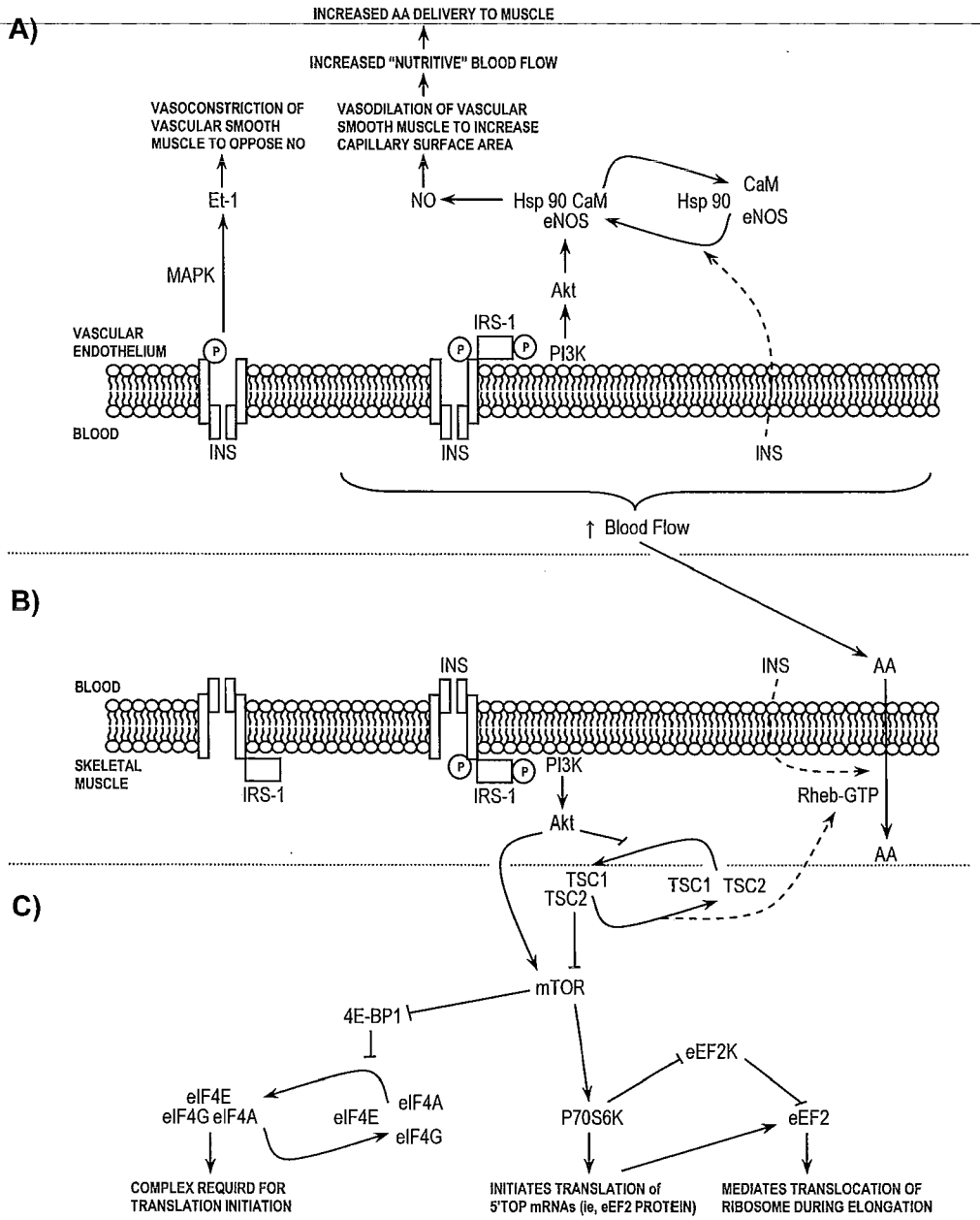

Figure 5. Mechanisms by which insulin may stimulate muscle protein synthesis. Indirect stimulation by increasing the delivery of amino acids to the muscle (A) and/or by facilitating an increased transport of amino acids into the muscle (B) and/or direct stimulation of the translational machinery (C).

in blood flow and amino acid delivery to the muscle (7), which may explain why the uptake of essential amino acids is not enhanced after resistance exercise, when insulin is unable to further stimulate blood flow (7). Therefore, it does not appear that the insulin-mediated regulation of amino acid transmembrane transport contributes largely to the insulin-mediated stimulation of MPS (Figure 5 B).

Insulin-mediated increases in blood flow, and thus amino acid delivery, are clearly integral to the stimulation of MPS by insulin; however, whether insulin stimulates MPS directly and the free amino acids are simply used as precursors, or whether insulin solely increases the provision of the essential amino acids that directly stimulate MPS remains unclear. That insulin is required, albeit at near basal concentrations, for maximal stimulation of MPS by amino acids at rest and after resistance exercise (35), suggests that insulin must have some role in priming or stimulating the muscle synthetic machinery. This is particularly true during the immediate post-exercise recovery period, when hyperinsulinemia is unable to further increase the flow of blood to the muscle.

1.5.2 Insulin and Muscle Protein Synthesis

At the cellular level, protein synthesis involves the coordinated processes of gene transcription, mRNA translation and the post-translational modifications of the polypeptide chains into complete and functional proteins (Figure 6). Increases in MPS measured within the first 4h of stimulus onset, such as during hyperinsulinemia with or without concurrent amino acid provision and resistance exercise, appear to be too rapid to be generated by an increased capacity for translation via transcription (77). Certainly, transient and gradual elevations or reductions in gene transcription may mediate the long-

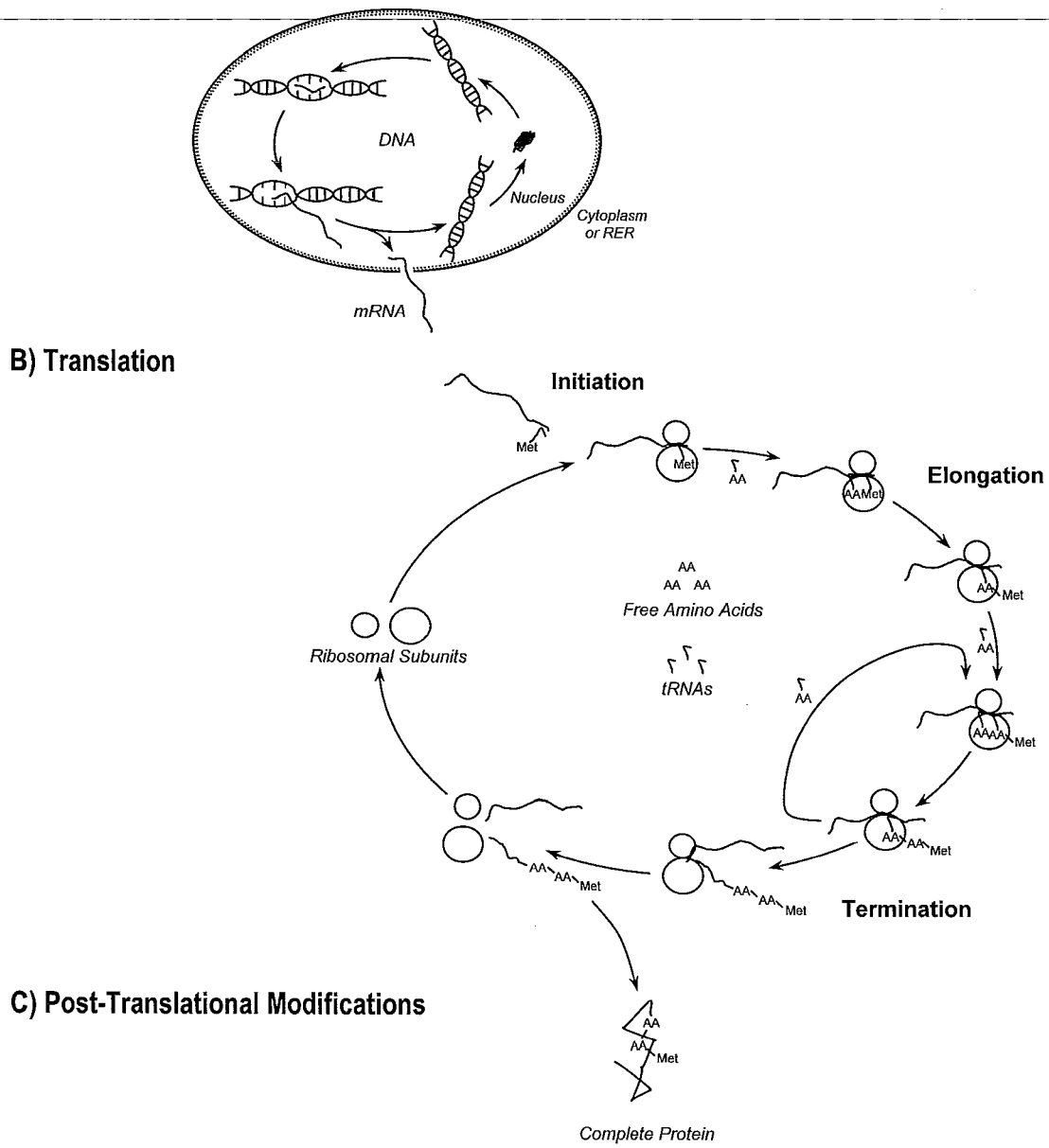

Figure 6. Overview of the key events of protein synthesis. The synthesis of proteins consists of the transcription of DNA into mRNA (A), the translation of mRNA into an amino acid polypeptide (B), and the post-translational modifications that transform the amino acid polypeptide into a complete protein (C).

term protein synthetic response (50), and the expression of hundreds of genes in muscle cells have been shown to be differentially regulated within 3h after exposure to insulin (82). The elevation of MPS measured following 3-4h after onset of hyperinsulinemia more likely reflects an enhanced translational efficiency; that is, an increase in the rate at which existing mRNAs are translated into proteins, which can occur via increases in the content or activity of the ribosomes (66, 108).

The mammalian target of rapamycin (mTOR) has been elucidated as a signalling molecule that is centrally important and necessary for contraction-induced increases in MPS (26) and muscle hypertrophy (8). In fact, mTOR appears to be a convergence point for a number of growth stimuli including feeding, exercise, and hormones (21, 25). The activation of mTOR mediates hypertrophy by enhancing mRNA translation, because several downstream targets of mTOR up-regulate the translation of mRNAs that encode for proteins that are required for the synthesis and activity of ribosomes (21, 25). When active, mTOR inhibits 4E-BP1, which permits the formation of the eIF4F complex (eIF4A-eIF4E-eIF4G) that binds mRNA to the ribosome during translation. Another downstream target of mTOR, p70S6K, initiates the translation of a series of mRNAs that encode for proteins that comprise the ribosome, elongation factors, eIF4G, and poly(A) binding protein (21, 25). The proteins translated by activated p70S6K independently enhance translational efficiency (i.e., the rate of protein synthesis per mRNA template). Another effect of activated p70S6K is to remove the inhibition of eukaryotic elongation factor 2 (eEF2), the activation of which mediates the translocation of the ribosome to reveal the next sequence of bases to be translated during elongation (21, 25).

In vitro and ex vivo studies have characterized an insulin-sensitive signalling pathway in skeletal muscle that converges on the mTOR signalling pathway (25, 108). By interacting with the insulin surface receptor on muscle cells, insulin initiates a signalling cascade involving phosphotidylinositol-3 kinase (PI3K) that phosphorylates protein kinase B or Akt (Figure 5 C). Phosphorylation of Akt appears to directly stimulate mTOR, as well as remove the TSC1-TSC2 complex-mediated inhibition of mTOR by preventing the formation of the TSC1-TSC2 complex (25, 108). The coordinated stimulation of the insulin- and mTOR-signalling pathways has recently been confirmed in vivo in humans (23, 24, 31, 35). Locally induced hyperinsulinemia stimulated Akt (37) and p70S6K (37, 41, 53) but did not appear to stimulate 4EBP1 (41) in healthy humans at rest. If amino acid concentrations and delivery decreased during systemic hyperinsulinemia, insulin signalling through Akt was maintained, but insulin was unable to stimulate MPS or stimulate mTOR, p70S6K, or eEF2 (24). Replacement of amino acids to basal concentrations permitted an insulin-mediated stimulation of mTOR and p70S6K, demonstrating that amino acids were necessary for the insulin-mediated stimulation of mRNA translation (24). Leucine provision during hyperinsulinemia at rest did not stimulate Akt more than hyperinsulinemia alone, but stimulated p70S6K to an extent that was greater than the sum of the individual effects of hyperinsulinemia and leucine alone, suggesting that insulin and amino acid signalling can act synergistically to increase translation efficiency, although MPS was not measured to verify whether the increased translation efficiency was expressed in the rate of MPS (37). The co-ingestion of carbohydrates and protein increased MPS and the stimulation of Akt, mTOR, 4EBP1,

and p70S6K at rest (31) and after resistance exercise (23), whereas eEF2 phosphorylation was only decreased (indicating an enhanced rate of protein chain elongation) in the resting condition (31). Stimulation of the mTOR signalling pathway by insulin during the provision of amino acids appears to be minimal compared to the amino acid-mediated stimulation of mTOR, because the increased stimulation of Akt did not increase the phosphorylation of TSC2 at rest (31) or after exercise (23), and therefore did not prevent formation of the mTOR-inhibiting TSC1-TSC2 complex. However, the direct stimulation of mTOR by Akt was not concurrently investigated and so could not be discounted. Interestingly, when the rate of MPS was saturated by the provision of amino acids, plasma insulin concentrations greater than basal did not stimulate MPS further, but insulin increased phosphorylation of Akt and p70S6K in a dose-dependent manner (35). Phosphorylation of mTOR, 4EBP1, and eEF2 was also increased by plasma insulin greater than basal concentrations, but appeared to saturate at an insulin concentration of 30 $\mu U \cdot ml^{-1}$ (35). Therefore, insulin does not appear to further stimulate MPS when amino acid provision has saturated MPS, in spite of increasing the stimulation of the translational machinery to increasing doses of insulin (Figure 7). Overall, it is clear that amino acids require insulin to stimulate MPS and to stimulate the translational machinery; however, the required concentration of insulin is naturally present at rest and in the post-absorptive state in healthy, young humans.

1.5.3 Insulin and Muscle Protein Breakdown

Few studies have directly measured MPB during hyperinsulinemia in humans, and even fewer studies have concurrently measured muscle proteolytic signalling or

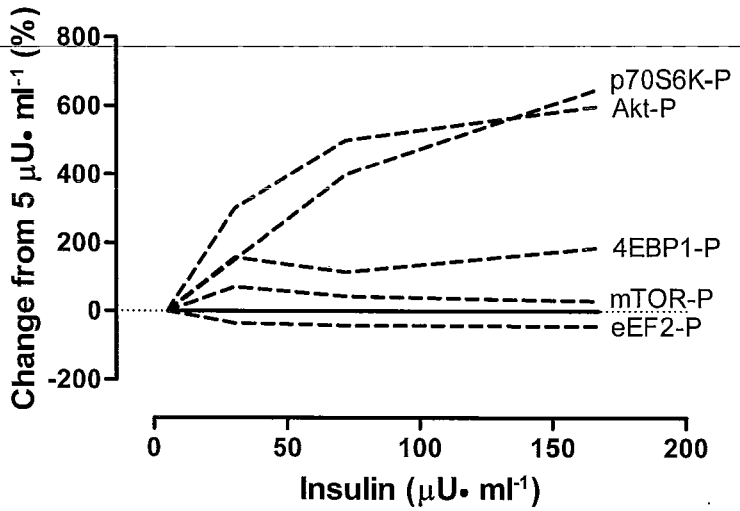

Figure 7. Dose-response curve for plasma insulin concentration on muscle protein synthesis, limb blood flow, and muscle anabolic signalling at rest, when sufficient amino acids are consumed to saturate the muscle protein synthesis rate (20, 63). Values during exposure to 30, 67, and 172 $\mu U \cdot ml^{-1}$ plasma concentrations of insulin have been expressed relative to the values during exposure to 5 $\mu U \cdot ml^{-1}$. The fractional synthesis rate of mixed muscle protein (FSR), a direct measure of MPS, did not significantly change with plasma insulin concentrations greater than 5 $\mu U \cdot ml^{-1}$ and is represented by the solid line. Limb blood flow did not significantly change with plasma insulin concentrations greater than 5 $\mu U \cdot ml^{-1}$, and is also represented by the solid line. All non-significant changes in FSR and blood flow were less than a 15% change from the values during exposure to plasma insulin concentrations of 5 $\mu U \cdot ml^{-1}$. Data are from Greenhaff et al. (35)

activity of proteolytic components. The insulin-mediated inhibition of MPB after resistance exercise, but not at rest, might be expected on the basis of studies that have elucidated the mechanism and insulin sensitivity of the proteolytic pathways, as well as the contribution of each proteolytic pathway to resting and post-exercise MPB. Protein breakdown in resting human muscle is primarily driven by the ubiquitin-proteasome pathway (27), which is a pathway that is not sensitive to acute changes in insulin (44).

This is consistent with the lack of change in MPB observed in humans during acute hyperinsulinemia at rest. The lysosomal pathway is insulin sensitive but contributes very little to bulk MPB at rest. Resistance exercise stimulates the activity of the lysosomal pathway (43), which is consistent with the inhibition of MPB by hyperinsulinemia after resistance exercise in humans (7). The selective inhibition of lysosomal protein breakdown by hyperinsulinemia also helps to resolve the observation that hyperinsulinemia inhibited urinary 3-MH excretion after resistance exercise (84) but not at rest (60).

The insulin-mediated stimulation of Akt has been observed to inhibit the activity of the FOXO family of transcription factors, which appear to stimulate the transcription of genes that code for proteins of the ubiquitin-proteasome system (45, 91). In a study of the effects of hyperinsulinemia on resting human muscle when sufficient amino acids were provided to maximally stimulate MPS, insulin caused dose-dependent increases in Akt phosphorylation and dose-dependent decreases in the expression of the ubiquitin ligases and C2 proteasome unit proteins, without causing changes in the mRNA expression of the ubiquitin ligases (35). Insulin also inhibited LPB in this study, but the inhibition of LPB was fully manifested at insulin concentrations that were much lower than those that decreased the proteolytic pathway protein expression (35). It is difficult to comment on this discrepancy in light of our current state of knowledge, and clearly, more in vivo work is needed to elucidate the regulation of the proteolytic pathways in human muscle.

1.6 RATIONALE FOR RESEARCH

Basal concentrations of insulin appear to be required for the maximal stimulation of MPS by amino acid and/or protein feeding at rest and after resistance exercise in healthy adult humans (35, 47); however, plasma insulin at greater than basal concentrations does not further stimulate MPS when the provision of exogenous amino acids is sufficient to maximally stimulate MPS (35, 47). These observations were made during hyperaminoacidemia that was sustained by the constant infusion of mixed amino acids (46) or the feeding of multiple boluses of protein (35, 47). Whether plasma insulin at greater than basal concentrations can further stimulate MPS after the feeding of a single bolus of whole protein remains unexplored, but may occur if the concentration of amino acids in the blood does not saturate the delivery of amino acids to the muscle. It would be of greater physiological relevance to explore how MPS responds to a bolus of food because this would be a more representative of 'real life' nutrient delivery than amino acid infusion and repeated feeding of small doses of protein. In the single bolus feeding scenario, hyperinsulinemia may stimulate MPS by increasing blood flow and amino acid delivery to the muscle and/or by directly stimulating the muscle translational machinery.

1.7 HYPOTHESES

We implemented a within-person repeated measures design to test the central question of whether carbohydrate, when added to protein, would further stimulate MPS than rates seen after protein ingestion alone. We hypothesized that when a bolus dose of whey protein, containing sufficient protein to maximally stimulate the process of MPS (25g), is consumed at rest and after resistance exercise, the co-ingestion of maltodextrin (50g) and

the subsequent plasma hyperinsulinemia will further stimulate MPS at rest but not after resistance exercise. At rest we hypothesized that carbohydrate ingestion would bring about an insulin-mediated increase in blood flow to the limb and a synergistic stimulation of anabolic signalling of the translational machinery producing a greater stimulation of MPS by protein-carbohydrate co-ingestion than by protein ingestion alone. In contrast, hyperinsulinemia would not further increase blood flow to the limb after resistance exercise, and therefore protein-carbohydrate co-ingestion would not stimulate MPS greater than protein ingestion alone after resistance exercise, in spite of a possible synergistic stimulation of anabolic signalling to the translational machinery.

CHAPTER II: Concurrent ingestion of carbohydrate and protein does not augment protein-stimulated muscle protein synthesis at rest or after resistance exercise in young men

2.1 INTRODUCTION

A primary adaptation to resistance exercise is the hypertrophy of skeletal muscle. Emerging evidence suggests that this process is principally driven by local rather than systemic mechanisms (102, 104). Resistance exercise alters the phosphorylation status of a variety of muscle signalling molecules involved in the regulation of mRNA translation initiation and elongation, which subsequently appears to be responsible for accelerating MPS and mediating muscle hypertrophy (25, 46, 75). In the fasted state, resistance exercise alone does not result in a net anabolic protein balance (51, 73), suggesting that the ability of resistance exercise to produce chronic gains in skeletal muscle mass is intrinsically limited, perhaps by the availability of amino acid precursors (9, 32, 89), a hormonal mechanism (88), or the energy demands of protein synthesis (15, 83). It has been clearly established that protein and/or essential amino acid (EAA) provision is required to promote muscle protein accretion during the acute recovery period after resistance exercise (51, 87, 95). Whether hyperinsulinemia stimulated by the co-ingestion of carbohydrates with protein would provide an even greater stimulus for muscle accretion remains unclear.

No study to date has examined the response of muscle protein synthesis (MPS) to the consumption of protein with and without carbohydrates in a bolus meal that would presumably mimic the most practical or 'real life' feeding pattern. In previous work (47), the repeated small aliquot feeding pattern employed may have continuously saturated plasma amino acid concentrations, which in turn may have masked any insulin-mediated

stimulation of local blood flow and amino acid delivery and/or amino acid uptake by the muscle.

The purpose of this study was to test whether the co-ingestion of carbohydrates (50g) would augment the muscle protein synthetic response to a bolus of high quality protein (25g) previously demonstrated to maximally stimulate MPS (20, 63). We hypothesized that insulin, if it were to be stimulatory for MPS, might stimulate limb blood flow and enhance amino acid delivery to the limb at rest (4); however, insulin would not further stimulate limb blood flow or MPS after resistance exercise followed by adequate protein consumption (7). We also proposed that the stimulatory effect of insulin on MPS may be enhanced or directly caused by insulin-mediated signalling to the translational machinery via protein kinase B (Akt). A unilateral exercise model was employed so that the nutritional manipulation could be examined under resting and exercised conditions in the same individual with the same systemic environment (102, 104).

2.2 METHODS

2.2.1 Participants

Nine young, recreationally active males (age: 23.0 ± 1.9 y; weight: 80.3 ± 8.5 kg; BMI: 24.2 ± 2.1 $kg \cdot m^{-2}$) were recruited to participate in the study. All participants were informed about the purpose of the study, the procedures, the risks, and their rights prior to providing written consent. Participants were assessed as being healthy based upon their responses to a routine medical questionnaire, which was used to screen for conditions

precluding participation. Participants were instructed not to change their activity or dietary habits immediately before or between trails. This study conformed to the Helsinki Declaration, and was approved by the local Research Ethics Board of McMaster University and the Hamilton Health Sciences.

2.2.2 Pre-testing

Unilateral leg extension testing was performed for each leg on two separate occasions, at least seven days apart, to determine the single leg 1 repetition maximum (RM) and the 8-12 RM. In this way we familiarized the participants with proper lifting techniques and ensured the reproducibility of their single leg strength assessments. Emphasis was placed on lifting with control through the full range of motion and lifting until voluntary failure, which was operatively defined as a lift that was not performed with control or through the full range of motion.

2.2.3 Diet and Activity Prior to Trials

Participants were asked to refrain from heavy leg exercise for 72h prior to each of the trials, and to refrain from alcohol, caffeine, and other drugs for 24h prior to each of the trials. Participants kept a food record for the day before their first trial and were asked to replicate the diet and approximate eating times before the second trial. Participants were only permitted to consume water during the 10h before each trial, and they were asked to obtain a full night of sleep on the evenings before each trial.

2.2.4 Trial Design

Participants performed two trials, separated by at least seven days. Each trial consisted of a bout of unilateral knee extension resistance exercise (4 sets, 8-12 RM to failure) and the consumption of a protein or protein-carbohydrate drink post-exercise. The participant's dominant leg (based on strength) was randomly assigned, in a counterbalanced fashion, to rest or exercise in each of the trials. After completing the resistance exercise protocol, participants consumed one of two drinks. One drink consisted of 25g of whey protein isolate (PRO; Protient Inc., St. Paul, MN), the quantity of which has previously been shown to maximize the rate of MPS at rest and following resistance exercise (20, 63). The other drink consisted of 25g of whey protein isolate and 50g of maltodextrin carbohydrate (PRO+CARB; Roquette Frères, Lestrem, France). Drinks were administered in a double-blind, randomized, cross-over fashion, with the flavour and appearance of the drinks matched by the addition of cocoa and artificial sweetener (Splenda®, Johnson & Johnson Inc., Guelph, ON). The drinks were also enriched with L-[*ring*-$^{13}C_6$]-phenylalanine tracer to 6% of the phenylalanine content of the whey protein, to ensure a relatively constant isotopic enrichment of the blood and intracellular precursor pools (see below).

2.2.5 Infusion and Sampling Protocol

Upon arrival to the laboratory, the participant's height and weight were recorded. The participant then lay supine on the bed for 15 minutes prior to preparation for the ultrasound measures to eliminate any residual blood flow effects due to transportation to the lab. Six electrodes (Kendall Meditrace®, Tyco/Healthcare, Mansfield, MA) were placed on the participant's chest for continuous heart rate monitoring. A MLE1054-V

Finometer® MIDI with medium finger cuff (Finapres Medical Systems, Amsterdam, The Netherlands) was used to monitor blood pressure during the ultrasound measures. The ultrasound probe was placed on the surface of the skin over the femoral artery, and a baseline image and a ten heart cycle sequence of the femoral artery was recorded for each leg. A polyethylene catheter was then inserted in an antecubital vein of each forearm. After taking a baseline blood sample, a primed (2.0 $\mu mol \cdot kg^{-1}$), continuous infusion (0.05 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) of L-[*ring*-$^{13}C_6$]-phenylalanine (Cambridge Isotopes Inc., Andover, MA) was started in one catheter. The isotope was dissolved in 0.9% saline, filtered through a 0.2-μm filter, and infused using a calibrated syringe pump (Harvard Apparatus, Holliston, MA). The blood sampling catheter was kept patent throughout the trial using a 0.9% saline drip.

Participants were moved to a leg extension machine, and warmed-up the leg to be exercised by performing 10-15 repetitions with a light weight (~40% of 1RM). Four minutes of recovery were given between the warm-up and the first trial set. The participant completed the first of four 8-12 RM sets, with four minutes of recovery between the sets. Each set was performed to failure, with the weight adjusted between sets to ensure that failure occurred between 8 and 12 repetitions. After the fourth set, the participant was moved back to the bed to commence a 180 minute recovery period. At 15 min post-exercise, another blood sample was taken, a 10-heart cycle sequence was recorded of the femoral artery of both legs, and one of the drinks was consumed. Additional blood samples were taken at 30, 60, 90, 120, 135, 150, 165, 180 min post-

exercise, and additional ultrasound sequences were recorded for both legs at 30, 60, 90, 120, 180 min post-exercise (Figure 8).

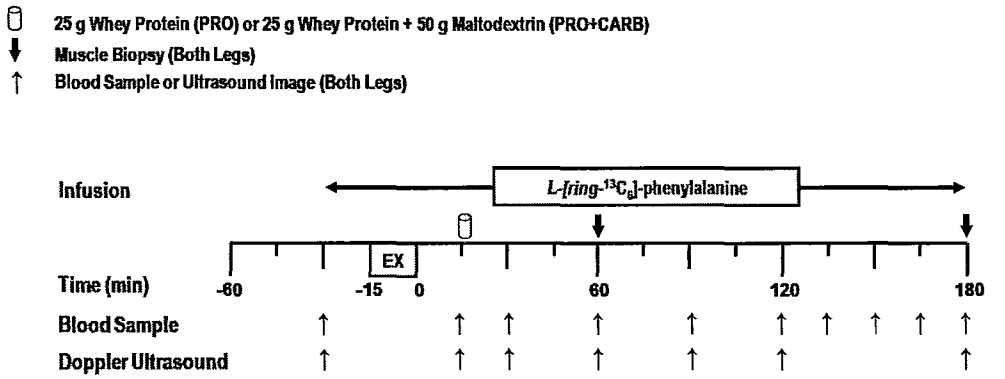

Figure 8. Protocol Schematic

Percutaneous muscle biopsies of the vastus lateralis of both the rested and the exercised legs were taken at 60 min and 180 min post-exercise, using a Bergström needle (modified for manual suction) under 2% xylocaine local anaesthesia. Muscle biopsies were taken from separate incisions, each approximately 4-5 cm proximal from the first incision site. In this protocol we elected to take only post-exercise biopsies since the effects were all within a single subject (i.e., an all within design). In this manner any resting pre-exercise value for MPS would have been relevant only if it differed between legs (i.e., intra-leg variability). Since, in previous studies (63, 103) we observed a CV of resting MPS rate between legs of less than 10% with values of 0.041±0.006 $\%\bullet h^{-1}$, we view our MPS values (all greater than 0.06 $\%\bullet h^{-1}$) and the magnitude of difference

between the rested and exercised legs (28±3%) as real effects representing true differences from a rested fasted state.

2.2.6 Analysis

2.2.6.1 Blood Analyses

All blood samples were collected into 4 ml evacuated containers containing lithium heparin (Vacutainer®, Becton, Dickinson and Company, Franklin Lakes, NJ). Vacutainers were gently inverted 3-5 times to ensure thorough mixing prior to being placed on ice. Within 5 min of blood collection, whole blood was drawn from the vacutainer and the blood glucose concentration was measured on a OneTouch® Ultra® 2 blood glucose meter (Lifescan Inc., Milpitas, CA). A perchloric acid (PCA) protein-extracted blood sample was subsequently collected by adding 100 µl of the whole blood to 500 µl of cold 0.6M PCA and a norleucine internal standard. The PCA-whole blood solution was briefly vortexed to ensure mixing and left on ice for subsequent processing. The whole blood and the PCA-whole blood solution were processed as previously described (61) and the recovered plasma and PCA extracts were stored at -80°C. Plasma insulin concentrations were measured using a commercially-available immunoassay kit (ALPCO™ Diagnostics, Salem, NH). To isolate plasma amino acids, 50 µl of plasma was added to 500 µl of ice-cold acetonitrile. The solution was subjected to vortex and centrifugation and the supernatant was collected and dried under nitrogen (N_2). The enrichment of the plasma amino acids was determined by examining the heptafluorobutyryl isobutyl derivative of the phenylalanine fragment on the gas chromatogram-mass spectrometer (GC-MS; models 6890 GC and 5973 MS; Hewlett-

Packard, Palo Alto, CA). Whole blood amino acid concentrations were analyzed by HPLC as previously described (61) and this method achieved separation of all EAA.

2.2.6.2 Blood Flow Analyses

At each blood flow collection time point, the diameter of the femoral artery was determined from digital B-mode ultrasound images, and the mean blood velocity in the femoral artery (MBV) was measured by using pulsed-Doppler ultrasonography (10 MHz linear array probe, model System 5; GE Medical Systems, Horten, Norway) while the ultrasound probe was placed on the skin surface 2–3 cm proximal to bifurcation of the femoral artery into the superficial and profundus segments. Digital images were acquired at a frame rate of 11 frames•s^{-1}. The diameters were determined using electronic callipers (Echopac v, GE medical Systems, Horten, Norway) on the end-systolic and end diastolic frames. The value at each time point represents the average of 5 consecutive end-systole and 5 consecutive end-diastole measurements. For determination of velocity, the ultrasound gate was maintained at full width to ensure that the entire vessel cross-section was exposed to complete insonation with constant intensity, and the signal was adjusted for insonation angle (34). The audio signal from the pulse wave Doppler was processed through an external spectral analyzer (Neurovision 500M TCD, Multigon Industries, Yonkers, NY) which generated the continuous mean voltage signal, proportional in magnitude to the mean blood velocity. This signal was analogue-to-digital converted and sampled at 200 Hz (Powerlab model ML795, ADInstruments, Colorado Springs, USA), and stored on a computer for subsequent analysis (Chart v5.5.3, ADInstruments,

Colorado Springs, USA). The average MBV was calculated by integrating the total area under the MBV profile for ten subsequent heart cycles at each time point.

2.2.6.3 Mixed Muscle Protein Synthesis

Upon excision, muscle biopsy samples were cleaned from any visible blood, fat, and connective tissue, and were rapidly frozen in liquid nitrogen. Muscle tissue was stored at -80°C until subsequent analyses. One piece of the muscle sample was homogenized in cold acetonitrile using a Teflon® pestle, and centrifuged to form an insoluble protein pellet. The supernatant was removed and dried under nitrogen (N_2) to extract the amino acids of the intracellular protein fraction. The isotopic enrichment of the intracellular protein fraction was measured by examining the heptafluorobutyryl isobutyl derivative of the phenylalanine fragment on the GC-MS. The insoluble protein pellet was washed with double-distilled water (dd-H_2O) and 70% ethanol. Amino acids were released from the insoluble protein pellet by hydrolysis overnight in 6M HCl at 110°C. Distilled water (1.5 ml) was added to the hydrolyzed pellet, and the amino acids were isolated and purified by ion exchange chromatography using cation exchange columns prepared with Dowex® 50W-X8-200 Ion Exchange Resin (Sigma Aldrich Inc., St. Louis, MO). The isolated amino acids were then concentrated on a rotary evaporator. The incorporation of L-[*ring*-$^{13}C_6$]-phenylalanine into the mixed muscle protein-bound samples was determined by converting the free amino acids to their N-acetyl-n-propyl ester derivatives prior to analysis by gas chromatography combustion-isotope ratio mass spectrometry (GC-C-IRMS: Hewlett Packard 6890; IRMS model Delta Plus XP, Thermo Finnigan, Waltham, MA), as previously described (64).

2.2.6.4 Muscle Signalling Molecule Analyses

A second piece of muscle was homogenized in ice-cold extraction buffer (10 µl•mg^{-1}; 50 mM Tris-HCL [pH 7.5], 1 mM EDTA, 1 mM EGTA, 0.1% Triton-X) containing protease and phosphatase inhibitors (10 mM β-glycerophosphate, 50 mM NaF, 0.5mM activated sodium orthovanadate, 1 complete protease inhibitor tablet; protease inhibitor: Roche Diagnostics Ltd, Burgess Hill, UK, and all other chemicals: Sigma Aldrich, Poole, UK) using a Teflon® pestle. The muscle-buffer solution was exposed to vortex and centrifugation to pellet the insoluble proteins from the supernatant. The supernatant was removed and the protein concentration of the homogenate was determined by bicinchoninic acid protein assay (Thermo Scientific, Rockford, IL). All samples were diluted to a standard concentration of 1 mg • ml^{-1} through addition of Laemmli loading buffer, in order to measure relative phosphorylated protein concentrations of protein kinase B (Akt^{Ser473}), eukaryotic elongation factor (eEF2^{Thr56}), 70 kDa S6 protein kinase (p70S6K^{Thr389}), eukaryotic initiation factor 4E binding protein 1 (4EBP1$^{Thr37/46}$), acetyl coA carboxylase-β (ACC^{Ser729}), extracellular-signal regulated kinase 1/2 (ERK$^{Thr202/Tyr204}$; all antibodies from New England Biolabs, UK), and α-actin (Sigma Aldrich, Poole, UK). The samples were mixed and heated at 95°C for 7 min before 15µg of protein/lane was loaded on to Criterion XT Bis-Tris 12% SDS-PAGE gels (Bio-Rad, Hemel Hempstead, UK) for electrophoresis at 200V for ~60 min. After equilibration (30 min) of the gels in transfer buffer (25mM Tris, 192mM glycine, 10% methanol), proteins were electroblotted on to 0.2 µm PVDF membranes (Bio-Rad) at 100V for 30 min. Blocking was performed for 1 h with 5% low-fat milk in TBS-T (Tris Buffered Saline and 0.1% Tween-20; both

Sigma-Aldrich, Poole, UK). The membranes were then rotated overnight with primary antibody against the aforementioned phosphorylation targets at a concentration of 1:2000 at 4°C. Membranes were washed (3×5 min) with TBS-T and incubated for 1 h at room temperature with HRP-conjugated anti-rabbit secondary antibody (New England Biolabs, UK). The membranes were subsequently washed (3×5 min) with TBS-T, and incubated for 5 min with ECL reagents (Enhanced Chemiluminescence kit, Immunstar; Bio-Rad, Hemel Hempstead, UK). Blots were imaged and quantified by assessing the peak density of the aforementioned target bands, after it was ensured that the bands were within the linear range of detection using the Chemidoc XRS system (Bio-Rad, Hemel Hempstead, UK). The phosphorylation of target signalling proteins was corrected for the amount of protein loaded by referencing to α-actin.

2.2.7 Calculations

Mean limb blood flow (Q) was calculated from the mean velocity and area using the formula: $Q\ (ml \bullet min^{-1}) = MBV \bullet \pi \bullet r^2 \bullet 60$, where MBV is the mean velocity of the blood ($cm \bullet s^{-1}$), r is the mean radius of the artery during the cardiac cycle (cm), and 60 is a constant ($s \bullet min^{-1}$) to convert the calculated flow in $ml \bullet s^{-1}$ to $ml \bullet min^{-1}$. The mean radius of the artery was calculated from the diameter assuming the artery is circular in shape ($r = d/2$). The mean diameter was calculated from the maximum diameter of the artery during systole (d_{max}; cm) and minimum diameter of the artery during diastole (d_{min}; cm) weighted to the percentage of time spent at each diameter in the cardiac cycle (ie, $d = 1/3\ d_{max} + 2/3\ d_{min}$).

The mixed muscle protein synthesis (MPS) was calculated at isotopic plateau using the standard precursor-product method. Briefly, MPS ($\%\cdot h^{-1}$) = $\Delta E_m/E_p \cdot 1/t \cdot 100$, where ΔE_m is the change in the isotopic enrichment of the mixed muscle proteins between biopsies, E_p is the mean isotopic enrichment of the intracellular amino acid precursor pool, t is the incorporation time between biopsies (h), and 100 is a constant to convert the calculated proportion of muscle synthesized per hour to a percentage of muscle synthesized per hour.

2.2.8 Statistics

This study was a within-subject repeated-measures design. Analyses of variance (ANOVA) were carried out with relevant pre-planned comparisons using STATISTICA© v5.1 software (StatSoft, Inc., Tulsa, USA). Strength (1 RM, 8-12 RM), external work (set, trial), and the area under the curve (AUC; glucose, insulin) data were analyzed using paired t-tests. Cardiovascular data and blood metabolites, hormones, and enrichments were analyzed using a two-factor (drink x time) repeated-measures ANOVA. Mixed MPS, intracellular amino acid enrichments, signalling molecule phosphorylation data, and bulk blood flow data were analyzed using a three-factor [drink (PRO and PRO+CARB) x activity (rest and exercise) x time] repeated-measures ANOVA. Where significant differences were observed in the ANOVA, a Tukey post hoc test was manually performed to determine differences between values. For all analyses, statistical significance was set at $P \leq 0.05$. Values are presented as means±standard error of the mean (SEM).

2.3 RESULTS

2.3.1 Strength and External Work

There were no differences in the pre-testing 1 RM (PRO: 84kg vs. PRO+CARB: 83kg; $P = 0.85$) or 8-12 RM (PRO: 62kg vs. PRO+CARB: 61kg; $P = 0.35$) between legs. External work was approximated by multiplying the number of repetitions performed by the mass lifted on each repetition, since each repetition was performed over the same range of motion and at the same cadence (and thus the same velocity). The total external work completed by each leg during their respective exercise trial was identical between conditions (PRO: 2235 kg•reps vs. PRO+CARB: 2242 kg•reps; $P = 0.91$).

2.3.2 Blood Metabolites and Hormones

The plasma glucose and plasma insulin responses were greater for PRO+CARB than PRO ($P < 0.05$), with differences at 30-135 min ($P < 0.05$) and 30-120 min ($P < 0.05$) for glucose and insulin respectively (Figure 9, A and B). Total exposure to plasma glucose and insulin, as determined by AUC analysis, were 17.5-fold and 5-fold greater for PRO+CARB than PRO ($P < 0.05$ each). Plasma concentrations of leucine, branched-chain amino acids, and EAA (Figure 9 C) were greater for PRO than PRO+CARB at 60 and 90 min ($P < 0.05$). Plasma concentrations of phenylalanine increased following consumption of the drink ($P < 0.05$) and thereafter declined to baseline values, but with no differences between drinks ($P = 0.69$). Plasma isotopic enrichments were not different between drinks (PRO: 0.068% vs. PRO+CARB: 0.069%; $P = 0.52$), and the slope of the plasma enrichment curve was not significantly different from 0, indicating a prerequisite isotopic steady state was achieved.

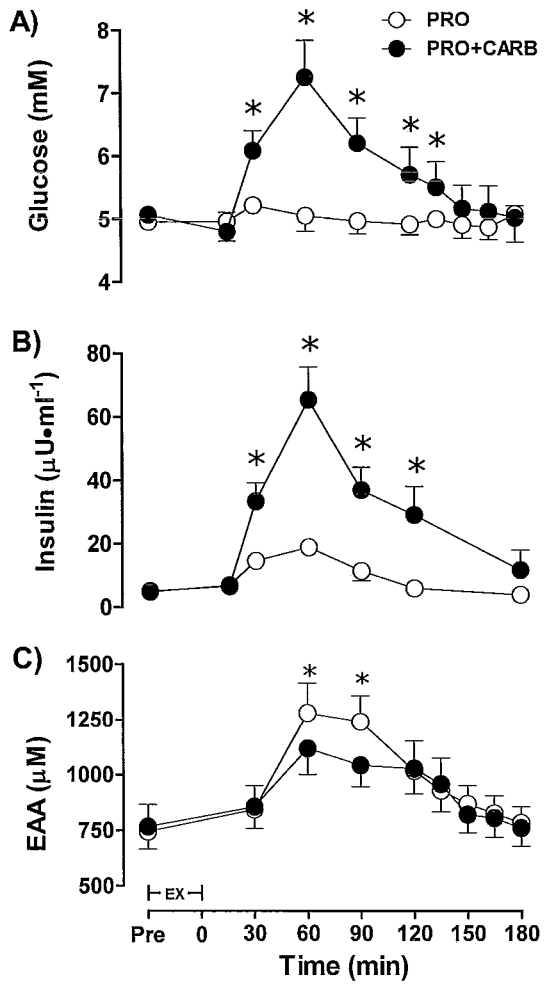

Figure 9. Plasma glucose (A), insulin (B), and essential amino acid (EAA; C) concentrations. An * indicates a significant group-by-time interaction and a significant pair-wise difference between values at the same time point in the other drink condition ($P<0.05$). Values are means±SEM.

2.3.3 Femoral Artery Blood Flow

Mean heart rate and mean arterial pressure were not different between drink conditions ($P = 0.49$ and 0.63 respectively). The mean blood velocity was greater in the exercised than

the rested legs ($P < 0.05$), with no differences according to the nature of the drinks ($P = 0.45$). No differences were found as a result of the drink ($P = 0.75$) or activity ($P = 0.18$) for mean arterial diameter. Femoral artery blood flow was elevated above rest from 15-60 min following exercise ($P < 0.05$), but there were no differences between drinks ($P = 0.49$; Figure 10).

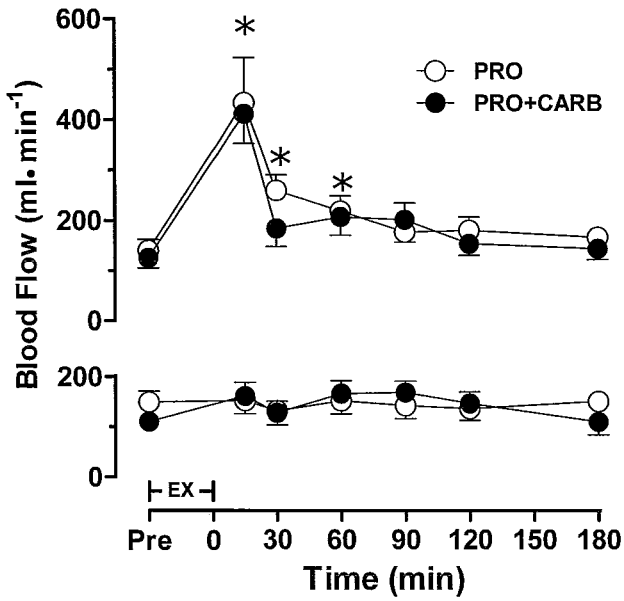

Figure 10. Femoral artery blood flow to the exercised (top panel) and the contralateral rested leg (bottom panel). An * indicates a significant group-by-time interaction and a significant pair-wise difference between values at the same time point in the rested leg ($P<0.05$). Values are means±SEM.

2.3.4 Mixed Muscle Protein Synthesis

Intracellular isotopic enrichments were not different as a result of the different drinks (PRO: 0.045% vs. PRO+CARB: 0.046%; $P = 0.38$). MPS was increased by ~30% after

exercise compared to rest ($P < 0.05$), but there were no differences between the PRO and PRO+CARB trials at rest ($0.070\%\cdot h^{-1}$ vs. $0.069\%\cdot h^{-1}$; $P = 0.99$) or after exercise ($0.091\%\cdot h^{-1}$ vs. $0.089\%\cdot h^{-1}$; $P = 0.99$; Figure 11).

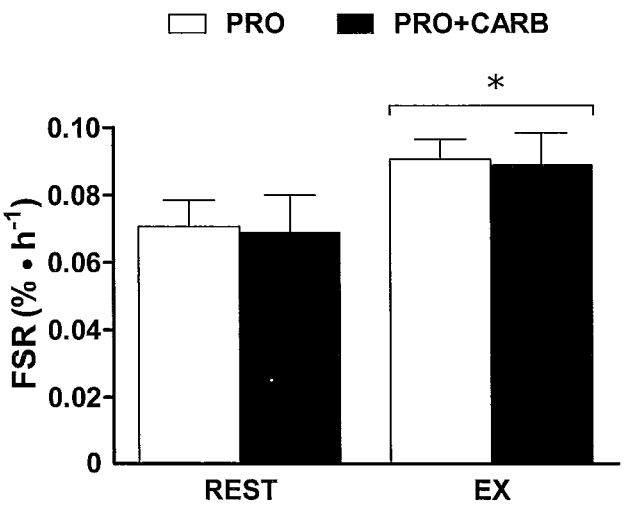

Figure 11. Mixed muscle protein fractional synthesis rate (FSR). The * indicates a significant main effect of exercise versus rest ($P<0.05$). Values are means+SEM.

2.3.5 Anabolic Signalling Molecule Phosphorylation

Phosphorylation of Akt was greater in the PRO+CARB than the PRO trial ($P < 0.05$), and was increased in the exercised leg ($P = 0.05$). Phosphorylation of ACC, a surrogate marker for AMP-activated protein kinase, was greater after exercise than at rest ($P < 0.05$), but with no differences as a result of the different drinks ($P = 0.42$). No differences were observed as a result of the different drinks or exercise for ERK, 4EBP1, p70S6K, or eEF2 (Figure 12).

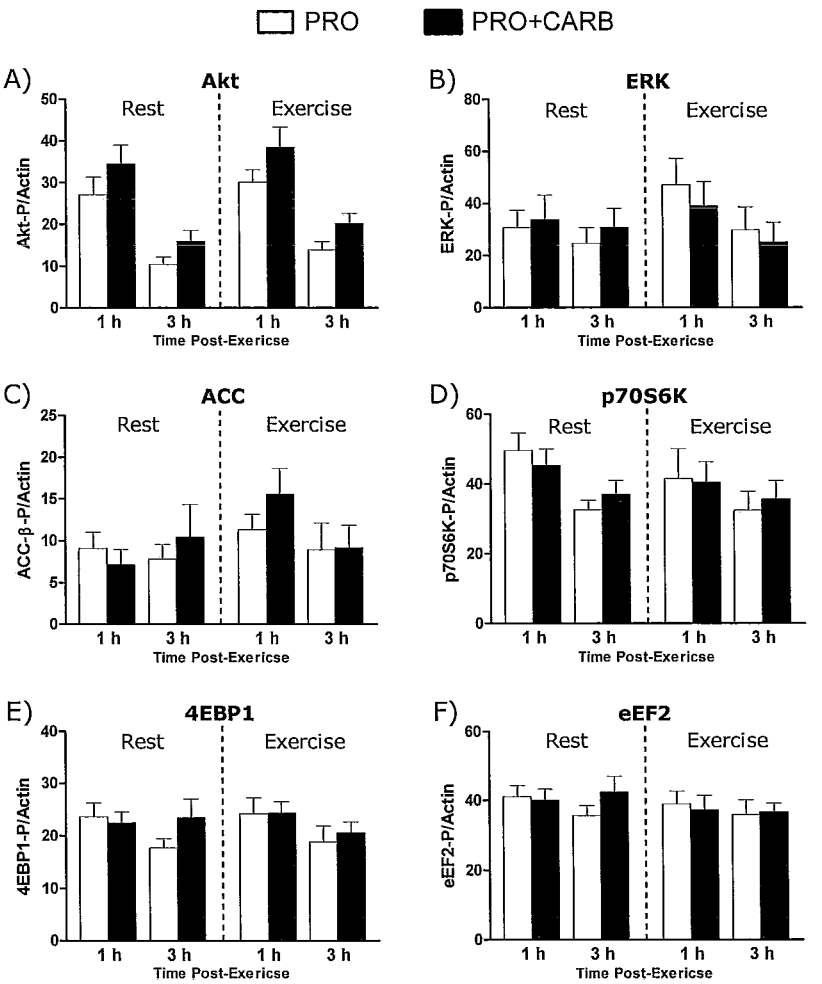

Figure 12. Phosphorylation of the anabolic signalling molecules: Akt (A), ERK (B), ACC (C), p70S6K (D), 4EBP1 (E), and eEF2 (F). For Akt, a significant main effect for PRO+CARB greater than PRO ($P<0.05$), and a significant main effect of EX greater than REST (<0.05) were observed. For ACC, a significant main effect of EX greater than REST (<0.05) was observed. Values are means±SEM.

2.4 DISCUSSION

We hypothesized that insulin might augment protein-stimulated MPS at rest by increasing limb blood flow and amino acid delivery (4) and by stimulating the

translational machinery via signalling through Akt. We also hypothesized that insulin would not further stimulate limb blood flow or MPS after resistance exercise and adequate protein consumption (7), in spite of continued stimulation of the translational machinery by insulin. Contrary to our hypotheses, we saw no augmentation of protein-stimulated MPS by hyperinsulinemia at rest or after resistance exercise, when the protein was consumed as a single meal-like bolus. This accords with the results of a previous study that had employed a repeated aliquot feeding pattern; Koopman and colleagues observed no differences in protein-stimulated MPS when carbohydrates were delivered at a low (~20 $\mu U \cdot ml^{-1}$) and a high dose (~70 $\mu U \cdot ml^{-1}$) to maintain hyperinsulinemia than when the amino acids were consumed alone (47). In our study, neither muscle anabolic signalling (4EBP1, p70S6K, eEF2) nor blood flow to the quadriceps were different between the PRO and PRO+CARB conditions. These results suggest that the plasma aminoacidemia and insulinemia in response to 25g of whey protein is sufficient to maximize insulin-mediated stimulation of MPS. Clearly, any insulin-mediated mechanisms that normally stimulate MPS at rest (eg. after a meal) are without any further effect after ingestion of protein at doses known to maximally stimulate MPS (20, 63).

Our results are consistent with those from Greenhaff and colleagues, who measured the dose-response of insulin on MPS during hyperaminoacidemia (35). These workers observed that leg blood flow and MPS were saturated at basal plasma insulin concentrations of ~5 $\mu U \cdot ml^{-1}$, and insulin concentrations always exceeded 5 $\mu U \cdot ml^{-1}$ in our study. The evidence is mounting to suggest that very low perhaps near basal concentrations of insulin are required to obtain a maximal amino acid-induced increase in

MPS (9, 35, 90). Our data extend the previous work by further demonstrating that physiological hyperinsulinemia fails to augment MPS in healthy individuals after the ingestion of an amount of high quality protein (i.e., 25g) that is sufficient to maximally stimulate MPS (20, 63). Naturally, more research is required to assess whether insulin-mediated local blood flow can increase MPS when amino acid delivery to the muscle is limiting, such as it would be after the ingestion of a quantity of protein (i.e., < 20g) that is insufficient to maximally stimulate synthesis (20, 63). It has been reported that locally-infused insulin increases blood flow, amino acid delivery, and intracellular amino acid availability, and can subsequently stimulate MPS above fasting rates in the absence of amino acid feeding or infusion (32). Therefore, insulin may be permissive or stimulatory for MPS depending on the quantity of protein ingested and the subsequent aminoacidemia.

The lack of an insulin-mediated augmentation of protein-stimulated MPS was reflected in the lack of differential phosphorylation of several muscle signalling molecules that are thought to be primary regulators of protein translation initiation and elongation; for review see Kimball (46). In particular, proteins downstream of mTOR such as 4EBP1, p70S6K, and eEF2 were not differentially phosphorylated up to 3h after hyperinsulinemia/aminoacidemia and/or exercise. The lack of differences in these particular signalling molecules is surprising given the exercise-stimulated increase in MPS observed in this study, and the abundance of insulin- and exercise-mediated effects reported in the literature (25, 46, 75). This dissociation of signalling and MPS supports accumulating evidence that the extent of phosphorylation of anabolic signalling

molecules is not always as predictive of changes in rates of MPS as might be expected (35, 63). This appears to be particularly true when the muscle is exposed to nutritional and contraction-mediated anabolic stimuli under physiological conditions, and likely reflects the high degree of convergence of the muscle signalling pathways. It is possible that the amino acids and/or exercise simply saturated the signalling transduction pathway such that any potential regulatory activity of these molecules was masked or other mechanisms of the synthetic pathway became rate-controlling between the resting and exercised conditions; we have reported this previously (63). However, we cannot discount the possibility that a divergent signalling response could have occurred at time points later than 3h, especially with respect to an exercise effect. Nonetheless, we propose that, on the basis of the evidence from this and other studies, the component of the stimulus-to-product pathway for MPS that is rate controlling is not simply signalling molecule activation (i.e., phosphorylation) but can vary according to a number of factors, including the combination of stimuli [e.g., feeding (32, 35, 63), exercise (52, 103), etc.], the magnitude of each stimulus [e.g., sub-optimal/optimal (47, 63), non-fatiguing/fatiguing (30, 52), etc.], and the properties of the muscle exposed to the stimuli [e.g., untrained/trained (103), aged (52), etc.].

In summary, carbohydrate co-ingestion with high quality protein does not further augment protein-stimulated MPS at rest or after resistance exercise when the protein is consumed as a single bolus dose known to maximize MPS (20, 63). Instead, the lack of differences in leg blood flow, anabolic signalling molecule phosphorylation, and MPS after pronounced hyperinsulinemia suggests that insulin is not further stimulatory for

MPS during hyperaminoacidemia. Insulin appears, in our view, to be permissive but not stimulatory of MPS in adult human muscle from healthy persons, when sufficient protein is consumed to maximize MPS.

S.1 SUPPLEMENTARY DATA

S.1.2 Introduction

It is becoming increasingly clear that the insulin-like growth factor (IGF) gene is differentially spliced in response to muscle contraction, and as such, the local production of the muscle IGF-I isoforms may also be mediators of muscle growth (36, 40, 58). Whether nutrition, which has been demonstrated to enhance skeletal muscle growth, might alter the splicing of the IGF gene has not been investigated in humans. We examined the expression of IGF-1 mRNA splice variants as a potential local locus of regulation for MPS after exercise and nutrient provision.

S.1.3 Muscle RNA Isolation and Analyses

RNA was isolated from a piece of muscle (from the 3h post-exercise biopsy only) that was homogenized using the TRIzol/RNeasy method, as previously described (58). Briefly, ~25 mg of each muscle sample was homogenized in a total of 1.0 mL of TRIzol Reagent (Invitrogen Corporation, Mississauga, ON) using a glass homogenizer. The RNA of the homogenate was purified by using the RNeasy mini kit, following the manufacturer's instructions (Qiagen Sciences, Valencia, CA). The RNA was quantified and purity was assessed using a spectrophotometer (NanoDrop 1000, Thermo Scientific, Waltham, MA). In 0.2 mL Eppendorf tubes, individual samples were reverse transcribed (RT) in 20 μL reactions using a commercially available kit (Applied Biosystems High Capacity cDNA Reverse Transcription Kit; Applied Biosystems Inc, Foster City, CA) according to the manufacturer's instructions. Briefly, 10 μL of RNA was diluted to 100

ng• μL^{-1} and added to 10 μL of a master mixture containing 2.0 μL of 10x RT buffer, 0.8 μL of 25x dNTP, 2.0 μL of 10x RT random primers, 1 μL of MultiScribe reverse transcriptase and 4.2 μL of nuclease-free H_2O. The cDNA synthesis reaction was carried out using an Eppendorf Mastercycle ep*gradient* thermal cycler (Eppendorf, Mississauga, ON). Following RT, samples were stored at -80°C until further analysis. Individual 25 μL reactions were set up in 0.2 mL Stratagene PCR tubes (Stratagene, La Jolla, CA) and run in duplicate for each time-point. Primer sequences are in Table 4.

Table 4. qRT-PCR Primer Sequences

Gene name	Forward sequence	Reverse sequence
IGF-1 Ea	5'-GCCTGCTCACCTTCACCAGC-3'	5'-TCAAATGTACTTCCTTCTGGGTCTTG-3'
IGF-1 Eb	5'-GCCCCCATCTACCAACAAGAACAC-3'	5'-CAGACTTGCTTCTGTCCCCTCCTTC-3'
MGF	5'-GCCCCCATCTACCAACAAGAACAC-3'	5'-CGGTGGCATGTCACTCTTCACTC-3'
GAPDH	5'-CCACCCATGGCAAATTCC-3'	5'-TGGGATTTCCATTGATGACAA-3'

IGF-1, Insulin-like growth factor; MGF, mechano growth factor (IGF-1 Ec); GAPDH, glyceraldehydes 3-phosphate dehydrogenase.

In each reaction tube, 1.0 μL of cDNA and 7.5 μL of dd-H_2O were added to 16.5 μL of a master mix containing 12.5 μL of RT^2 Real-Time SYBR Green / Rox PCR master mix (SuperArray Bioscience Corp., Frederick, MD) along with 2 μL of the specific forward and reverse primers. qRT-PCR reactions were carried out using a Stratagene Mx3000P real-time qPCR System (Stratagene, La Jolla, CA) using Stratagene MxPro qPCR Software Version 3.00 (Stratagene, La Jolla, CA). Fold changes in gene expression were calculated using the Δ-Δ Ct method (54). GAPDH expression was not

different between groups or conditions. Unpaired t-tests were used to determine differences between values, with significance set at $P \leq 0.05$.

S.1.4 Results: Expression of IGF-1 Splice Variant mRNA

At 3h post-exercise, IGF-1 Ea expression was not different between the PRO and the PRO+CARB trials within the resting or exercised legs. In contrast, the Ea isoform expression was increased by exercise in the PRO+CARB ($P < 0.05$) but not the PRO condition (Figure 13). Expression of IGF-1 Eb was greater for PRO+CARB than PRO within the resting leg ($P < 0.05$) but this difference was not observed in the exercised leg. Expression of IGF-1 Eb was greater in the exercised than the rested leg for both the PRO and PRO+CARB conditions ($P < 0.05$) with no difference between conditions. Expression of IGF-1 Ec mRNA, or so called mechanogrowth factor (MGF), was not present in detectable levels at 3h post-exercise.

S.1.5 Discussion

We provide here, for the first time in humans, evidence that the expression of the IGF-1 Eb splice variant can be acutely up-regulated by contraction and carbohydrate feeding and/or insulin secretion. It is difficult to reconcile the nutritional manipulation of IGF-1, because the function and regulation of local IGF-1 production is not well-understood; however, the time-course of the up-regulation of IGF-1 Eb expression in this study is consistent with a role in the MPS. This is not inconsistent with the emerging roles of each of the skeletal muscle isoforms of IGF-1. The MGF (IGF-1 Ec) isoform appears to be primarily responsible for the activation of satellite cells, whereas the IGF-1 Ea isoform

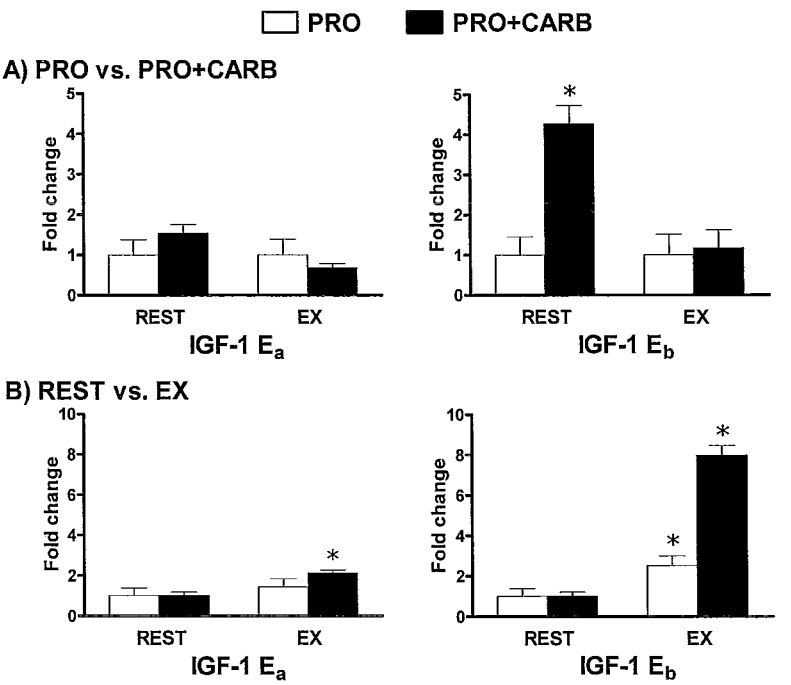

Figure 13. Expression of the IGF-1 Ea and the IGF-1 Eb mRNA in response to the drink (A) and the exercise (B). The * indicates a significant difference between the PRO+CARB and PRO within the rested leg in panel A ($P<0.05$), whereas the * indicates a significant difference between REST and EX within the same drink condition in panel B ($P<0.05$). Values are means±SEM.

appears to stimulate protein synthesis via the PI3K-Akt pathway (106, 110). Nonetheless, the role, regulation, and significance of the IGF-1Eb expression in the context of the current study requires further investigation.

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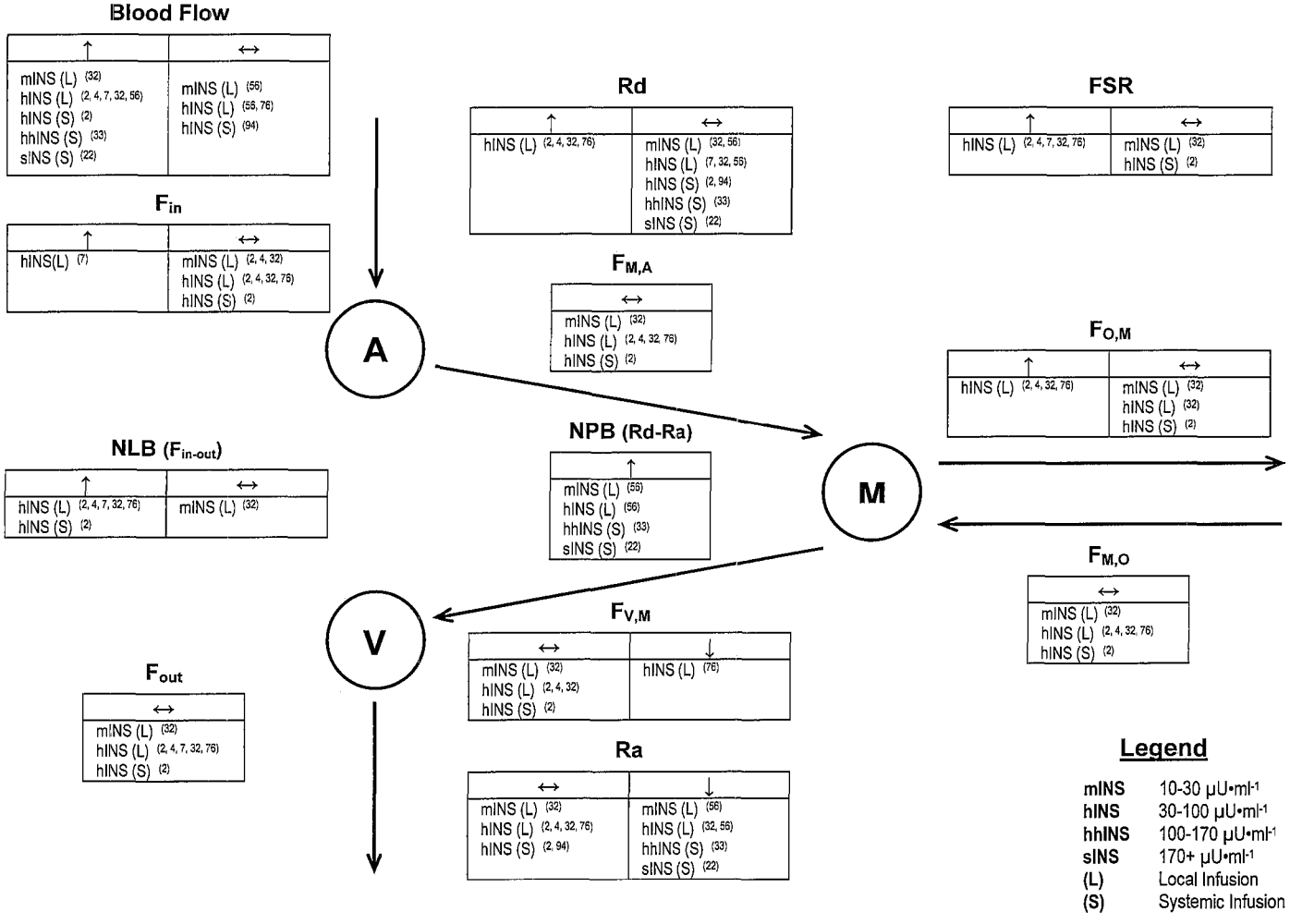
Appendices

Appendix A: Literature Summary Figures

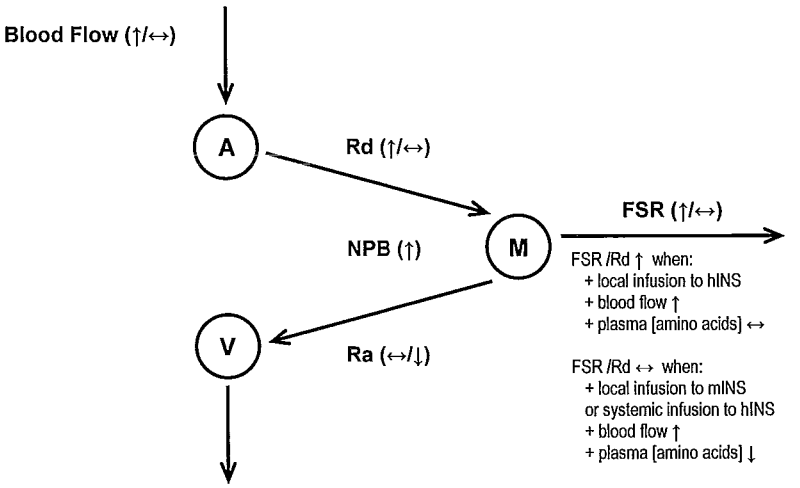


(Figure Caption on Page 79)

Continued from Page 78.

Figure A.1. Plasma insulin and limb blood flow, limb protein turnover, and the synthesis of mixed muscle proteins at rest without amino acid replacement. The statistics and findings reported in the literature were used to designate a significant increase (↑), significant decrease (↓), or no significant difference (↔) between basal kinetics and kinetics during increased exposure to insulin. All participants were young, healthy humans studied in the post-absorptive state at rest. All three-pool amino acid kinetics were measured using a phenylalanine tracer. The details of each study are summarized in the literature summary tables of Appendix A. The schematic and amino acid kinetics were adapted from the three-pool amino acid model originally proposed by Biolo *et al.* (4). FSR is the fractional synthesis rate of mixed muscle proteins.

A) Insulin at rest



B) Insulin at rest with amino acid provision

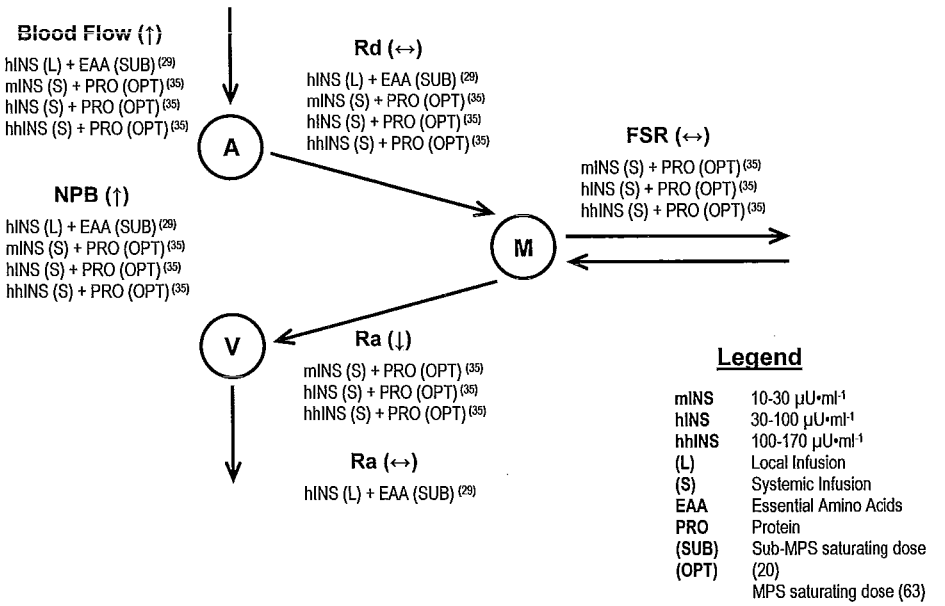
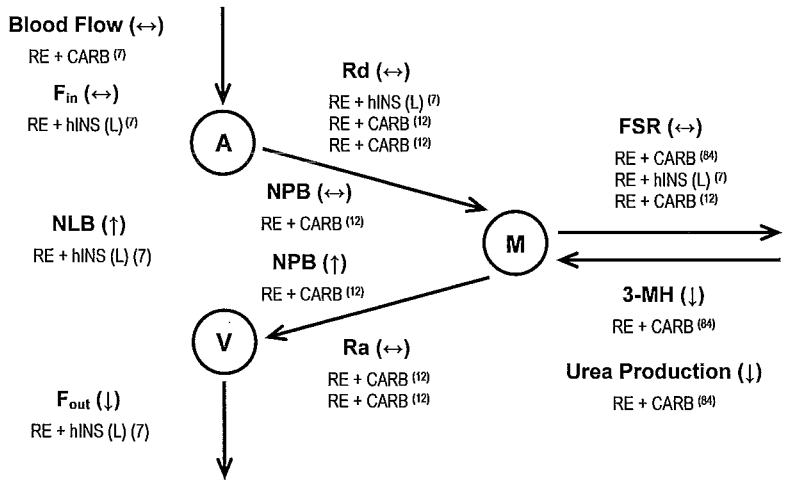


Figure A.2. Plasma insulin and limb blood flow, limb protein turnover, and the synthesis of mixed muscle proteins at rest. Panel A summarizes the effects of insulin-mediated kinetics vs basal kinetics (see Figure 2), whereas panel B summarizes the effects of insulin-mediated kinetics vs amino acid/protein-fed kinetics. The statistics reported in the literature were used to designate a significant increase (↑), significant decrease (↓), or no significant difference (↔) between (A) basal kinetics and kinetics during increased exposure to insulin, and between (B) amino acid/protein-fed kinetics and amino acid/protein-fed kinetics during increased exposure to insulin. All participants were young, healthy humans studied at rest. The three pool amino acid kinetics were measured using a phenylalanine tracer. The references for Panel A are summarized in the literature summary figure A.1 of Appendix A. The schematic was adapted from the three-pool amino acid model originally proposed by Biolo *et al.* (4).

A) Insulin after resistance exercise without amino acid replacement



B) Insulin after resistance exercise with amino acid replacement

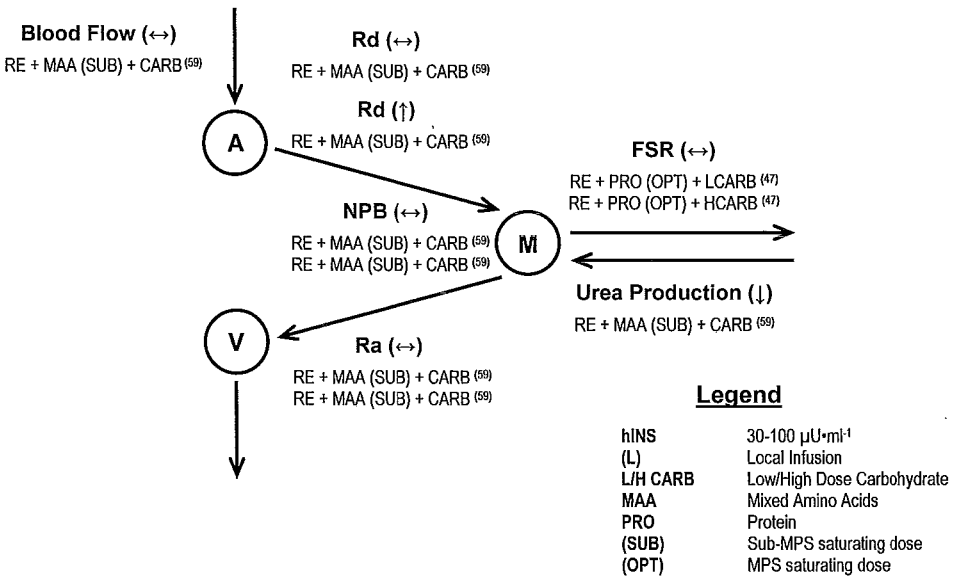


Figure A.3. Plasma insulin and limb blood flow, limb protein turnover, and the synthesis of mixed muscle proteins after resistance exercise. Panel A summarizes the effects of insulin-mediated kinetics vs fasted kinetics, whereas panel B summarizes the effects of insulin-mediated kinetics vs amino acid/protein-fed kinetics. The statistics reported in the literature were used to designate a significant increase (↑), significant decrease (↓), or no significant difference (↔) between (A) post-exercise fasted kinetics and post-exercise kinetics during increased exposure to insulin, and between (B) post-exercise amino acid/protein-fed kinetics and post-exercise amino acid/protein-fed kinetics during increased exposure to insulin. All participants were young, healthy humans studied after an intense bout of leg resistance exercise. The three-pool amino acid kinetics were measured using a phenylalanine tracer. The schematic was adapted from the three-pool amino acid model originally proposed by Biolo *et al.* (4).

Appendix B: Consent Form and Description of Medical Procedures

McMaster University

Department of Kinesiology 1280 Main Street West Hamilton, Ontario, Canada L8S 4K1 Phone 905.525.9140 Fax 905.523.6011 http://kinlabserver.mcmaster.ca

EXERCISE METABOLISM RESEARCH GROUP
DEPARTMENT OF KINESIOLOGY, MCMASTER UNIVERSITY

INFORMATION AND CONSENT TO PARTICIPATE IN RESEARCH

THE ROLE OF POST-EXERCISE NUTRITION ON MUSCLE BLOOD FLOW AND PROTEIN SYNTHESIS

Does additional carbohydrate promote muscle blood flow, in turn improving muscle protein synthesis, after exercise?

Funding for this study is provided by the National Science and Engineering Research Council (NSERC) of Canada.

INVESTIGATORS	DEPARTMENT	CONTACT
Stuart M. Phillips, Ph.D.	Kinesiology, IWC 219B	x24465
Maureen MacDonald, Ph.D.	Kinesiology, IWC 212	x23580
Steven Baker, MD	Medicine, HSC 2H24	x76939
Kyra Pyke, Ph.D.	Kinesiology, IWC AB124	x21385

You are being invited to participate in a research study being conducted by the investigators listed below. Prior to participating in this study you are asked to read this form, which outlines the purpose and testing procedures and describes the medical procedures (see Description of Medical Procedures) used in this study. In addition, you must answer some questions regarding your health, which are included in the attached forms (see Subject Screening Questionnaire). Unless otherwise stated all testing and experimental procedures will be conducted in the Exercise Metabolism Research Laboratory, Room A103, Ivor Wynne Centre.

PURPOSE

Resistance exercise (i.e., weightlifting) and feeding can stimulate the synthesis (i.e., making) of new proteins within your muscle. These new proteins can increase the size of the fibres within your muscle to build a larger muscle; a process termed muscle hypertrophy. However, in order to induce hypertrophy, muscle tissue requires the delivery of amino acids (the building blocks of protein) via the bloodstream. Just as they

stimulate muscle growth, exercise and feeding also promote an increase in what is referred to as 'nutritive' blood flow to muscle tissue; that is, blood flow that supplies the muscle with the nutrients it needs to build and repair itself optimally. It is suspected that the hormone insulin, which the body releases into the bloodstream after a carbohydrate (sugar)-rich meal, promotes the opening (dilation) of capillary beds and the medium sized vessels that control large artery flow. Such dilation would improve the supply of amino-acid-rich blood to the nearby muscle tissue. Thus, a post-exercise meal that initiates a large insulin response from the body may trigger greater protein synthesis than a meal that initiates a smaller insulin response. Our study aims to investigate the role of insulin in the promotion of blood flow to muscle after exercise, and whether this will in turn improve muscle protein synthesis.

Since the consumption of carbohydrates promotes insulin release, our hypothesis is that a post-exercise beverage containing protein and carbohydrates will induce greater blood flow to the exercised muscle than a beverage containing protein alone. In turn, the body's insulin response from the carbohydrate-containing drink will promote greater opening of small blood vessels within the muscle itself, thus promoting increased nutritive flow and subsequently enhancing muscle protein synthesis. The value of this investigation lies in the fact that many disease states, including type 2 diabetes, feature both muscle wasting and impaired blood flow as symptoms. A better understanding of the processes underlying muscle growth and blood flow will allow researchers to investigate interventions that will minimize these negative processes. In addition to affecting blood flow, insulin may also alter the stiffness of the large elastic arteries. Arterial stiffening is an important factor in cardiovascular disease progression and is increased with type 2 diabetes. For this reason, arterial stiffness will also be assessed before and after beverage consumption.

If you consent to participate in this research you will be one of ten participants.

DESCRIPTION OF TESTING PROCEDURES

Prior to the commencement of the study you will participate in a familiarization session with the exercise equipment and testing procedures to be used in the study. The study itself will consist of two ~4h experimental trial sessions, separated by at least 5 days. Each session consists of a bout of resistance exercise, the consumption of a protein-containing beverage, and a three-hour resting measurement collection period (see Figure 1). The only difference between the two trials will be the post-exercise beverage: on one occasion, the drink will contain 25g whey protein; in the other session, the drink will contain 25g whey protein plus 75g maltodextrin (a carbohydrate, similar to sugar) to induce an increase in your blood's insulin concentration.

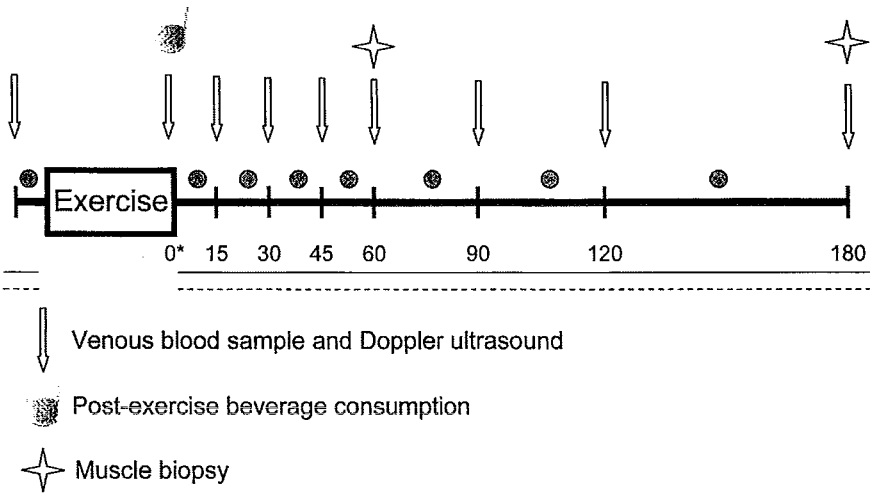
On the evening before each trial date, we will ask that you refrain from eating/drinking after 10:00 pm. Drinking water is fine, but other beverages (e.g. coffee, juice, pop) are not to be consumed. When you arrive in the laboratory the next morning, you will be asked to lie down in order to prepare for the forthcoming measurements. First, six electrodes will be placed on your chest and attached to a recording system to allow us

to monitor your heart rate. Second a blood pressure cuff will be placed around your arm and a sensor secured to your wrist. This will allow us to monitor your blood pressure. Then, a small plastic catheter (flexible plastic tube) will be inserted into a vein (blood vessel) in each forearm using a small needle, to allow for repeated blood sampling An infusion (long slow injection) of an amino acid that contains a special 'stable isotope' will also begin through a catheter. Note that the needle will not remain in your arm, but only the flexible plastic tube. Next, you will receive resting blood flow measurements using standard Doppler ultrasound (we will need to gain access to your artery that flows into your leg as it passes through your groin) to determine the flow within the large femoral artery. The probe will be placed over your femoral artery (in your groin) for ~1 minute. This procedure will be performed in both legs. Also, the collection of a 6mL blood sample will occur at this time. In order to measure arterial stiffness the ultrasound probe will be placed on your carotid artery. On the other side of your neck a pen-like probe placed over your carotid pulse will monitor your carotid pressure. The same pen-like probe will also be placed over your femoral artery to measure femoral artery pressure. A small square shaped infrared (IR) probe will be secured over an artery in the top of your foot (dorsis pedis) OR a small disk shaped piezo-electric probe will be fixed to your big toe to obtain a blood pressure measurement. The probe that provides the best signal will be chosen. This measurement will take less than 10 min.

Following these three measurements, you will proceed to conduct the exercise bout. This will consist of four sets of knee extension, with each set containing 8-10 repetitions, but you will lift the weight with only one leg. Immediately after completion of the exercise, you will return to the supine (lying down) position and receive another blood sample and Doppler ultrasound measurement. You will be given a protein beverage to consume, but neither you nor the experimenters will know which drink you are consuming. Further, this will mark the onset of the stable-isotope-labelled amino acid infusion through an existing catheter in your arm.

For the following three hours, we will conduct additional measurements (blood sampling and Doppler ultrasound blood flow measurements (both legs)) at 15, 30, 45, 60, 90, 120, and 180 minutes from the time of drink consumption. Arterial stiffness measurements (carotid artery ultrasound and carotid, foot/toe and femoral pressure) will be performed in the interval between these blood sampling and Doppler ultrasound femoral blood flow measurements. The foot/toe probe operation is "hands free" and will remain secured to your foot/toe for the duration of the protocol. We will take a muscle biopsy from each of your legs after 60 minutes of the infusion. Finally, at 180 minutes, we will collect a 2nd muscle biopsy from both legs. At this point, the venous catheter will be removed from your arm and the trial will be over. For this study you will complete 2 trials, have two biopsies per leg in each trial, for a total of 8 biopsies (4 in each leg).

Figure 1: Trial Timeline



POTENTIAL RISKS AND DISCOMFORTS

Please refer to the attached form entitled "Description of Medical Procedures" for a complete description of the medical procedures to be performed during the study and the potential risks associated with these procedures.

BENEFITS AND REMUNERATION

In participating in this study you will receive no direct benefit. You will receive an honorarium of $400 upon the completion of the study to compensate you for your time commitment.

PLEASE NOTE: Remuneration will be in the form a cheque issued by McMaster University. The cheque will be mailed to you at the completion of the study for the full amount or at any time should you decide to withdraw from the study for the amount commensurate with the portion of the study you have completed. While we will attempt to have the remuneration forwarded to you as soon as possible, but it may take up to three weeks following the completion of the study for to receive remuneration.

CONFIDENTIALITY

All data collected during this study will remain confidential and stored in offices and on computers to which only the investigators have access. You should be aware that the results of this study will be made available to the scientific community through publication in a scientific journal, although neither your name nor any reference to you will be used in compiling or publishing these results. Additionally, if you are interested you will have access to your own data, as well as the group data, when it becomes available.

PARTICIPATION WITHDRAWAL

At any time you can choose whether to participate in this study or not. You should be aware that your participation in this study will in no way affect your academic performance in any course offered within the Department of Kinesiology. You may exercise the option of removing yourself or your data from the study at any time if you wish. You may also refuse to answer any questions posed to you during the study and still remain as subject in the study. The investigators reserve the right to withdraw you from the study if they believe that circumstances have arisen that warrant doing so.

RIGHTS OF RESEARCH PARTICIPANTS

Funding for this study is provided by the National Science and Engineering Research Council (NSERC) of Canada. This agency is government funded and all research carried out under the auspices of this agency is covered by the Tri-Council Policy Statement, which is the over-riding national policy relating to research involving human participants. A copy of this policy statement can be viewed at http://www.pre.ethics.gc.ca/english/policystatement/policystatement.cfm. As a condition of the investigators listed on this application receiving funding from this agency, this research proposal has been reviewed by the Hamilton Health Sciences/Faculty of Health Sciences Research Ethics Board using the strict application of the ethical principles and the articles of this policy.

You will receive a completed (i.e., signed) copy of this ethics form. You may withdraw your consent to participate in this study at any time, and you may also discontinue participation at any time without penalty. In signing this consent form or in participating in this study you are not waiving any legal claims or remedies. Finally, please realize that this study has been reviewed by the Hamilton Health Sciences Research Ethics

Board. If you have any further questions regarding your rights as a research participant please feel free to contact:

The Office of the Chair of the Hamilton Health Sciences/Faculty of Health Sciences REB at (905) 521-2100 x 42013

INFORMATION

You will be able to contact Dr. Stuart Phillips at 905-525-9140 (x27037 lab or x24465 office) or 905-524-1262 regarding any questions you may have about the study.

I HAVE READ AND UNDERSTAND THE ABOVE EXPLANATION OF THE PURPOSE AND PROCEDURES OF THE PROJECT. I HAVE READ AND UNDERSTOOD THE ATTACHED FORM ENTITLED "DESCRIPTION OF MEDICAL PROCEDURES" AND COMPLETED THE ATTACHED FORM ENTITLED "SUBJECT SCREENING QUESTIONNAIRE" AND AGREE TO PARTICIPATE AS A SUBJECT. I HAVE ALSO RECEIVED A COPY OF THE INFORMATION AND SIGNED CONSENT FORM. MY QUESTIONS HAVE BEEN ANSWERED TO MY SATISFACTION AND I AGREE TO PARTICIPATE IN THIS STUDY. I WILL RECEIVE A SIGNED COPY OF THIS CONSENT FORM.

SIGNATURE DATE

PRINTED NAME OF PARTICIPANT

PERSON OBTAINING CONSENT DATE

PRINTED NAME OF WITNESS

INVESTIGATOR

In my judgement, the participant is voluntarily and knowingly giving informed consent and possesses the legal capacity to give informed consent and participate in this research study.

SIGNATURE OF INVESTIGATOR DATE

PRINTED NAME OF INVESTIGATOR

McMaster University

Department of Kinesiology | 1280 Main Street West, Hamilton, Ontario, Canada L8S 4K1 | Phone 905.525.9140, Fax 905.523.6011, http://kinlabserver.mcmaster.ca

EXERCISE METABOLISM RESEARCH GROUP
DEPARTMENT OF KINESIOLOGY, MCMASTER UNIVERSITY

DESCRIPTION OF MEDICAL PROCEDURES

The study in which you are invited to participate involves four procedures which require medical involvement: muscle biopsy sampling, Doppler ultrasonography, heart rate measurement, blood pressure measurements, and venous/arterialized blood sampling. Prior to any involvement, you are asked to read this form, which outlines the potential medical risks inherent to this procedure. In addition, you must also complete the "Subject Screening Questionnaire" which is designed to identify any medical reason that might preclude your participation.

MUSCLE BIOPSY SAMPLING

This procedure involves the removal of a small piece of muscle tissue using a sterile hollow needle. The primary investigator Dr. Stuart Philips Ph.D. (under approval by Dr. Mark Tarnopolsky M.D. Ph.D.) has extensive biopsy experience and will perform the procedure. He will clean an area over your vastus lateralis muscle (on the outside of your thigh) and inject a small amount of local anaesthetic ("freezing") into and under the skin. He will then make a small incision (~4-5 mm) in the skin in order to create an opening through which to put the biopsy needle into your leg. There is a small amount of bleeding from the incision, but this is minimal. He will then quickly cut off a very small piece of muscle (~50-100 mg; about the size of the eraser on the end of a pencil) and remove the needle from your leg. During the time that the sample is being taken (~5 s), you may feel the sensation of deep pressure in your thigh and on some occasions this is moderately painful. However, the discomfort quickly passes and you are quite capable of performing exercise and daily activities immediately after the procedure.

Following the biopsies, the incisions will be closed with sterile suture (stitch), and wrapped with a tensor bandage. You should refrain from excessive muscle use for the

remainder of the day. Once the anaesthetic wears off, your leg may feel tight and often there is the sensation of a deep bruise or "charlie horse". Analgesics (pain killers) such as Tylenol or Ibuprofen (Motrin) are acceptable if you experience significant pain associated with the biopsy. It is also beneficial to periodically apply an ice pack to the biopsy site the following day, as this will help to reduce any swelling and any residual soreness. The following day your leg may feel uncomfortable when doing squatting activities. The tightness in the muscle usually disappears within 2 days and subjects routinely begin exercising at normal capacity within a day. In order to allow the incisions to heal properly and minimize any risk of infection, you should avoid prolonged submersion in water for 2-3 days. Daily showers are acceptable, but baths, swimming, saunas, etc. should be avoided for at least 4 days following the biopsy procedure.

Potential Risks. The biopsy technique is routinely used in physiological research and complications are rare provided that proper precautions are taken. However, there is a risk of internal bleeding at the site of the biopsy, which can result in bruising and temporary discoloration of the skin. On occasion a small lump may form under the site of the incision, but this normally disappears within 2-3 months. As with any incision there is also a slight risk of infection, however this risk is virtually eliminated through proper cleansing of the area and daily changing of wound coverings. If the incision does not heal within a few days or you are in any way concerned about inflammation or infection, please contact us immediately. In very rare occasions there can be damage to a superficial sensory nerve, which will result in temporary numbness in the area. There is also an extremely remote chance that you will be allergic to the local anaesthetic; the real incidence of lidocaine allergy is unknown.

In past experience with healthy young subjects, approximately 1 in 2,200 have experienced a local skin infection; 1 in 500 have experienced a small lump at the site of the biopsy (in all cases this disappeared within ~4-6 wk using local massage); 1 in 1,500 have experienced a temporary loss of sensation in the skin at the site of incision (an area of numbness about the size of two $2 coins which lasted up to 3 months), and 1 in 30 have experienced bruising around the site of incision which lasted for ~4-5 days. While there is also a theoretical risk of damage to a small nerve branch that is used to allow your muscle to move, this has never been seen in any biopsies performed at McMaster University in the Ivor Wynne Centre (~2500 total biopsies). Hence, the risk of damaging a small motor nerve branch is impossible to estimate.

STABLE-ISOTOPE-LABELLED AMINO ACID INFUSION

You will receive, through a small catheter placed in your arm, an infusion (slow measured amount) of an amino acid (a small component of protein) solution. The amino acid will be dissolved in saline (a salt solution similar to your blood). The amino acid will be labelled with a stable isotope of carbon, hydrogen, or nitrogen. An isotope is slightly heavier form of these elements. Since the isotope is stable (i.e., non-radioactive) it poses no health risk to you due to radioactive exposure. Also, a certain fraction of all of the carbon, hydrogen, and nitrogen within your body is already in the same form as that of the stable isotope. Hence, the infusion of the stable isotope-labeled amino acid will

simply result in a slight increase in the amount of stable isotope within your body; we refer to this as "enriching" the amount of stable isotope within your body. This enrichment will not remain high, however, and will be back to pre-infusion levels within a few days. All of the infused solutions are prepared under sterile conditions and are filtered through a very selective filter prior to entering your body. All solutions that enter your body do not contain, except for the amino acid, anything that will affect your health.

Potential Risks. Despite all precautions there is a theoretical risk (less than 1 in 1,000,000) that you could have a rapid drop in blood pressure due to some small bacterial contamination of the infusion solution (infusate). This has never occurred in our experience.

DOPPLER ULTRASOUND ARTERIAL DIAMETER AND BLOOD VELOCITY MEASUREMENTS

Doppler ultrasound will be used to measure femoral arterial diameters and blood velocity. These procedures will take approximately one minute per measurement. Doppler ultrasound uses high frequency sound waves emitted from a probe placed on the surface of your artery. Blood vessel diameter is determined by sound waves deflecting off the blood vessel wall. Red blood cells flowing through the artery deflect transmitted sound waves back to the probe, allowing for measurement of blood velocity.

Specifically, femoral artery measurements will be recorded using a 10MHz probe. Conductive gel will be spread over the skin surface and a small wand, which emits high frequency sound waves, will be pressed against the skin over the gel covered area. The wand will be moved around until the best position is found. This procedure causes little to no discomfort and will only require that you lay very still. The wand will be placed on the upper thigh. For measurement of arterial stiffness, the carotid artery in the neck will be assessed in the same way.

HEART RATE MEASUREMENT

Heart rate is continuously monitored by an electrocardiogram (ECG) through 6 spot electrodes on the skin surface. The electrodes are placed on the chest and they can detect the electrical activity that makes your heart beat.

Potential Risks: In a small group of participants, a slight rash might develop as a reaction to the adhesive or the gel on the electrodes. This is unlikely to cause any real discomfort

BLOOD PRESSURE MONITORING

A cuff that can be inflated with air is wrapped around your upper arm, just as would occur if you had your blood pressure measured at the doctor's office. This cuff is inflated to a pressure higher than your systolic blood pressure (the pressure in your

blood vessels when the heart beats), and gradually deflated over a number of seconds to measure systolic blood pressure and diastolic (the pressure in your blood vessels when the heart is relaxed) blood pressure. Meanwhile, your wrist is secured in a wrist brace and a small pressure sensor is placed over your radial artery at the wrist. This pressure sensor is able to detect the increases and decreases in size of your radial artery that occur with each heart beat, and what the pressure sensor measures is compared to the pressure that the upper arm cuff measures (this calibrates the sensor). From then on, the pressure sensor at the wrist measures blood pressure continuously, while the upper arm cuff may be inflated intermittently.

In order to measure arterial stiffness blood pressure will also be monitored in your carotid artery while it is imaged with ultrasound and in your femoral artery following carotid imaging. Pressure measurements in the carotid and femoral arteries will be obtained using the pencil-like probe over your pulse. Pressure measurements from your foot will be obtained continuously from an infrared probe secured over your dorsis pedis artery (top of foot) OR from a piezo-electric probe secured to your big toe. You should feel no discomfort at any time during these procedures.

CATHETERIZATION AND VENOUS/ARTERIALIZED BLOOD SAMPLING

At the onset of each trial, a small catheter (flexible plastic tube) will be inserted into a forearm vein by a physician or a trained and qualified member of the laboratory group. The catheter will be introduced with the assistance of a small needle, which is subsequently removed. The discomfort of this procedure is transient and is very similar to having a needle injection or donating blood. Once the needle is removed you should feel no sensation from the catheter.

During the trial sessions, blood will be drawn before exercise, immediately after exercise and at 15, 30, 45, 60, 90, 120, and 180 minutes thereafter from the catheter. Before drawing some of the samples your hand will be wrapped in a heating blanket to warm the hand and arm. The purpose of this procedure is to "arterialize" the venous blood samples so that they are more representative of arterial blood. A total of ~54 ml of blood will be taken during each trial. This is approximately $1/10^{th}$ of the volume removed during a blood donation and should not affect your physical performance in any way.

After each sample is taken, the catheter is "flushed" with a sterile saline solution in order to prevent blood from clotting in the catheter. This is a salt solution similar in composition to your own blood and it will not affect you. Following removal of the catheter, pressure will be placed on the site in order to minimize bleeding and facilitate healing.

Potential Risks. The insertion of catheters for blood sampling is a common medical practice and involves few risks if proper precautions are taken. The catheters are inserted under completely sterile conditions; however, there is a theoretical risk of

infection. There is also a chance of internal bleeding if adequate pressure is not maintained upon removal of the catheter. This may cause some minor discomfort and could result in bruising/skin discoloration, which could last for up to a few weeks. In very rare occasions, trauma to the vessel wall could result in the formation of a small blood clot, which could travel through the bloodstream and become lodged in a smaller vessel. However, we have never experienced such a complication after several thousand catheter placements.

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EXERCISE METABOLISM RESEARCH GROUP
DEPARTMENT OF KINESIOLOGY, MCMASTER UNIVERSITY

SUBJECT SCREENING QUESTIONNAIRE

Your responses to this questionnaire are confidential and you are asked to complete it for your own health and safety. If you answer "YES" to any of the following questions, please give additional details in the space provided and discuss the matter with one of the investigators. You may refuse to answer any of the following questions.

Name:_____ Date: _____

1. Have you ever been told that you have a 'heart problem' (including a cardiac shunt) or experienced chest pain when exercising?

   YES NO

2. Have you ever been told that you have a breathing problem, such as asthma?

   YES NO

3. Have you ever been told that you sometimes experience seizures?

   YES NO

4. Have you ever had any major joint instability or ongoing chronic pain such as in the knee or back?

   YES NO

5. Have you ever been told that you have 'kidney problems'?

   YES NO

6. Have you had any allergies to any medications (including 'over-the-counter' medicines such as aspirin or Tylenol™)?

   YES NO

7. Have you had any allergic reactions to food (i.e., fish, nuts, wheat gluten) or environmental factors (dust, pollen, or mould)?

YES NO

If YES, please describe the degree of the allergic reaction: mild, moderate, or severe

8. Have you had any stomach problems, such as ulcers?

YES NO

9. When you experience a cut do you take a long time to stop bleeding?

YES NO

10. When you receive a blow to your muscle, do you develop bruises easily?

YES NO

11. Are you currently taking any medication (including aspirin) or have you taken any medication in the last two days?

YES NO

12. Is there any medical condition with which you have been diagnosed and are under the care of a physician (e.g. diabetes, high blood pressure)?

YES NO

13. Have you previously participated in a study under the supervision of Dr.'s Stuart Phillips, Mark Tarnopolsky, or Martin Gibala that involved having muscle biopsies taken?

YES NO

14. Are you aware, through your own experience or a doctor's advice, of any other reason why you should not take part in this study, without medical approval?

YES NO

Appendix C: Methods – Mixing Isotope for Infusion

C.1 Mixing Isotope for Infusion

- Note: at time of writing, standard protocol for isotope solution preparation was to add 5 g of isotope to 500 ml of distilled water (dH2O) to make a 10 mg/ml isotope solution. This stock isotope solution was infused at a rate that was calculated for each participant based upon the participant's mass.
- Turn on the air and light of the clean cabinet at least 10 min in advance of working.
- Sterilize the entire clean cabinet surface with Virox wipes. Permit to dry.
- Mark a 500 ml volume line on the side of a culture flask, using a permanent marker and the pre-measured culture flask template as a guide.
    - *Note: use the blue lid culture flasks, because they seal tighter than the other lids.*
- Hang 2 bags of 0.9% NaCl solution (saline) from the rings in the clean cabinet, and puncture one of the bags with an intravenous (IV) drip line.
- Empty one bag of saline into the culture flask.
    - *Note: can increase the flow rate of the saline by holding the end of the IV line into the culture flask and concurrently squeezing the saline bag.*
- Peel the silicone tape off of the bottle of isotope. Remove the lid from the isotope bottle, and carefully pour the isotope into the culture flask. Bottle should insert snugly into the opening of the culture flask, permitting tapping of the end of the isotope bottle to remove the isotope.
    - *Note: the powdered isotope is massed by the manufacturer to 1, 5, 10, or 20 g vials.*
- Add saline to the isotope bottle to dissolve any of the residual isotope in the bottle. Screw the lid back on the isotope bottle, shake vigorously, unscrew the lid, and pour the saline solution into the culture flask. Repeat as necessary to transfer all isotope, from as many isotope bottles as needed.
- Fill the culture flask with saline to the 500 ml mark. Place the lid on the culture flask, and agitate vigorously.
    - *Note: must dissolve all the flakes of isotope in the saline.*
    - *Note: ensure a tight seal between the lid and the flask to prevent spilling of isotope solution.*
    - *Note: if lid seal is not tight, place fabric tape around the lip underneath the lid.*
    - *Note: pre-warm the saline bags to promote dissolution of the isotope.*
    - *Note: dissolution may take 5-30 min.*
- Peel the aluminum seal off of an evacuated container, and hang the evacuated container from the rings in the clean cabinet.
- Attach a 16G or 18G needle to a stopcock, and attach a 10 ml syringe to the stopcock. Uncap the needle, insert the needle into the rubber seal of the evacuated container, and withdraw the residual saline in the evacuated container. Close the stopcock off to the evacuated container to prevent the loss of the vacuum. Remove the syringe from the stopcock, leaving the needle in the rubber seal of the evacuated container, and the stopcock attached to the needle. Discard the syringe and saline.
    - *Note: important to produce as few punctures of the evacuated container rubber seal as possible, to prevent loss of the vacuum. The vacuum prevents the growth of aerobic organisms (bacteria).*
- Attach a 0.22 μm Millipore filter to the stopcock attached to the needle, and attach a second stopcock on the other end of the filter. Remove the plunger from a 60 ml syringe, and attach the 60 ml syringe to the other end of the second stopcock (see diagram below).
    - *Note: the second stopcock permits stopping during filling of the evacuated container to change a filter, if a filter fails and prevents filling, but there remains isotope solution in the syringe.*

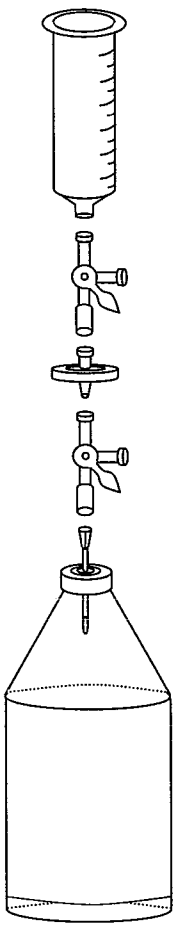
- Reinforce the needle and stopcock by adhering Tegaderms and fabric tape around the junction between the evacuated container, needle, and first stopcock.
    - *Note: this will help to prevent pulling the needle from the evacuated container, and losing the vacuum, when withdrawing isotope to use in a trial.*

- Fill the 60 ml syringe with the isotope solution, and close the first stop cock off to the environment. The vacuum will draw the isotope solution through the syringe, through the filter, and into the evacuated container. Pour isotope solution into the syringe for continuous filling.
    - *Note: if the filter is exposed to air, the filter will fail and prevent flow. Therefore, must change the filter if the isotope solution runs through the syringe before the syringe is filled again.*
    - *Note: change the filter every 100-130 ml, to ensure the filter does not become faulty during use.*

- To change the filter, permit the isotope remaining in the syringe to flow through the filter, and then turn the first stopcock off to the evacuated container. Detach the system at the junction between the first stopcock and the filter, replace the filter, and re-attach the new filter to the first stopcock.

- When all the isotope solution has been filtered into the evacuated container, remove the filter from the first stopcock, and place the end caps on the first stopcock.

- Fill out and affix a WHIMIS handling label, and keep in the food fridge @ 4°C until required.



60 ml Syringe

Stopcock (safeguard vs. filter failure)

0.22 µm Filter

Stopcock (regulate vacuum)

16G or 18G Needle

Evacuated Container

Appendix D: Methods – Preparation before Infusion Trial Day

D.1 Preparing the Infusion Pump and the Isotope Prime Syringe

- Place an absorbent pad on top of an infusion cart, and place an infusion pump on top of the pad.
- Remove the bottle of isotope from the food fridge @ 4°C, and hang on an intravenous (IV) administration pole.
- Draw the volume of isotope required for the trial into a 60 ml syringe.
    - *CAUTION: remove all air bubbles from the isotope in the syringe.*
- Load the 60 ml syringe into the appropriate slot of the infusion pump. Lock the syringe in place.
- Attach a 0.22 μm Millipore filter to the 60 ml syringe.
- Attach a straight extension infusion line to the filter, but keep the other end of the line in sterile packaging.
    - *Note: use a Y-extension infusion line when 2 syringes (each with a filter) are required for infusion.*
- Prime the filter and the infusion line by manually depressing the plunger on the syringe. Stop when the infusate reaches the end of the infusion line.
    - *CAUTION: ensure that there is not an air bubble in the infusion line.*
- Set the pump to the required specifications: set the rate of infusion and the diameter of the syringe.
- Draw the volume of isotope required to prime the amino acid pool into a 5 or 10 ml syringe. Place the prime syringe back into its sterile packaging, seal the package, and place on the infusion cart.
- Place the bottle of isotope back into the food fridge @ 4°C.

D.2 Preparing the Saline Intravenous Drip

- Open a bag of 0.9% NaCl solution (saline), and hang on an intravenous (IV) administration pole.
- Open an IV drip line, and turn the flow-regulating valve to the "off" position.
- Remove the plastic sleeve from the IV drip line needle.
- Remove the rubber cap from the needle insertion sleeve on the bag of saline.
- Push the IV drip line needle into the needle insertion sleeve on the bag of saline, until the needle punctures the seal.
- Turn the flow valve to the "on" position, and permit saline to flow to the end of the IV drip line.
    - *CAUTION: remove all air bubbles from the IV drip line.*
    - *Note: can be easier to remove bubbles if a stopcock is placed on the end of the IV drip line, and saline is run through the line and through the stopcock.*
- Keep the end of the IV drip line in sterile packaging, and seal the packaging.

D.3 Preparing Eppendorfs with PCA

- Label all eppendorfs for blood and plasma collection, and place eppendorfs in a Styrofoam eppendorf tray.
    - *Note: standard procedure: 2 eppendorfs for plasma extract, 2 eppendorfs for PCA-extracation procedure, and 2 eppendorfs for the PCA-extract*
- Uncap the labelled PCA-extraction eppendorfs.
- Add 500 µl of perchloric acid (PCA) to each labelled eppendorf using the repeater pipette and the PCA tip.
- Add 15.625 µl of norleucine standard to each labelled eppendorf. Vortex all eppendorfs.
- Cap eppendorfs, and keep the tray of eppendorfs in the blood fridge @ 4°C before and during the trial.

Appendix E: Methods – Preparation on Infusion Trial Day

E.1 Preparing a Venous Catheter Insertion Tray

- Disinfect the working surface and a blue cafeteria tray using a Virox wipe. Permit to dry.
- Place an absorbent pad on top of the tray.
- Place the following on top of the absorbent pad:
  - tourniquet
  - alcohol prep pads (70% isopropyl)
  - 2 venous catheters
  - 2 stopcocks
  - non-sterile 2x2 gauze
  - 2 tegaderms
  - fabric tape
- Fold the edges of the absorbent pad to cover the contents of the tray until catheter insertion.

E.2 Preparing a Muscle Biopsy Tray

- Disinfect the working surface and a blue cafeteria tray using a Virox wipe. Permit to dry.
- Carefully open up a pack of operating room (OR) towels, keeping track of which of the packaging flaps were inside the bundle, and therefore sterile.
- Grab the visible corner of the folded OR towel on top of pile of towels, and lift clear of any obstructions. Shake the folded OR towel loose, being certain to avoid contaminating the towel. Grab another free corner of the unfolded OR towel, and cover the tray with the towel.
  - *CAUTION: do not contaminate.*
  - *CAUTION: be certain not to touch the surface of the OR towel.*
  - *CAUTION: be certain not to lean into the towel when placing the towel or other items on the tray.*
- Remove the packaging from and place the following on top of the OR towel:
  - 22G needle
  - 26G needle
  - 10 ml syringe
  - 2 sterile 4x4 gauze pads/incision site
  - 1 biopsy blade/incision site
  - 1 suture kit/2 incision sites
  - *Note: peel the packaging from the corner and let item fall onto the tray to prevent contamination.*
- Cover the items on the tray with another OR towel/incision site, moving the towel as per above.
- Repackage the pack of unused OR towels, placing the sterile packaging flaps on the inside of the bundle. Essentially fold the packaging flaps in the reverse order of which they were unfolded.

E.3 Preparing the Muscle Biopsy Cart

- Disinfect the surface of the biopsy cart using a Virox wipe. Permit to dry.
- Carefully place the prepared biopsy tray on top of the biopsy cart.
- Fill 2 aluminum dishes with chlorhexidine gluconate antiseptic solution: Baxedin® or Vap®.

- Place several O-rings in one of the aluminum dishes containing antiseptic.
- Place several non-sterile 4x4 gauze pads in the other aluminum dish containing antiseptic.
- Place the following on top of the cart:
    - bottle of sodium bicarbonate solution
    - bottle of xylocaine anaesthetic
    - bottle of xylocaine anaesthetic with epinephrine
    - package of vasoline
    - 60 ml syringe
    - labelled cryotubes for the muscle biopsy samples
- Place a complete muscle biopsy needle kit on top of the biopsy tray.
    - *Note: ensure that all pieces of the needle are inside the packaging.*
- Fill a working dewer with liquid nitrogen, and keep in a safe place in the procedures room.
    - *CAUTION: keep dewer away from foot traffic.*

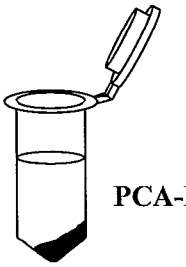
Appendix F: Methods – Analysis of Blood Samples

F.1 Processing Blood – During Trial

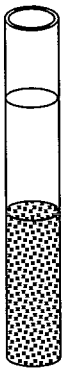
- Gently invert the vacutainer 5 times after taking a blood sample to ensure thorough mixing of the whole blood and the anticoagulant.
- Place the vacutainer in a Styrofoam container filled with ice.
- Within 5 minutes of taking the blood sample, place a OneTouch glucose strip into the OneTouch glucose meter. When prompted by the display, remove the cap off the vacutainer, withdraw 15 µl of whole blood, and dispense the blood into the loading window of the glucose strip. Record the blood glucose reading.
- Remove the PCA-containing eppendorfs (D.3) corresponding to the time point of the blood sample from the Styrofoam eppendorf tray in the blood fridge. Uncap the eppendorfs and place in the ice of the Styrofoam container. Withdraw 100 µl of whole blood, clean the residual blood off the outside of the pipette tip with a Kim wipe, and dispense into the PCA of one of the eppendorfs. Draw some of the PCA back into the pipette tip, dispense back into the eppendorf, repeat, and recap the eppendorf (wash of the pipette tip). Repeat for each eppendorf.
  - *Note: try to keep the whole blood from sticking to the sides of the eppendorf (prevents exposure to PCA) or creating large clumps of blood (center not exposed to PCA), by injecting the blood as thin streams into the PCA. Want to promote the PCA action on the blood.*
- Vortex all eppendorfs. Return eppendorfs to the blood fridge @ 4°C until the end of the trial. Recap the vacutainer, and leave on ice until the end of the trial.
  - *Note: ensure entire solution is homogenous, fine-grained, brown fluid.*
  - *Note: avoid vortexing too vigorously to prevent splashing the lid of the eppendorf.*

F.2 Processing Blood – After Trial

- Remove the PCA-containing eppendorfs from the blood fridge.
- Centrifuge all PCA-containing eppendorfs using the 8848 rotor: 15000 rpm, 3 min, @ 4°C.
- Remove the transparent supernatant (PCA-extract) from an eppendorf using a plastic transfer pipette, and dispense into the correspondingly labelled PCA-extract eppendorf (see diagram below). Repeat for all samples.
  - *Note: do not draw up any of the dark, solid precipitate. Re-spin if necessary.*
- Place all of the PCA-extract eppendorfs back into the blood fridge @ 4°C, and discard the PCA-extraction eppendorfs in the biohazardous waste.
- Remove the vacutainers from the ice container.
- Centrifuge all vacutainers using the 8947 rotor: 4500 rpm, 10 min, @ 4°C.
  - *Note: split runs in two if there are too many or there is too much weight for the rotor.*
- Remove the transparent supernatant (plasma) from a vacutainer using a plastic transfer pipette, and split into the correspondingly labelled plasma extract eppendorfs (see diagram below). Repeat for all samples.
  - *Note: do not draw up any of the red, whole blood cells. Re-spin if necessary.*



PCA-Extract



Plasma

Blood Cells

- Place all of the plasma extract eppendorfs back into the blood fridge @ 4°C, and discard the vacutainers in the biohazardous sharps waste.
- Store all plasma and PCA-extract eppendorfs in a freezer at -20°C or -80°C.

F.3 Insulin ELISA

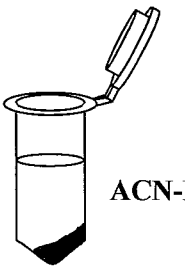
- NOTE: for best results, run within 1-2 months after collection of blood samples.
- NOTE: for best results, run on samples that have not previously been thawed.
- Remove the plasma eppendorfs from the -20°C or -80°C freezer, and place in a Styrofoam eppendorf tray (F.2).
- Permit plasma samples to thaw. Vortex all samples. Centrifuge all samples using the 8848 rotor: 15000 rpm, 3 min, @ 4°C. Place in a Styrofoam container filled with ice.
- Open the insulin ELISA kit packaging.
- Dilute 1 part enzyme conjugate concentrate (11x) with 10 parts enzyme conjugate buffer in a Falcon tube. Need 100 µl of enzyme conjugate/well.
- Reconstitute the diabetes antigen controls, by adding 0.6 ml of dH_2O to each of the control vials. Gently swirl the vials, and let sit for 30 min prior to use.
- Dilute 1 part wash buffer concentrate (21x) with 20 parts dH_2O in a glass or plastic stock bottle. Fill a wash bottle with the stock wash buffer solution. Need enough wash buffer to rinse the microplate 5-6 times during the wash stage (~400 ml).
- Open the insulin ELISA microplate.
  - *Note: let the microplate warm to room temperature before plating.*
  - *Note: the plates are coated with the insulin antibody during the manufacturing process.*
- Pipette 25 µl of each standard/control/plasma sample into their corresponding wells. Repeat in duplicate or triplicate if desired. Be certain to leave 1-3 wells empty to serve as a blank.
  - *Note: always prime each new tip – the reader is very sensitive. Draw once from sample, expel back into sample. Draw again from sample, expel into well. Draw again from sample, expel into well. Remove tip for next sample. Repeat for all samples.*
- Fill a multi-channel reservoir with the enzyme conjugate.
- Pipette 100 µl of enzyme conjugate into each of the wells using the multi-channel pipette.
  - *Note: ensure that all multi-channel tips are finger-tight on the pipette before using – never assume they are tight enough – a tip falling off mid-addition can ruin several wells.*

- Incubate the microplate for 60 min on an orbital microplate shaker: 800 rpm @ room temperature.
- Wash the microplate with the wash buffer, by filling each of the wells with wash buffer, and inverting the microplate over the sink to dump out the wash buffer. Place the inverted microplate on a paper towel, and tap on the microplate to shake the wash buffer droplets off the microplate. Repeat for a total of 5-6 washes of the microplate.
  - *Note: be certain to lock the microplate before inverting, to prevent accidental removal of individual lanes during inversion.*
  - *Note: try to limit the number of bubbles introduced to wells of the microplate – keep wash buffer bottle full and upright so that foam in the wash bottle is not squirted onto the microplate.*
- Fill a multi-channel reservoir with enzyme substrate.
- Pipette 100 µl of enzyme substrate into each of the wells using the multi-channel pipette.
- Incubate the microplate for 15 min on an orbital microplate shaker: 800 rpm @ room temperature.
- Fill a multi-channel reservoir with stop solution.
- Pipette 100 µl of stop solution into each of the wells using the multi-channel pipette.
- Gently mix the wells for 15 s on an orbital microplate shaker: 350 rpm @ room temperature.
  - *Note: remove any large bubbles in the wells by poking with a clean pipette or needle tip.*
- Read the microplate within 30 min of addition of the stop solution on a spectrophotometer. Spectrophotometer settings for the insulin ELISA are:
  - Absorbance at: 450 nm
  - Reference Wavelength: 620-650 nm
  - Preferred Calibration Curve Calculation Method: Linear
  - Plot Standard Curve on: Log-Log Scale
  - Special Instructions: Subtract the 0 standard/blank from each well
  - *Note: for best results, read @ 10-15 min after addition of stop solution.*
- Return the plasma eppendorfs to the -20°C or -80°C freezer.

F.4 Plasma Amino Acid Extraction

- Note: may be run on plasma samples that have previously been thawed.
- Remove the plasma eppendorfs from the -20°C or -80°C freezer, and place in a Styrofoam eppendorf tray.
- Permit plasma samples to thaw. Vortex all samples. Centrifuge all samples using the 8848 rotor: 15000 rpm, 3 min, @ 4°C. Place in a Styrofoam container filled with ice.
- In the fumehood, pull acetonitrile (ACN) from the stock bottle, and dispense into a Falcon tube with a flip cap. Will require 500 µL of ACN/sample.
  - *Note: 50:500 ratio of plasma:ACN yielded the same enrichments as other ratios, with a suitable abundance of amino acids, and this ratio used the least consumables.*
- Place the Falcon tube containing ACN into a Styrofoam container filled with ice.
  - *CAUTION: do not inhale ACN vapours if working outside fumehood.*
- Pull an eppendorf out of the ice, uncap, and add 500 µl of ACN. Recap and place on ice.
  - *Note: blood will instantly form a dark, non-sticky, solid mass.*
- Repeat for all samples.

- Vortex all samples.
  - *Note: try to suspend the solid muscle mass to thoroughly mix solvent*
- Centrifuge all samples using the 8848 rotor: 15000 rpm, 3 min, @ 4°C
- Place eppendorfs back on ice.
- Uncap an eppendorf, remove the ACN extract, and dispense the ACN extract into a labelled 2 ml crimp top vial. Repeat for all samples.



ACN-Extract (Plasma AA)

- Dry down the 2 ml crimp top vials containing the 500 µl of ACN extract, by heating on a block at 70°C under N_2 *(g)* blow in the fumehood.
- Remove vials from heating block when all ACN solution has evaporated. Permit vials to cool, firmly seal crimp top vials with Parafilm, and store at room temperature. Derivatize and run on the GC-MS as per the directions for the intracellular amino acids (G.7).
- Return the plasma eppendorfs to the -20°C or -80°C freezer.

Appendix G: Methods – Analysis of Muscle Tissue – Part 1

G.1 Storing Muscle Biopsy Samples

- Place excised muscle biopsy sample into a labelled cryovial, cap the cryovial, and place immediately into liquid nitrogen [N_2 *(l)*].
- Store the cryovials containing the muscle biopsy samples in a freezer at -80°C.

G.2 Preparing Muscle Biopsy Samples for Processing

- Pour N_2 *(l)* from the storage dewer into 2 working dewers.
- Remove the cryovials containing muscle biopsy samples from the freezer at -80°C, and place in one of the dewers containing N_2 *(l)*. The muscle samples must remain chilled throughout the procedure.

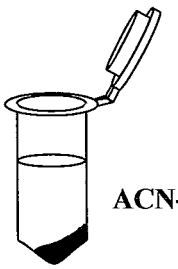
G.3 Chipping Muscle Biopsy Samples

- Chill an aluminum dish by pouring N_2 *(l)* from the dewer into an aluminum dish.
- Uncap a cryovial, and transfer the muscle biopsy sample from the cryovial into the chilled aluminum dish.
- Chip a small piece of muscle off of the muscle biopsy sample using a clean razor blade.
  - *Note: cover dish with opposite hand when chipping to prevent muscle sample from exiting the dish.*
  - *CAUTION: to prevent frost bite, do not keep fingers in dish too long and try to warm fingers between samples.*
- Tare the electronic balance with a labelled eppendorf on the balance.
- Chill the labelled eppendorf by placing the end in the aluminum dish containing N_2 *(l)*.
- Use tweezers to remove the chipped piece of muscle from the dish, and place into the chilled eppendorf.
- Mass the chipped piece of muscle using the electronic balance, and then cap the eppendorf and place the eppendorf into the dewer containing N_2 *(l)*. Record the mass of the chipped muscle sample.
- Use the tweezers to return the remaining muscle biopsy sample to the labelled cryovial, cap the cryovial, and return the cryovial to the dewer containing N_2 *(l)*.
- Pour the residual N_2 *(l)* from the aluminum dish into a waste container, and use a Kim wipe to remove any muscle fragments from the side of the dish.
- Repeat procedure for all muscle biopsy samples.
- Return the cryovials containing the muscle biopsy samples to the freezer, and pour the residual N_2 *(l)* from the working dewers back into the storage dewer.

G.4 Extraction of the Intracellular (IC) Fraction

- Continued from G.3.
- Mass of muscle sample required: 15-25 mg

- In the fumehood, pull acetonitrile (ACN) from the stock bottle, and dispense into a Falcon tube with a flip cap. Will require 1 mL of ACN/sample.
- Place the Falcon tube containing ACN into a Styrofoam container filled with ice.
  - *CAUTION: do not inhale ACN vapours if working outside fumehood.*
- Pull an eppendorf out of the dewer containing N_2 *(l)*, and immediately place on ice.
- Uncap the eppendorf, add 500 µl of ACN, and homogenize muscle with the Teflon pestle.
  - *Note: continue homogenizing until muscle is a matte, non-sticky, solid mass.*
- Cap the eppendorf, and place on ice.
- Repeat for all samples.
- Vortex all samples.
  - *Note: try to suspend the solid muscle mass to thoroughly mix solvent.*
- Centrifuge all samples using the 8848 rotor: 10000 rpm, 5 min, @ 4°C.

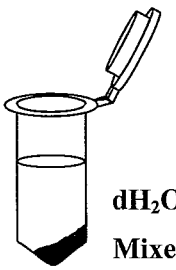


ACN-Extract (IC)

- Place eppendorfs back on ice.
- Uncap an eppendorf, remove the ACN-extract, and dispense the ACN-extract into a labelled 2 ml crimp top vial.
- Add 500 µl of ACN to the eppendorf and recap the eppendorf.
- Repeat for all samples.
- Vortex all samples.
  - *Note: try to suspend the solid muscle mass to thoroughly mix solvent.*
- Centrifuge all samples using the 8848 rotor: 10000 rpm, 5 min, @ 4°C.
- Place the eppendorfs back on ice.
- Uncap an eppendorf, remove the ACN-extract, and dispense the ACN-extract into the labelled 2 ml crimp top vial that contains the ACN-extract from the previous steps. Recap the eppendorf, and keep on ice for the wash steps (G.5).
- Dry down the 2 ml crimp top vials containing the 1 ml of ACN-extract, by heating on a block at 70°C under N_2 *(g)* blow in the fumehood.
- Remove vials from heating block when all ACN solution has evaporated. Permit vials to cool, firmly seal crimp top vials with Parafilm, and store at room temperature until ready to derivatize (G.7).

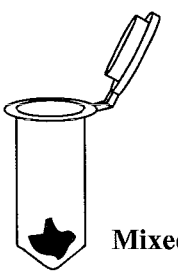
G.5 Washing the Mixed Muscle Pellet

- Continued from G.4.
- Uncap an eppendorf, add 500 µl of distilled water (dH_2O), and recap eppendorf.
- Repeat for all samples.
- Vortex all samples.
  - *Note: try to suspend the solid muscle mass to thoroughly mix solvent.*



dH_2O/etOH Wash
Mixed Muscle Proteins

- Centrifuge all samples using the 8848 rotor: 10000 rpm, 5 min, @ 4°C.
- Place eppendorfs back on ice.
- Uncap an eppendorf, remove the dH_2O solution, and dispense the dH_2O into a labelled waste beaker.
- Add 500 µl of 70% ethanol (etOH) and recap eppendorf.
- Repeat for all samples.
- Vortex all samples.
  - *Note: try to pull suspend the solid muscle mass to thoroughly mix solvent.*
- Centrifuge all samples using the 8848 rotor: 10000 rpm, 5 min, @ 4°C.
- Place eppendorfs back on ice.
- Uncap an eppendorf, remove the etOH solution, and dispense the etOH into a labelled waste beaker.
- Recap the eppendorf, and poke a hole in the cap using a 16G or 18G needle.
- Repeat for all samples.
- Lyophilize samples on the vacuum-suctioned, freeze drier.
- When muscle pellets are dry (~1 h), remove eppendorfs, and proceed to hydrolysis (G.6).



Mixed Muscle Protein Pellet

G.6 Hydrolysis of the Mixed Muscle Pellet

- Continued from G.5.
- In the fumehood, pull 6M hydrochloric acid (HCl) from the stock bottle, and dispense into a labelled beaker. Will require 1.5 mL of HCl/sample.
- Uncap eppendorf, and transfer the dried muscle pellet from the eppendorf into a 4 ml screw cap vial.
  - *Note: can mass the dried pellet during this step if required.*
  - *Note: ~18% of wet muscle mass is retained in the dry muscle mass*
    - *wet-dry mass correlation: $r^2 = 0.72$*
- Repeat for all samples.
- Using the repeater pipette with the HCl tip, add 1.5 ml of HCl to each of the 4 ml screw cap vials.
- Screw caps onto the 4 ml screw cap vials.
  - *CAUTION: ensure an air tight seal to prevent drying of the sample, but do not over-tighten, because the pressure required to remove an over-tightened cap will break the vial.*
  - *CAUTION: if a cap is too tight to remove easily with the fingers, place vial in the fumehood, wedge the tips of a pair of tweezers between the edge of the cap and the lip of the vial, and pry cap away from vial. Gradually fracture the cap away from the vial until the cap is loose enough to remove.*
- Place 4 ml screw cap vials in a rack with the middle shelf lined with aluminum foil to create a platform for the vials. Place the rack in the oven heated to 100°C.
- Boil the 4 ml screw caps @ 100°C for 24 hr. Ensure that the pellet is completely hydrolyzed before removing the vials from the oven.
- Store 4 ml screw cap vials at room temperature until prepared to column (G.9).

G.7 Derivatization of the Intracellular Fraction

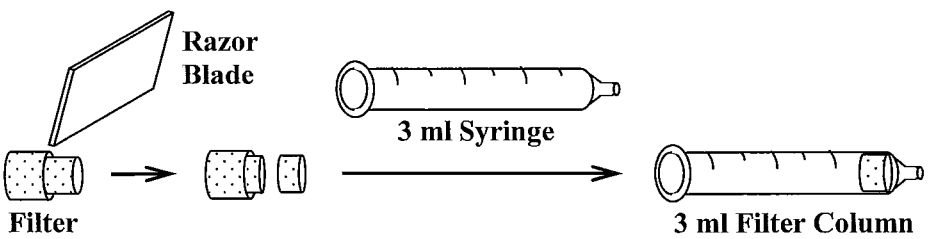
- Continued from G.4.
- CAUTION: All steps to be completed in the fumehood.
- Remove the Parafilm from the 2 ml crimp top vials containing the IC fraction.
- Pull a standard from the freezer, and add an appropriate volume of standard to a labelled 2 ml crimp top. Dry down the 2 ml crimp top vial containing the standard, by heating on a block at 70°C under N_2 *(g)* blow in the fumehood. Derivatize the standard with the samples.
- Add 200 µl of dichloromethane to each of the 2 ml crimp top vials.
  - *Note: pull directly from the stock bottle, but ensure pipette tip remains uncontaminated during procedure.*
- Repeat for all samples.
- Vortex all samples.
- Dry down the 2 ml crimp top vials containing the 200 µl of dichloromethane solution, by heating on a block at 70°C under N_2 *(g)* blow in the fumehood.
- In a suitable vesicle, prepare a (5:1) 2-methyl-1-propanol:acetyl chloride solution – will need 200 µl of 5:1 solution/sample. Place the vesicle in a beaker containing ice, add 5 parts 2-methyl-1-propanol to the vesicle and then cap the vesicle. Uncap, add 1 part acetyl chloride, drop-by-drop, to the chilled solution in the vesicle, and then cap the vesicle.

- *Note: an expired 10 ml sterile vacutainer (red cap) is the perfect size and has own rubber stopper.*
- *Note: pull directly from the stock bottle, but ensure pipette tip remains uncontaminated during procedure.*
- *CAUTION: highly exothermic reaction – do not hold solution vesicle when mixing.*
- *CAUTION: use a vesicle that is large enough to permit the bubbling of the exothermic reaction.*

- When dry, add 200 µl of (5:1) 2-methyl-1-propanol:acetyl chloride solution to a 2 ml crimp top, and place a cap over the 2 ml crimp top. Repeat for a total of 4-8 samples, cap the solution vesicle, and then crimp the tops onto the 4-8 crimp top vials.
  - *Note: lightly twist caps on sealed crimp top vials, to ensure cap is snug. Recap if necessary.*
- Uncap the storage vesicle, and repeat until all samples are treated with the (5:1) 2-methyl-1-propanol:acetyl chloride solution.
- Vortex all samples.
- Place the 2 ml crimp tops in a heating block in the fumehood – boil @ 110°C for 20 min.
- Permit the 2 ml crimp tops to cool, uncrimp caps from the vials, and dry down the 2 ml crimp top vials containing the 200 µl of 5:1 solution, by heating on a block at 70°C under N_2 *(g)* blow in the fumehood.
- Break the glass cap off a bottle of heptafluorobutyric acid anhydride (HFB), and use a glass transfer pipette to transfer the HFB from the stock bottle to a 2 ml crimp top vial. Crimp top on the vial until ready to use the HFB.
- When dry, uncrimp the cap from the HFB vial, and add 100 µl of HFB to a 2 ml crimp top vial, and place a cap on the 2 ml crimp top. Repeat for a total of 4-8 samples, place a cap over the HFB vial, and then crimp the tops onto the 4-8 crimp top vials.
  - *Note: lightly twist caps on sealed crimp top vials, to ensure cap is snug. Recap if necessary.*
- Uncap the HFB vial, and repeat until all samples are treated with HFB.
  - *Note: after all samples have been treated with HFB, the residual HFB can be stored by crimping a top onto the HFB vial, wrapping the vial in aluminum foil, labelling the foil vial with appropriate info, and pacing in the appropriate cabinet.*
- Vortex all samples.
- Place the 2 ml crimp tops in a heating block in the fumehood – boil @ 110°C for 10 min.
- Permit the 2 ml crimp tops to cool, uncrimp caps from the vials, and dry down the 2 ml crimp top vials containing the 100 µl of HFB, by heating on a block at 70°C under N_2 *(g)* blow in the fumehood.
  - *CAUTION: check samples every minute, to prevent the loss of the volatile amino acids by over-drying of the sample.*
- When dry, add 75-100 µl of ethyl acetate to a 2 ml crimp top vial, briefly vortex, draw the ethyl acetate solution into a pipette tip, place a 200 µl insert into the 2 ml crimp top vial, and dispense the ethyl acetate solution into the insert. Crimp cap onto the crimp top vial.
  - *Note: perform these steps in succession for one sample, before beginning the next sample.*
  - *Note: ensure that the insert is perfectly vertical in the vial after crimping.*
- Repeat for all samples.
- Run samples on the gas chromatogram-mass spectrometer (GC-MS) within 1 week.
  - *Note: for phenylalanine tracers, ions are: m+0 = 316, m+1 = 317, m+6 = 322.*
  - *Note: interested in the ratio between the enriched ion and the non-enriched ion.*

G.8 Constructing Filter Columns

- Wipe the work surface clean to ensure it is free of amino acids and/or isotope.
- Pull the plunger out of a 3 ml syringe.
- Cut the end off of a 1 ml pipette filter using a clean razor blade.
  - *Note: the small end of the filter is the end that fits inside the 3 ml syringe.*
  - *Note: cut the small end in half, or longer – if too small/light, it will not stay at bottom of syringe.*
  - *Note: cutting the small end longer will not decrease flow rate.*
- Place the manufacturing cut edge of the filter face down inside the syringe, and press the filter to the bottom of the syringe using the plunger.
  - *Note: fastest/most consistent flow rates are achieved by limiting the air space between the filter and the bore exiting the syringe.*
- Remove the plunger.
- Ensure that the filter remained at the bottom of the syringe.
  - *Note: best/most consistent flow rates are achieved by limiting the air space between the filter and the bore exiting the syringe.*
- Repeat to construct as many filter columns as required.



G.9 Purification and Isolation of the Mixed Muscle Amino Acids

- Continued from G.6.
- When cool or after storage, screw caps off the 4 ml screw cap vials containing the HCl solution.
- Add 2 ml of dH_2O to each of the 4 ml screw cap vials using a repeater pipette and the dH_2O tip.
  - *Note: brings acidity to level appropriate for loading onto column.*
- Place the 3 ml filter columns (G.8) in a rack that only has the top shelf, and place the rack of columns over a Styrofoam collection container.
- Add 3 ml of dH_2O to each of the columns to prime the filter.
  - *Note: may have to force water through the column with a plunger to permit flow*
  - *Note: may also press finger over the lip of the column, to generate air pressure to force water through the column.*
- Repeat as necessary to achieve continuous flow.
- Remove the Dowex Ion Exchange Resin from the fridge. Stir with a 5 ml pipette, draw 1 ml into the pipette tip, and dispense into a column. Permit the solution to run through the column. Repeat for all columns.

- *Note: ensure that the resin solution is adequately mixed prior to each addition to a column, because the resin settles rapidly – top up a column in which the resin is at a low level.*

- In fumehood, pour 2M ammonium hydroxide (NH_4OH) into a beaker.
  - *CAUTION: do not inhale the NH_4OH vapours.*

- Add 1.5 ml of NH_4OH to each of the columns using the repeater pipette and the NH_4OH tip. Permit NH_4OH to run through columns. Add another 1.5 ml of NH_4OH to each of the columns. Permit NH_4OH to run through columns.
  - *Note: pH test of last drop should be very basic (deep blue) – this is a column and resin wash, because the resin releases amino acids when basic.*

- Add 2.5 ml of ddH_2O to each of the columns using the repeater pipette and the dH_2O tip. Permit the ddH_2O to run through columns. Add another 2.5 ml of ddH_2O to each of the columns, and permit to ddH_2O to run through the columns. Repeat addition of ddH_2O to each of the columns until each column is neutral.
  - *Note: pH test of last drop should be neutral (yellow) – this neutralizes the exchange column resin prior to charging of the resin.*

- Add 1.5 ml of 1M HCl to each of the columns using the repeater pipette and the HCl tip. Permit HCl to run through columns. Add another 1.5 ml of HCl to each of the columns. Permit HCl to run through columns.
  - *Note: pH test of last drop should be very acidic (deep red) – this is a column and resin charge, because the resin binds amino acids when acidic.*

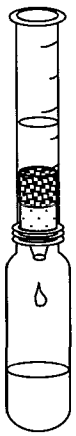
- Transfer each sample from the 4 ml screw cap vials to their respectively labelled columns using a glass transfer pipette. Ensure use of a new transfer pipette for every transfer.

- Add 2.5 ml of ddH_2O to each of the columns using the repeater pipette and the dH_2O tip. Permit the ddH_2O to run through columns. Add another 2.5 ml of ddH_2O to each of the columns, and permit to ddH_2O to run through the columns. Repeat addition of ddH_2O to each of the columns until each column is neutral.
  - *Note: pH test of last drop should be neutral (yellow) – this neutralizes the exchange column resin prior to washing of the resin.*

- Add 0.5 ml of NH_4OH to each of the columns using the repeater pipette and the NH_4OH tip. Permit NH_4OH to run through columns. Test with the pH paper. If not slightly basic (green), add another 0.5 ml of NH_4OH to each of the columns. Permit NH_4OH to run through columns. Continue addition of 0.5 ml of NH_4OH until slightly basic (green).
  - *Note: pH test of last drop should be basic (green) – this is a column and resin wash, because the resin retains the amino acids but releases other molecules until basic.*

- When all columns are beginning to turn basic, place glass culture tubes in a rack, and place the column rack over the culture tube rack. Align each column with its respectively labelled culture tube (see diagram below).

- Add 2.0 ml of NH_4OH to each of the columns using the repeater pipette and the NH_4OH tip. Permit NH_4OH to run through columns, collecting in the glass culture tubes. Add another 2.0 ml of NH_4OH to each of the columns, and permit NH_4OH to run through the columns.
  - *Note: pH test of last drop should be very basic (deep blue) – this is a column and resin wash, because the resin releases/elutes the amino acids when basic The purified and isolated amino acids are collected.*



3 ml Filter Column

NH_4OH

Ion Exchange Resin

Filter

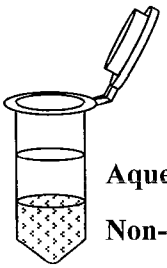
Eluted Amino Acids

- Dry down the NH_4OH eluent on the rotary evaporator on medium speed overnight (6-12 h)
- Store glass culture tubes at room temperature until prepared to derivatize the mixed muscle fraction (G.10), or, store at room temperature after reconstituting the amino acids by adding 500 µl of 0.1M HCl to each glass culture tube, vortexing each culture tube, and transferring the HCl-extract to a labelled eppendorf. The amino acids are stable for months/years at this stage.

G.10 Derivatization of the Mixed Muscle Amino Acids

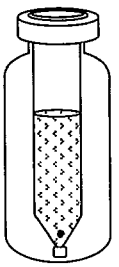
- Continued from G.9.
- CAUTION: All steps to be completed in the fumehood.
- Transfer 200-250 µl of the columned hydrolysate (HCl-extract) from the eppendorf to a labelled 2 ml crimp top vial.
- Pull a corresponding standard from the freezer, and add an appropriate volume of standard to a labelled 2 ml crimp top. Derivatize the standard with the samples.
- Dry down the 2 ml crimp top vials containing the sample hydrolysates and the standard, by heating on a block at 70°C under N_2 *(g)* blow in the fumehood.
- When dry, add 200 µl of propyl acetate to each of the 2 ml crimp top vials. Vortex all samples.
  - *Note: pull directly from the stock bottle, but ensure pipette tip remains uncontaminated during procedure.*
- Add 100 µl of boron trifluoride propanol (in excess propoanol) to each of the 2 ml crimp top vials. Crimp caps onto all 2 ml crimp top vials.
  - *Note: pull directly from the stock bottle, but ensure pipette tip remains uncontaminated during procedure.*
- Vortex all samples.
- Place the 2 ml crimp tops in a heating block in the fumehood – boil @ 110°C for 30 min.
- Permit the 2 ml crimp tops to cool, uncrimp caps from the vials, and dry down the 2 ml crimp top vials by heating on a block at 70°C under N_2 *(g)* blow in the fumehood.

- Add 100 µl of boron trifluoride propanol (in excess propoanol) to each of the 2 ml crimp top vials. Crimp caps onto all 2 ml crimp top vials.
  - *Note: pull directly from the stock bottle, but ensure pipette tip remains uncontaminated during procedure.*
- Vortex all samples.
- Dry down the 2 ml crimp top vials containing the boron trifluoride propanol solution, by heating on a block at 70°C under N_2 *(g)* blow in the fumehood.
  - *Note: it is crucial that all water is removed during this step – ensure vial is dry.*
- Add 50 µl of ACN (anhydrous) to each of the 2 ml crimp top vials. Vortex all samples.
  - *Note: pull directly from the stock bottle, but ensure pipette tip remains uncontaminated during procedure.*
- Add 25 µl of 1,4-dioxane (anhydrous) to each of the 2 ml crimp top vials. Vortex all samples.
  - *Note: pull directly from the stock bottle, but ensure pipette tip remains uncontaminated during procedure.*
- Add 37.5 µl of triethylamine to each of the 2 ml crimp top vials. Vortex all samples.
  - *Note: pull directly from the stock bottle, but ensure pipette tip remains uncontaminated during procedure.*
- Add 22.5 µl of acetic anhydride to a 2 ml crimp top vial, and place a cap on the 2 ml crimp top. Repeat for 4-8 samples, and then crimp the tops onto the 2 ml crimp top vials.
  - *Note: pull off a small amount from the stock bottle, and place in beaker for use.*
- Vortex all samples.
- Place the 2 ml crimp tops in a heating block in the fumehood – boil @ 55°C for 15 min.
- Permit the 2 ml crimp tops to cool.
- Transfer 50 µl of chloroform into labelled eppendorfs.
  - *Note: pull off a small amount from the stock bottle, and place in beaker for use.*
- Uncrimp the cap from a 2 ml crimp top vial, and transfer the cocktail solution from the 2 ml crimp top vial into the labelled eppendorf containing the chloroform. Draw some of the cocktail solution back up the pipette tip, and dispense to wash the pipette tip.
  - *Note: perform these steps in succession for one sample, before beginning the next sample.*
- Repeat for all samples.
- Place 200 µl inserts into the 2 ml crimp top vials. Transfer 1 grain of molecular sieve from the storage Falcon tube to each of the 200 µl inserts.
  - *Note: these are the same crimp top vials used throughout the derivatization process.*
- Add 75 µl of 0.001 $NaHCO_3$ to an eppendorf, cap the eppendorf, tap the eppendorf on the ledge. Repeat for the same sample. Pull off the non-aqueous phase from the eppendorf, and transfer to the insert in the crimp top (see diagram below). Crimp cap onto the 2 ml crimp top vial.
  - *Note: perform these steps in succession for one sample, before beginning the next sample.*
  - *Note: ensure that the insert is perfectly vertical in the vial after crimping*



Aqueous Phase

Non-Aqueous Phase



Non-Aqueous Phase

Grain of Molecular Sieve

- Repeat for all samples.
- Run samples on the gas chromatogram – combustion – isotope ratio mass spectrometer (GC-C-IRMS) within 1 week.
  - *Note: for L-[$^{13}C_6$]-phenylalanine tracer, interested in the ratio between ^{13}C and ^{12}C at the peak in the samples that corresponds to the retention time of the peak observed in the standard.*

Appendix H: Methods – Analysis of Muscle Tissue – Part 2

H.1 Mixing Stock Homogenization Buffer

- NOTE: make fresh stock every 30 days.
- Place a stir bar inside a glass beaker, and place the beaker on the stir platform.
- Add the following to the beaker:
  - 100 ml dH_2O
  - 0.788 g Tris-HCl (FW: 157.6, Concentration: 50 mM)
  - 0.0372 g EDTA (FW: 372.2, Concentration: 1 mM)
  - 0.0380 g EGTA (FW: 380.4, Concentration: 1 mM)
  - 0.1 ml Triton X-100 (Concentration: 0.1%)
- Place Parafilm over the top of the beaker. Stir solution until all solids are fully dissolved.
- Split the stock homogenization buffer into 2 labelled Falcon tubes, and store in fridge @ 4°C.

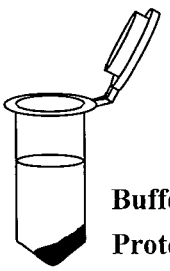
H.2 Mixing Working Homogenization Buffer

- NOTE: only good for 4-6 h after addition of inhibitor tablets.
- NOTE: directions to prepare 10 ml of working buffer.
- Place a stir bar inside a glass beaker, and place the beaker on the stir platform.
- Remove stock homogenization buffer (H.1) from the fridge @ 4°C, vortex, and place on ice.
- Transfer 9.8 ml of stock homogenization buffer to a glass beaker using a 5 ml pipette.
- Return stock homogenization buffer to the fridge @ 4°C.
- Place Parafilm over the top of the beaker. Stir solution.
- Use the pH meter to verify the acidity of the solution. Adjust pH to 7.5.
  - *Note: if too acidic, add 0.6 M NaOH using a glass transfer pipette.*
  - *Note: add 0.1 M NAOH using a glass transfer pipette to fine-tune pH.*
  - *Note: if too basic, add 0.1 M HCl using a glass transfer pipette.*
- Add 0.108 g of β-glycerophosphate (FW: 216.0, Concentration: 10 mM) to the beaker.
  - *Note: use glycerol-2-phosphate disodium salt hydrate.*
  - *Note: provides excess P_i, which is the dephosphorylation product.*
- Remove an eppendorf of 200 mM sodium orthovanadate from the -20°C freezer, and place on a heating block: boil at 95°C for 10 min.
  - *Note: 200 mM stock made by adding 184 mg of dry stock to 5 ml of dH_2O – then aliquoted into eppendorfs and placed in -20°C freezer.*
- Add 50 µl of 200 mM sodium orthovanadate (FW:183.91, Concentration: 1 mM) to the beaker.
- Add 0.021g of sodium fluoride (FW: 41.99, Concentration: 50 mM) to the beaker.
  - *Note: Ser/Thr phosphatase inhibitor.*
- Add a complete protease inhibitor tablet (Roche).
  - *Note: inhibits the activity of proteases.*
- Place Parafilm over the top of the beaker. Stir solution until all solids are fully dissolved.

- Use the pH meter to verify the acidity of the solution. Adjust pH to 7.5.
  - *Note: if too acidic, add 0.6 M NaOH using a glass transfer pipette.*
  - *Note: add 0.1 M NAOH using a glass transfer pipette to fine-tune pH.*
  - *Note: if too basic, add 0.1 M HCl using a glass transfer pipette.*
- Transfer the working homogenization buffer to a Falcon tube, and place in a Styrofoam container filled with ice.

H.3 Extraction of the Sarcoplasmic/Non-Myofibrillar Fraction

- Continued from G.3.
- CAUTION: keep homogenization buffer (H.2) and homogenized samples on ice at all times.
- Mass of muscle sample required: 25 mg+
  - *Note: mass of muscle sample required depends on subsequent analysis – require 25 mg of muscle for sarcoplasmic FSR, 25 mg for Western buffers, or 50 mg for both.*
- Pull an eppendorf out of the dewer containing N_2 *(l)*, and immediately place on ice.
- Uncap the eppendorf, add 7.5 µl of buffer/mg of muscle, and homogenize the muscle with the Teflon pestle, keeping the eppendorf on the ice.
  - *Note: continue homogenizing until solution is a homogenous pinkish-red muscle fluid.*
  - *Note: break up any residual muscle pieces as best as possible.*
- Cap the eppendorf, and place on ice.
- Repeat for all samples.
- Vortex all samples.
- Centrifuge all samples using the 8848 rotor: 3000 rpm, 10 min, @ 4°C.



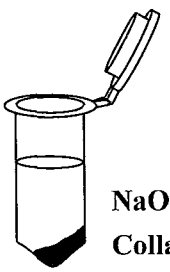
Buffer-Extract (SARC)

Protein Pellet (MYO and Collagen)

- Place eppendorfs back on ice.
- Uncap a sample-containing eppendorf, remove the homogenization buffer-extract, and dispense the buffer-extract into a labelled eppendorf. Recap both eppendorfs.
- Repeat for all samples.
- Immediately place the eppendorfs containing the buffer-extract in a freezer at -80°C, until ready to prepare for Western analysis or Sarcoplasmic FSR.

H.4 Extraction of the Myofibrillar Protein Fraction

- Continued from H.3.
- Uncap an eppendorf containing the myofibrillar-collagen protein pellet, add 500 µl of dH_2O, and recap eppendorf.
- Repeat for all samples.
- Vortex all samples.
  - *Note: try to suspend the solid muscle mass to thoroughly mix.*
- Centrifuge all samples using the 8848 rotor: 3000 rpm, 10 min, @ 4°C.
- Place eppendorfs back on ice.
- Uncap an eppendorf, remove the dH_2O, and dispense the dH_2O into a labelled waste beaker. Add 1 ml of 0.3M NaOH to the eppendorf, and recap.
- Repeat for all samples.
- Vortex all samples.
- Place the eppendorfs in a heating block in the fumehood – boil @ 50°C for 30 min. Vortex all samples every 10 min during the heating. Permit to cool.
- When cool, vortex all samples, and centrifuge all samples using the 8848 rotor: 10000 rpm, 5 min, @ 4°C.
- Uncap an eppendorf, remove the NaOH-extract, and dispense the NaOH-extract into a correspondingly labelled 4 ml screw top vial. Add 1 ml of 0.3M NaOH to the eppendorf and recap. Repeat for all samples.
  - *Note: avoid drawing up the collagen pellet when drawing the NaOH-extract.*

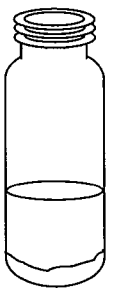


NaOH-Extract (MYO)
Collagen Protein Pellet

- Vortex all samples.
- Centrifuge all samples using the 8848 rotor: 10000 rpm, 5 min, @ 4°C.
- Uncap an eppendorf, remove the NaOH-extract, and dispense the NaOH-extract into the correspondingly labelled 4 ml screw top vial containing the NaOH-extract from the previous step. Repeat for all samples. Store the eppendorf containing the collagen pellet in the fridge at 4°C.
  - *Note: avoid drawing up the collagen pellet when drawing the NaOH-extract.*
  - *Note: collagen pellet may not be useful if a Phe tracer was infused, because the Phe amino acid is not abundant enough in the collagen tissue to detect changes during a short incorporation period.*
  - *Note: to study collagen synthesis, infuse a Leu or Pro tracer.*
- Add 1 ml of 1M PCA to the 4 ml screw top vials containing the NaOH-extract.
- Vortex all samples.

- Centrifuge all samples using the 8890 rotor: 3000 rpm, 10 min, @ 4°C.
  - *CAUTION: spin the 4 ml screw top vials without the lids for the vials.*
  - *CAUTION: to load the vials into the buckets for centrifugation, carefully lower the 4 ml screw top vials into the bucket slots using a pair of tongs.*

- Remove the NaCl water-extract from a 4 ml screw top vial, and dispense into a labelled waste beaker. Carefully add 1 ml of 70% etOH to the 4 ml screw top vial. Repeat for all samples.
  - *Note: avoid drawing up the myofibrillar pellet when drawing the NaCl water-extract.*
  - *Note: to add etOH, tip vial on side, and dispense etOH slowly onto the side wall of the vial, and let etOH slowly run down to the bottom of the vial.*
  - *CAUTION: do not disturb or re-suspend the myofibrillar pellet.*
  - *CAUTION: DO NOT VORTEX.*



NaCl Water

Myofibrillar Pellet

- Centrifuge all samples using the 8890 rotor: 3000 rpm, 10 min, @ 4°C.
  - *CAUTION: spin the 4 ml screw top vials without the lids for the vials.*
  - *CAUTION: to load the vials into the buckets for centrifugation, carefully lower the 4 ml screw top vials into the bucket slots using a pair of tongs.*

- Remove the etOH-extract from a 4 ml screw top vial, and dispense into a labelled waste beaker. Carefully add 1 ml of 70% etOH to the 4 ml screw top vial. Repeat for all samples.
  - *Note: avoid drawing up the myofibrillar pellet when drawing the etOH-extract.*
  - *Note: to add etOH, tip vial on side, and dispense etOH slowly onto the side wall of the vial, and let etOH slowly run down to the bottom of the vial.*
  - *CAUTION: do not disturb or re-suspend the myofibrillar pellet.*
  - *CAUTION: DO NOT VORTEX.*

- Centrifuge all samples using the 8890 rotor: 3000 rpm, 10 min, @ 4°C.
  - *CAUTION: spin the 4 ml screw top vials without the lids for the vials.*
  - *CAUTION: to load the vials into the buckets for centrifugation, carefully lower the 4 ml screw vials into the bucket slots using a pair of tongs.*

- Remove the etOH-extract from a 4 ml screw top vial, and dispense into a labelled waste beaker. Add 2 ml of 6M HCl to the 4 ml screw top vial. Repeat for all samples.
  - *Note: avoid drawing up the myofibrillar pellet when drawing the etOH-extract.*

- Screw caps onto the 4 ml screw cap vials.
  - *CAUTION: ensure an air tight seal to prevent drying of sample, but do not over-tighten, because the pressure required to remove an over-tightened cap will break the vial.*
  - *CAUTION: if a cap is too tight to remove easily with the fingers, place the vial in the fumehood, wedge the tips of a pair of tweezers between the edge of the cap and the lip of the vial, and pry the cap away from the vial. Gradually fracture the cap away from the vial until the cap is loose enough to remove by finger.*

- Place 4 ml screw cap vials in a rack with the middle shelf lined with aluminum foil to create a platform for the vials. Place the rack in the oven heated to 100°C.

- Boil the 4 ml screw cap vials @ 100°C for 24 hr. Ensure that the pellet is completely hydrolyzed before removing the vials from the oven.
- Store 4ml screw cap vials at room temperature until prepared to column the myofibrillar muscle fraction. Column and derivatize the myofibrillar fraction as per the directions for the mixed muscle fraction (G.9 and G.10).

Appendix I: Data Table – Participant Characteristics

	Age (y)	Height (m)	Weight (kg)	BMI ($kg \cdot m^2$)
1	27	1.92	82	22.4
2	23	1.84	75	22.2
3	20	1.94	100	26.4
4	24	1.76	72	23.3
5	23	1.73	69	23.2
6	23	1.76	82	26.6
7	21	1.89	79	22.1
8	23	1.86	83	24.1
9	23	1.71	81	27.7
Mean	**23**	**1.82**	**80**	**24.2**
SEM	1	0.03	3	0.7

Appendix J: Data and t-Test Tables – Strength and External Work

J.1 Strength: Mass Lifted on 1-RM (kg)

	PRO	PRO+CARB
1	230	220
2	155	135
3	200	237
4	200	170
5	192	180
6	202	202
7	150	150
8	192	192
9	140	162
Mean	**185**	**183**
SEM	10	11

T-test for Dependent Samples (new.sta)
Marked differences are significant at p < .05000

	Mean	Std.Dv.	N	Mean Diff.	Std. Dv. Diff.	t	df	p
VAR1	184.7222	29.67123						
VAR2	183.3333	33.16625	9	1.388889	20.77074	0.200603	8	0.846015

J.2 Strength: Mass Lifted on 8-12 RM (kg)

	PRO	PRO+CARB
1	155	150
2	112	100
3	175	180
4	137	137
5	135	125
6	145	150
7	120	112
8	135	135
9	110	115
Mean	**136**	**134**
SEM	7	8

T-test for Dependent Samples (new.sta)
Marked differences are significant at p < .05000

	Mean	Std.Dv.	N	Mean Diff.	Std. Dv. Diff.	t	df	p
VAR1	136.1111	20.77074						
VAR2	133.8889	24.24198	9	2.222222	6.782842	0.982872	8	0.354457

J.3 Approximate External Work (kg•reps)

	PRO	PRO+CARB
1	2516	2643
2	1955	1977
3	3558	3156
4	2426	2483
5	2450	2336
6	2073	2353
7	1741	1702
8	2085	2110
9	1312	1419
Mean	**2235**	**2242**
SEM	209	172

T-test for Dependent Samples (new.sta)
Marked differences are significant at p < .05000

	Mean	Std.Dv.	N	Mean Diff.	Std. Dv. Diff.	t	df	p
VAR1	2235.164	626.0922						
VAR2	2242.172	516.137	9	-7.00758	189.3182	-0.11104	8	0.914317

Appendix K: Data and ANOVA Tables – Plasma Metabolites and Hormones

K.1 Plasma Glucose Concentrations (mM)

	PRO										PRO+CARB									
	Pre	**15**	**30**	**60**	**90**	**120**	**135**	**150**	**165**	**180**	**Pre**	**15**	**30**	**60**	**90**	**120**	**135**	**150**	**165**	**180**
1	5.1	5.1	5.0	5.0	5.1	5.2	4.9	4.9	4.9	5.3	5.2	4.7	6.1	6.1	5.9	4.6	4.7	4.7	4.8	4.8
2	5.0	5.1	4.8	4.3	4.9	4.7	5.2	4.9	4.9	5.0	5.4	5.2	6.2	6.3	6.1	6.4	6.3	5.5	5.1	5.2
3	4.8	4.6	5.4	4.3	4.2	4.7	4.9	4.7	4.7	5.1	4.9	4.8	5.0	4.8	4.6	4.7	4.7	4.5	4.8	4.9
4	4.5	4.6	5.1	5.2	4.8	4.7	4.5	4.3	4.4	4.9	4.6	4.3	4.9	7.9	6.9	6.3	5.4	4.6	4.4	4.7
5	5.0	4.7	5.2	5.1	5.2	4.9	4.9	4.9	4.9	5.2	5.4	5.1	7.1	8.4	6.1	5.2	5.1	5.1	5.3	4.5
6	5.3	5.2	5.3	5.3	4.9	5.2	5.2	5.2	5.2	5.1	4.6	4.5	5.6	9.1	5.5	6.0	5.1	4.2	3.9	3.7
7	4.3	NV	5.2	4.3	4.4	4.2	4.6	3.9	3.8	4.4	4.6	4.2	5.3	5.7	5.3	4.2	4.5	4.9	4.6	4.6
8	5.2	4.6	5.1	5.3	4.9	4.7	5.1	5.2	5.2	4.8	5.5	4.8	7.3	6.8	6.7	5.5	5.4	5.0	5.0	4.9
9	5.4	5.8	5.9	6.7	6.3	6.0	5.7	6.2	5.8	5.9	5.4	5.6	7.3	10	8.8	8.5	8.4	8.0	8.2	7.9
Mean	**5.0**	**5.0**	**5.2**	**5.1**	**5.0**	**4.9**	**5.0**	**4.9**	**4.9**	**5.1**	**5.1**	**4.8**	**6.1**	**7.3**	**6.2**	**5.7**	**5.5**	**5.2**	**5.1**	**5.0**
SEM	0.1	0.1	0.1	0.3	0.2	0.2	0.1	0.2	0.2	0.1	0.1	0.1	0.3	0.6	0.4	0.4	0.4	0.4	0.4	0.4

ANOVA - Summary of all Effects; design: (new.sta)
1-SUBSTRAT, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	16.28411	8	1.650959	9.863425	**0.013793**
2	9	2.8536	72	0.361039	7.903853	**5.13E-08**
12	9	2.288504	72	0.229382	9.976836	**8.97E-10**

K.2 Plasma Insulin Concentrations ($\mu U \cdot ml^{-1}$)

	PRO							PRO+CARB						
	Pre	**15**	**30**	**60**	**90**	**120**	**180**	**Pre**	**15**	**30**	**60**	**90**	**120**	**180**
1	1.7	4.1	6.7	9.3	4.9	3.2	1.5	1.8	2.9	24.5	16.9	10.2	1.3	1.3
2	4.6	6.8	18.5	11.4	8.2	5.7	4.3	4	4	44.8	71.5	33.1	41.9	8
3	9.5	8.6	17.1	18.6	9.5	6.5	4	7	10.8	25.2	42.6	28	14.7	4.8
4	1.8	4.4	7.9	13.7	5.3	2.7	1.7	2.5	6.3	15.7	73.5	47	47.1	14.8
5	2.4	6.4	12.6	15.7	9.3	4.6	4.4	3.2	6	30.8	106.4	22.8	8.8	1
6	1.7	3.7	14.1	22.7	8.6	4.9	3.2	1.5	3.5	15.6	94.8	29.7	38.2	4.3
7	4.3	NV	20.7	18.4	9	3.8	3.1	4.7	4.8	26.1	41.3	29.9	7	2.8
8	9.6	9.2	19.8	28.6	13.3	11.1	8	8.8	9	49.4	43.2	46.3	17.1	7.2
9	8.1	12.3	14	32.1	34.9	11.5	5.5	11.7	12.3	68.8	99	86	86.3	61.4
Mean	**4.9**	**6.9**	**14.6**	**18.9**	**11.4**	**6**	**4**	**5**	**6.6**	**33.4**	**65.5**	**37**	**29.2**	**11.7**
SEM	1.1	1	1.7	2.6	3	1.1	0.7	1.2	1.1	5.9	10.4	7.2	9	6.4

ANOVA - Summary of all Effects; design: (new.sta)
1-SUBSTRAT, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	7945.582	8	699.7358	11.35512	**0.009789**
2	6	2887.507	48	223.7348	12.90594	**1.27E-08**
12	6	1591.405	48	167.39	9.507167	**7.04E-07**

K.3.1 Glucose AUC (mM•180min)

	PRO	PRO+CARB
1	-11	27
2	-35	96
3	-23	26
4	47	221
5	6	125
6	-21	200
7	18	61
8	-29	89
9	120	505
Mean	**8**	**144**
SEM	16	52

K.3.2 Insulin AUC ($\mu U \cdot 180min \cdot ml^{-1}$)

	PRO	PRO+CARB
1	515	1184
2	678	5640
3	144	2418
4	645	6160
5	1016	4506
6	1317	5707
7	892	2380
8	925	3405
9	1763	10984
Mean	**877**	**4709**
SEM	156	973

K.3.1 Glucose AUC

T-test for Dependent Samples (new.sta)
Marked differences are significant at p < .05000

	Mean	Std.Dv.	N	Mean Diff.	Std. Dv. Diff.	t	df	p
VAR1	8.218889	49.2882						
VAR2	144.1778	155.7682	9	-135.959	116.5859	-3.49851	8	**0.008097**

K.3.2 Insulin AUC

T-test for Dependent Samples (new.sta)
Marked differences are significant at p < .05000

	Mean	Std.Dv.	N	Mean Diff.	Std. Dv. Diff.	t	df	P
VAR1	877.2222	469.3899						
VAR2	4709.333	2919.417	9	-3832.11	2582.03	-4.45244	8	**0.002132**

K.4 Plasma Leucine Concentrations (μM)

	PRO										PRO+CARB									
	Pre	**15**	**30**	**60**	**90**	**120**	**135**	**150**	**165**	**180**	**Pre**	**15**	**30**	**60**	**90**	**120**	**135**	**150**	**165**	**180**
1	198	NV	204	378	330	264	238	207	189	178	115	100	176	218	215	112	92	92	85	92
2	124	103	145	230	241	208	190	169	163	138	155	129	182	256	219	228	217	178	160	168
3	147	98	170	311	261	215	179	161	129	156	236	184	225	324	254	317	265	186	204	166
4	117	98	120	274	354	219	199	150	133	130	118	99	114	170	146	172	154	137	131	148
5	102	97	118	223	282	186	173	155	140	135	130	102	150	232	231	203	165	127	114	110
6	117	101	148	293	228	174	158	149	128	130	140	109	125	249	196	205	201	136	135	102
7	114	NV	195	288	244	205	182	149	142	131	119	107	145	230	239	171	172	172	153	119
8	65	54	96	144	133	86	77	87	66	64	57	48	83	102	112	88	73	60	58	49
9	75	64	69	128	155	117	110	108	108	96	76	67	108	128	104	111	100	96	110	101
Mean	**118**	**88**	**141**	**252**	**247**	**186**	**167**	**148**	**133**	**129**	**127**	**105**	**145**	**212**	**191**	**179**	**160**	**132**	**128**	**117**
SEM	13	7	15	27	24	18	16	12	11	11	17	13	15	23	19	24	21	14	14	13

ANOVA - Summary of all Effects; design: (new.sta)
1-SUBSTRAT, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	5813.918	8	8470.909	0.686339	0.431432
2	9	34754.88	72	763.0353	45.5482	**3.24E-26**
12	9	2238.328	72	513.6647	4.357566	**0.000147**

K.5 Plasma Phenylalanine Concentrations (μM)

	PRO										PRO+CARB									
	Pre	**15**	**30**	**60**	**90**	**120**	**135**	**150**	**165**	**180**	**Pre**	**15**	**30**	**60**	**90**	**120**	**135**	**150**	**165**	**180**
1	94	NV	118	131	120	117	104	97	91	94	58	62	78	80	82	61	55	60	54	56
2	57	57	71	79	73	66	69	59	58	54	79	94	91	97	93	98	88	85	77	73
3	62	80	77	92	78	71	63	59	62	59	100	94	108	118	87	117	105	77	93	68
4	55	68	76	94	119	87	59	54	50	53	62	71	81	83	73	89	74	73	71	78
5	61	87	78	91	95	79	76	71	65	66	66	62	77	86	102	93	83	70	64	64
6	60	61	74	91	74	67	64	61	57	58	64	70	72	89	78	81	76	64	63	55
7	62	NV	86	92	78	68	63	62	60	88	70	73	81	88	89	76	74	74	71	59
8	38	38	46	53	50	39	37	38	36	35	35	35	45	46	51	44	46	37	37	32
9	42	47	48	63	66	56	55	56	54	54	41	74	58	57	52	56	50	49	53	50
Mean	**59**	**63**	**75**	**87**	**84**	**72**	**66**	**62**	**59**	**62**	**64**	**71**	**77**	**83**	**79**	**79**	**72**	**65**	**65**	**59**
SEM	5	6	7	7	8	7	6	5	5	6	6	6	6	7	6	8	6	5	5	5

ANOVA - Summary of all Effects; design: (new.sta)
1-SUBSTRAT, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	286.3987	8	1691.319	0.169334	0.691508
2	9	1375.76	72	66.53867	20.67609	**1.08E-16**
12	9	111.4906	72	68.69378	1.623009	0.124902

K.6 Plasma Branched-chain Amino Acid Concentrations (μM)

	PRO										PRO+CARB									
	Pre	**15**	**30**	**60**	**90**	**120**	**135**	**150**	**165**	**180**	**Pre**	**15**	**30**	**60**	**90**	**120**	**135**	**150**	**165**	**180**
1	686	NV	701	1123	964	839	787	704	672	617	397	357	531	653	642	389	330	326	303	321
2	416	359	474	672	700	612	570	523	510	443	492	432	579	706	645	686	678	591	511	552
3	517	409	570	915	783	679	597	543	534	520	793	664	770	988	770	1006	879	635	690	561
4	381	336	412	807	965	653	567	476	442	427	381	328	376	525	463	514	476	433	417	457
5	351	318	388	652	786	562	529	489	437	417	425	331	465	668	681	602	522	405	370	353
6	360	317	432	819	655	518	475	457	406	407	390	348	381	706	566	614	601	429	432	339
7	390	NV	586	822	708	591	521	468	452	412	426	376	487	689	719	564	571	557	522	420
8	214	176	274	407	385	270	241	244	218	208	180	155	236	293	326	268	231	197	186	160
9	232	201	217	369	442	343	328	320	317	293	242	220	323	366	315	326	295	291	327	308
Mean	**394**	**302**	**450**	**732**	**710**	**563**	**513**	**469**	**443**	**416**	**414**	**357**	**461**	**622**	**569**	**552**	**509**	**429**	**417**	**386**
SEM	47	28	51	80	67	57	52	44	43	39	57	47	52	69	55	74	68	48	49	42

ANOVA - Summary of all Effects; design: (new.sta)
1-SUBSTRAT, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	34320.52	8	86365.88	0.397385	0.546024
2	9	219068.7	72	5204.211	42.0945	**3.43E-25**
12	9	15509.04	72	3639.642	4.261145	**0.000186**

K.7 Plasma Essential Amino Acid Concentrations (μM)

	PRO										PRO+CARB									
	Pre	**15**	**30**	**60**	**90**	**120**	**135**	**150**	**165**	**180**	**Pre**	**15**	**30**	**60**	**90**	**120**	**135**	**150**	**165**	**180**
1	1229	NV	1318	1976	1712	1555	1459	1345	1274	1206	752	714	1004	1234	1218	839	733	725	676	701
2	764	690	879	1124	1229	1086	1005	954	940	821	918	857	1071	1295	1191	1271	1276	1108	994	1074
3	943	750	999	1548	1331	1172	1034	946	965	907	1404	1219	1405	1755	1374	1797	1601	1162	1300	1121
4	753	707	840	1501	1742	1250	1075	935	874	859	695	641	736	1008	922	1024	959	865	831	914
5	737	716	785	1231	1401	1056	1003	959	839	805	829	637	858	1177	1278	1115	985	787	719	692
6	680	623	809	1429	1153	945	876	848	766	763	717	675	727	1204	1014	1072	1053	804	806	667
7	717	NV	1003	1308	1108	943	841	774	757	733	782	709	875	1188	1209	1000	996	978	939	768
8	444	376	530	738	719	529	475	466	438	413	368	345	455	572	629	543	484	434	409	353
9	440	397	430	673	775	622	593	595	581	548	440	468	570	641	555	587	536	530	583	563
Mean	**745**	**608**	**844**	**1281**	**1241**	**1018**	**929**	**869**	**826**	**784**	**767**	**696**	**856**	**1119**	**1043**	**1028**	**958**	**821**	**806**	**761**
SEM	80	52	87	135	119	104	95	83	79	74	99	82	94	118	96	125	117	81	86	81

ANOVA - Summary of all Effects; design: (new.sta)
1-SUBSTRAT, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	37416.85	8	280203.5	0.133535	0.724268
2	9	553141.8	72	14369.81	38.49334	**4.78E-24**
12	9	34632.47	72	9736.823	3.556856	**0.001064**

K.8 Plasma $^{13}C_6$-Phenylalanine Enrichments (%)

	PRO							PRO+CARB						
	Pre	**15**	**30**	**60**	**90**	**120**	**180**	**Pre**	**15**	**30**	**60**	**90**	**120**	**180**
1	0.004	0.051	0.065	0.059	0.063	0.064	0.069	0.004	0.060	0.058	0.068	0.070	0.081	0.084
2	0.005	0.056	0.060	0.062	0.066	0.072	0.060	0.005	0.056	0.062	0.066	0.069	0.071	0.065
3	0.006	NV	0.066	0.065	0.066	0.070	0.070	0.005	0.054	0.056	0.064	0.074	0.073	0.084
4	0.002	0.136	0.093	0.066	0.069	0.071	0.077	0.041	0.099	0.085	0.081	0.081	0.075	NV
5	0.003	NV	0.075	0.060	0.061	0.066	0.062	0.003	0.065	0.064	0.063	0.063	0.069	0.070
6	0.002	0.067	0.069	0.060	0.065	0.073	0.077	0.002	0.091	0.063	0.067	0.068	0.074	0.082
7	0.006	NV	0.066	0.064	0.073	0.074	NV	0.002	0.054	0.059	0.059	0.066	0.071	0.070
8	0.003	0.073	0.055	0.056	0.068	0.065	0.066	0.003	0.079	0.059	0.059	0.066	0.070	0.062
9	0.002	0.074	0.058	0.053	0.059	NV	0.052	0.002	0.101	0.065	0.074	0.065	0.074	0.072
Mean	**0.004**	**0.076**	**0.067**	**0.061**	**0.065**	**0.069**	**0.066**	**0.007**	**0.073**	**0.064**	**0.067**	**0.069**	**0.073**	**0.074**
SEM	0.001	0.010	0.004	0.001	0.001	0.001	0.003	0.004	0.006	0.003	0.002	0.002	0.001	0.003

ANOVA - Summary of all Effects; design: (new.sta) – Ran on data from 30-180 min.
1-SUBSTRAT, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	0.000253	8	4.83E-05	5.230167	0.101509
2	4	0.00018	32	4.15E-05	4.336818	**0.006477**
12	4	8.35E-05	32	1.76E-05	4.733813	**0.004092**

Correlations (new.sta) – Pooled PRO and PRO+CARB data because ANOVA determined no differences between substrates.
Marked correlations are significant at p < .05000

	Mean	Std.Dv.	r(X,Y)	r^2	t	p	N	Constant dep: Y	Slope dep: Y	Constant dep: X	Slope dep: X
Time	96	57.70615									
Enrich	0.067556	0.003164	0.794005	0.630444	2.262266	0.108698	5	0.063377	4.35E-05	-882.346	14482.01

Appendix L: Data and ANOVA Tables – Cardiovascular Data

L.1 Heart Rate (bpm)

	PRO													
	REST							EX						
	Pre	**15**	**30**	**60**	**90**	**120**	**180**	**Pre**	**15**	**30**	**60**	**90**	**120**	**180**
1	54	64	62	58	56	59	58	52	61	59	57	57	58	59
2	68	74	81	74	72	68	74	68	76	76	79	74	72	73
3	83	93	87	88	83	69	69	82	98	89	80	78	72	67
4	65	86	87	76	68	67	69	71	85	84	81	68	66	68
5	69	90	85	78	73	73	81	70	86	86	79	74	74	85
6	71	72	77	76	66	64	77	67	79	80	73	68	66	75
7	54	60	63	61	60	59	62	57	62	69	61	60	63	61
8	64	71	71	72	60	59	65	62	72	69	63	59	61	65
9	70	78	NV	75	80	75	75	69	82	NV	74	75	72	76
Mean	**66**	**76**	**77**	**73**	**69**	**66**	**70**	**67**	**78**	**77**	**72**	**68**	**67**	**70**
SEM	3	4	3	3	3	2	3	3	4	3	3	3	2	3

	PRO+CARB													
	REST							EX						
	Pre	**15**	**30**	**60**	**90**	**120**	**180**	**Pre**	**15**	**30**	**60**	**90**	**120**	**180**
1	54	65	70	67	62	59	57	55	64	70	64	61	55	58
2	66	81	82	78	83	79	81	64	75	78	81	78	76	73
3	84	89	98	82	78	70	81	71	97	99	83	82	66	71
4	73	88	97	80	80	78	82	69	89	92	85	78	79	78
5	73	98	99	99	96	81	87	73	98	97	103	90	80	81
6	68	73	81	77	68	80	70	72	71	78	81	69	76	73
7	50	59	62	58	55	54	50	49	61	59	53	54	54	50
8	53	73	77	60	67	68	57	51	73	77	61	68	63	57
9	64	75	77	71	64	67	68	62	75	76	72	64	65	68
Mean	**65**	**78**	**83**	**75**	**72**	**71**	**70**	**63**	**78**	**81**	**76**	**72**	**68**	**68**
SEM	4	4	4	4	4	3	4	3	5	4	5	4	3	4

L.2 Mean Arterial Pressure (mmHg)

	PRO													
	REST							EX						
	Pre	**15**	**30**	**60**	**90**	**120**	**180**	**Pre**	**15**	**30**	**60**	**90**	**120**	**180**
1	102	105	111	112	101	90	98	107	104	110	111	100	87	98
2	95	89	91	88	93	87	87	92	89	94	79	92	87	90
3	76	87	89	102	93	93	83	76	90	88	100	99	94	81
4	112	91	92	87	100	85	93	119	89	93	91	89	91	93
5	98	87	93	80	101	98	88	95	83	96	89	98	98	81
6	104	93	109	107	91	91	99	107	90	109	99	91	94	99
7	94	78	83	86	82	84	69	91	86	81	87	83	91	72
8	90	79	103	102	89	82	85	93	70	101	114	87	81	85
9	109	107	NV	108	122	114	118	109	114	NV	110	122	113	124
Mean	**98**	**91**	**96**	**97**	**97**	**92**	**91**	**99**	**91**	**96**	**98**	**96**	**93**	**91**
SEM	4	3	3	4	4	3	5	4	4	3	4	4	3	5

	PRO+CARB													
	REST							EX						
	Pre	**15**	**30**	**60**	**90**	**120**	**180**	**Pre**	**15**	**30**	**60**	**90**	**120**	**180**
1	87	84	96	88	100	98	87	88	83	87	85	89	96	87
2	102	92	97	104	123	87	90	104	99	106	107	128	90	92
3	106	73	123	107	97	81	109	97	76	125	110	98	82	107
4	118	118	112	91	102	142	105	113	124	119	98	104	141	109
5	96	85	85	81	87	113	85	94	88	78	79	97	108	84
6	113	109	104	111	103	111	104	94	115	97	111	106	94	105
7	77	78	85	79	73	66	84	71	76	79	83	73	65	85
8	90	83	92	87	89	91	88	89	88	84	89	88	89	86
9	109	101	NV	96	98	102	109	113	104	NV	106	100	101	106
Mean	**100**	**91**	**99**	**94**	**97**	**99**	**96**	**96**	**95**	**97**	**96**	**98**	**96**	**96**
SEM	5	5	4	4	5	7	4	4	6	6	4	5	7	4

L.3 Mean Arterial Diameter (cm)

	PRO													
	REST							EX						
	Pre	**15**	**30**	**60**	**90**	**120**	**180**	**Pre**	**15**	**30**	**60**	**90**	**120**	**180**
1	0.97	1.02	1.03	1.04	1.01	1.08	1.06	1.02	1.02	1.08	1.04	1.07	1.02	1.03
2	0.8	0.77	0.82	0.79	0.84	0.84	0.86	0.71	0.72	0.72	0.7	0.67	0.67	0.73
3	1.03	1.01	1.04	1.06	1.07	1.03	0.98	0.97	1.04	1	1.01	1	1.04	1.02
4	0.97	0.97	0.92	1.02	1.02	1.01	0.98	0.99	1.04	1.01	1.06	1.04	1.04	0.96
5	1.01	0.92	0.99	0.98	1.02	1.02	1.03	0.83	0.9	0.93	0.84	0.85	0.91	0.88
6	0.92	0.9	0.92	0.88	0.91	0.93	0.91	0.91	0.92	0.85	0.84	0.85	0.85	0.87
7	0.82	0.86	0.86	0.84	0.89	0.92	0.89	0.87	0.94	0.92	0.91	0.92	0.95	0.93
8	0.82	0.83	0.84	0.87	0.82	0.82	0.82	0.87	0.9	0.88	0.91	0.87	0.87	0.86
9	0.82	0.84	0.85	0.85	0.84	0.87	0.81	0.78	0.83	0.87	0.8	0.84	0.81	0.77
Mean	**0.91**	**0.9**	**0.92**	**0.93**	**0.93**	**0.95**	**0.93**	**0.88**	**0.92**	**0.92**	**0.9**	**0.9**	**0.91**	**0.89**
SEM	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.04	0.04	0.04	0.04	0.04	0.03

	PRO+CARB													
	REST							EX						
	Pre	**15**	**30**	**60**	**90**	**120**	**180**	**Pre**	**15**	**30**	**60**	**90**	**120**	**180**
1	1.02	1.03	1.02	1.04	1.03	1.06	1.05	0.97	1.09	0.94	0.98	1.08	0.95	1.12
2	0.74	0.65	0.72	0.69	0.75	0.75	0.74	0.77	0.85	0.81	0.85	0.81	0.77	0.79
3	1.02	0.97	0.99	0.98	1.03	1	0.98	1.02	1.04	1.04	1.06	0.97	1.03	1.03
4	1.01	0.94	1.03	0.96	0.98	1	0.99	0.99	1.03	0.94	0.98	1.12	1.09	1
5	0.92	0.87	0.89	0.9	0.92	0.94	0.91	1.02	1.01	1.01	1.01	0.98	1	0.95
6	0.9	0.86	0.8	0.79	0.83	0.89	0.9	0.91	0.95	0.93	0.91	0.91	0.94	0.92
7	0.9	0.91	0.92	0.88	0.91	0.91	0.93	0.83	0.92	0.86	0.92	0.89	0.92	0.89
8	0.88	0.9	0.9	0.89	0.91	0.89	0.85	0.87	0.92	0.9	0.9	0.88	0.86	0.87
9	0.77	0.74	0.76	0.75	0.71	0.76	0.72	0.81	0.84	0.8	0.84	0.84	0.82	0.79
Mean	**0.9**	**0.88**	**0.89**	**0.88**	**0.9**	**0.91**	**0.89**	**0.91**	**0.96**	**0.91**	**0.94**	**0.94**	**0.93**	**0.93**
SEM	0.03	0.04	0.04	0.04	0.04	0.03	0.04	0.03	0.03	0.03	0.02	0.04	0.03	0.04

L.4 Mean Blood Velocity ($cm \cdot s^{-1}$)

	PRO													
	REST							EX						
	Pre	**15**	**30**	**60**	**90**	**120**	**180**	**Pre**	**15**	**30**	**60**	**90**	**120**	**180**
1	4.05	4.78	3.17	3.27	2.82	3.28	3.16	4.19	12.49	5.6	4.32	3.07	4.27	5.34
2	3.31	3.09	2.48	3.96	3.1	2.72	4.07	3.72	9.81	7.95	9.3	8.63	4.63	3.35
3	5.84	6.4	5.98	4.2	3.9	4.1	3.1	6.31	19	9.09	4.16	3.51	6.32	4.93
4	3.25	3.81	2.07	3.58	3.17	3.2	3.13	2.22	10.7	6.33	8.06	4.44	4.06	3.69
5	3.62	3.09	2.02	2.15	2.37	3.09	4.5	3.56	4.77	5.94	3.59	5.67	2.91	4.89
6	2.66	1.98	1.66	2.18	1.76	3.81	2.67	2.88	10.71	5.22	4.92	2.63	3.49	3.5
7	2.01	3.11	2.2	1.57	3.72	2.06	2.49	2.05	4.6	4.01	3.36	2.38	4.56	3.96
8	4.1	2.98	5.83	4.27	2.35	1.5	3.81	3.99	14.86	8.13	5.8	5.48	3.2	4.04
9	4.74	5.23	7.08	8.94	9.23	6.38	7.53	5.13	10.39	9.57	8.76	8.45	8.72	5
Mean	**3.73**	**3.83**	**3.61**	**3.79**	**3.6**	**3.35**	**3.83**	**3.78**	**10.81**	**6.87**	**5.81**	**4.92**	**4.68**	**4.3**
SEM	0.38	0.46	0.69	0.72	0.74	0.46	0.51	0.45	1.5	0.63	0.77	0.79	0.6	0.25

	PRO+CARB													
	REST							EX						
	Pre	**15**	**30**	**60**	**90**	**120**	**180**	**Pre**	**15**	**30**	**60**	**90**	**120**	**180**
1	4.06	5.36	4.17	5.62	5.01	5.27	4.76	4.85	9.88	6.53	8.73	3.77	5.19	4.17
2	2.18	3.48	3.08	3.53	3.53	3.53	3.99	1.16	7.61	4.04	7.05	9.58	8.23	4.52
3	2.47	5.92	5.66	5.97	2.82	2.47	1.56	3.05	13.5	7.3	5.79	4.7	2.22	1.06
4	2.03	3.41	3.72	1.56	4.74	2.01	2.01	3.01	3.55	2.99	1.07	6.55	3.85	3.33
5	2.13	2.46	2.86	3.94	6.51	4.22	3.31	3.55	13.06	6.21	6.08	5.7	4.22	4.46
6	2.54	1.57	1.28	2.65	2.01	1.53	1.07	3.64	5.83	1.21	2.49	2.14	1.82	0.86
7	1.19	1.35	2.7	3.15	3.88	2.75	1.54	2.29	9.48	3.25	3.73	3.14	2.16	3.27
8	2.47	5.32	2.35	4.12	3.76	4.27	6.53	1.42	9.8	4.98	4.3	3.14	2.09	2.5
9	7.36	7.85	5.07	7.48	3.52	4.81	3.35	4.92	11.69	5.39	5.29	4.44	5.4	5.97
Mean	**2.94**	**4.08**	**3.43**	**4.22**	**3.98**	**3.43**	**3.12**	**3.1**	**9.38**	**4.66**	**4.95**	**4.8**	**3.91**	**3.35**
SEM	0.61	0.73	0.46	0.61	0.44	0.44	0.6	0.44	1.09	0.65	0.78	0.75	0.71	0.56

L.5 Femoral Artery Blood Flow ($ml \cdot min^{-1}$)

	PRO													
	REST							EX						
	Pre	**15**	**30**	**60**	**90**	**120**	**180**	**Pre**	**15**	**30**	**60**	**90**	**120**	**180**
1	181	233	159	165	135	180	168	207	617	309	220	164	210	265
2	101	86	79	116	103	90	142	88	239	194	217	184	98	84
3	289	304	306	223	211	203	140	277	960	428	200	164	319	242
4	144	170	83	175	154	153	141	102	550	306	428	227	206	159
5	175	122	93	98	115	150	225	114	181	240	119	194	115	179
6	107	75	66	80	68	155	104	113	422	177	162	90	118	124
7	63	109	77	52	139	82	92	72	191	161	130	94	195	162
8	131	96	195	153	74	47	120	144	569	294	225	197	114	142
9	151	173	239	306	308	229	234	146	334	338	263	281	268	138
Mean	**149**	**152**	**144**	**152**	**145**	**143**	**152**	**140**	**452**	**272**	**218**	**177**	**183**	**166**
SEM	22	25	29	26	25	20	17	22	84	29	31	20	26	19

	PRO+CARB													
	REST							EX						
	Pre	**15**	**30**	**60**	**90**	**120**	**180**	**Pre**	**15**	**30**	**60**	**90**	**120**	**180**
1	200	266	204	289	252	277	245	217	551	274	392	206	222	247
2	56	69	75	79	94	93	103	32	258	124	241	293	229	133
3	121	260	262	269	140	117	71	151	685	369	307	206	110	53
4	97	143	187	68	216	94	92	138	178	124	49	389	214	157
5	84	89	106	152	262	175	129	173	631	297	290	257	200	189
6	96	54	38	78	66	57	40	141	247	49	97	83	75	35
7	45	53	107	115	153	107	62	75	375	113	150	118	85	123
8	90	205	90	154	148	161	221	50	387	189	163	115	73	89
9	204	205	138	196	83	132	82	152	388	162	176	148	171	173
Mean	**110**	**149**	**134**	**155**	**157**	**135**	**116**	**125**	**411**	**189**	**207**	**202**	**153**	**133**
SEM	19	29	24	27	24	21	24	20	59	35	36	33	22	23

L.6 ANOVA Tables For Cardiovascular Data

Heart Rate

ANOVA - Summary of all Effects; design: (new.sta)
1-SUBSTRAT, 2-ACTIVITY, 3-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	162.571	8	218.6212	0.743619	0.413605
2	1	21.49517	8	3.673537	5.851357	**0.041918**
3	6	945.9153	48	46.33965	20.41266	**1.07E-11**
12	1	31.10733	8	5.369514	5.793324	**0.042712**
13	6	65.62815	48	25.7935	2.544368	**0.032103**
23	6	5.433616	48	5.842819	0.929965	0.482231
123	6	9.125434	48	6.0597	1.505922	0.196471

Mean Arterial Pressure

ANOVA - Summary of all Effects; design: (new.sta)
1-SUBSTRAT, 2-ACTIVITY, 3-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	208.7148	8	816.8688	0.255506	0.626854
2	1	0.006007	8	18.40601	0.000326	0.98603
3	6	177.4764	48	159.7832	1.110733	0.370151
12	1	4.88951	8	16.83019	0.29052	0.604559
13	6	64.85127	48	177.9867	0.36436	0.897846
23	6	14.58265	48	9.674651	1.507305	0.196018
123	6	24.22392	48	11.95356	2.026504	0.08024

Mean Arterial Diameter

ANOVA - Summary of all Effects; design: (new.sta)
1-SUBSTRAT, 2-ACTIVITY, 3-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	0.000363	8	0.003419	0.106118	0.752966
2	1	0.00572	8	0.002102	2.721392	0.137621
3	6	0.001579	48	0.001355	1.165864	0.340172
12	1	0.052353	8	0.019247	2.719998	0.137708
13	6	0.000856	48	0.000711	1.204614	0.320279
23	6	0.004206	48	0.001021	4.120893	**0.002047**
123	6	0.001255	48	0.000919	1.36598	0.247498

Mean Blood Velocity

ANOVA - Summary of all Effects; design: (new.sta)
1-SUBSTRAT, 2-ACTIVITY, 3-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	5.670592	8	8.913991	0.636145	0.448135
2	1	158.3228	8	1.733077	91.35358	**1.19E-05**
3	6	54.17267	48	4.434805	12.21534	**2.72E-08**
12	1	19.82529	8	4.007356	4.947225	**0.056806**
13	6	2.430197	48	3.796401	0.640132	0.697538
23	6	31.64892	48	1.841239	17.18893	**1.74E-10**
123	6	1.537498	48	2.406882	0.638792	0.698584

Femoral Artery Blood Flow

ANOVA - Summary of all Effects; design: (new.sta)
1-SUBSTRAT, 2-ACTIVITY, 3-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	9526.876	8	18235.07	0.522448	0.490388
2	1	318125.5	8	5455.11	58.31696	**6.09E-05**
3	6	99271.67	48	8682.803	11.43314	**6.68E-08**
12	1	5146.768	8	9309.357	0.55286	0.478417
13	6	4526.743	48	6098.131	0.742316	0.618292
23	6	73517.88	48	3776.671	19.46632	**2.34E-11**
123	6	2540.271	48	4011.586	0.633234	0.702927

Appendix M: Data and ANOVA Tables – Muscle Protein Synthesis

M.1 Intracellular $^{13}C_6$-Phenylalanine Enrichments (%)

	PRO REST		PRO EX		PRO+CARB REST		PRO+CARB EX	
	60	**180**	**60**	**180**	**60**	**180**	**60**	**180**
1	0.016	0.037	0.049	0.044	0.031	0.039	0.04	0.044
2	0.040	0.039	0.046	0.038	0.038	0.040	0.061	0.051
3	0.050	0.034	0.046	0.041	0.037	0.061	0.066	NV
4	0.040	0.042	0.059	0.041	0.041	0.045	0.049	0.052
5	0.044	0.044	0.064	0.053	0.041	0.036	0.057	0.048
6	0.035	0.050	0.062	0.061	0.039	0.042	0.061	0.052
7	0.038	0.042	0.057	0.042	0.034	0.051	0.043	0.056
8	0.045	0.045	0.049	0.050	0.036	0.054	0.055	0.049
Mean	**0.039**	**0.042**	**0.054**	**0.046**	**0.037**	**0.046**	**0.054**	**0.050**
SEM	0.004	0.002	0.003	0.003	0.001	0.003	0.003	0.001
Mean	**0.045**				**0.046**			
SEM	0.002				0.002			

ANOVA - Summary of all Effects; design: (new.sta)
1-SUBSTRAT, 2-ACTIVITY, 3-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	4.93E-05	7	5.63E-05	0.876148	0.380418
2	1	0.001675	7	4E-05	41.86047	**0.000344**
3	1	2.39E-08	7	4.49E-05	0.000533	0.982225
12	1	9.75E-07	7	3.3E-05	0.02954	0.868402
13	1	0.000105	7	5.24E-05	2.000589	0.200141
23	1	0.000552	7	3.3E-06	167.5735	**3.82E-06**
123	1	1.35E-06	7	7.48E-05	0.018114	0.896727

M.2 Mixed Muscle Fractional Synthesis Rate (FSR; %•h^{-1})

	PRO		PRO+CARB	
	REST	**EX**	**REST**	**EX**
1	0.039	0.099	0.064	0.049
2	0.101	0.098	0.088	0.086
3	0.055	0.069	0.134	0.123
4	0.058	0.112	0.067	0.114
5	0.063	0.091	0.031	0.119
6	0.099	0.099	0.049	0.082
7	0.085	0.063	0.046	0.067
8	0.064	0.094	0.072	0.071
Mean	**0.071**	**0.091**	**0.069**	**0.089**
SEM	0.008	0.006	0.011	0.010

ANOVA - Summary of all Effects; design: (new.sta)
1-SUBSTRAT, 2-ACTIVITY, 3-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	2.42E-05	7	0.00089	0.027167	0.873741
2	1	0.003207	7	0.000528	6.078981	**0.043113**
12	1	1.98E-10	7	0.000491	4.03E-07	0.999511

Appendix N: Data and ANOVA Tables – Muscle Anabolic Signalling

N.1 Akt (P:Actin)

	PRO REST		PRO EX		PRO+CARB REST		PRO+CARB EX	
	60	**180**	**60**	**180**	**60**	**180**	**60**	**180**
1	7.5	3.1	26.9	5.1	45.2	4.6	21.8	7.6
2	22	5.2	29.1	11	17.4	15.7	14.7	13.1
3	48.3	9.9	19	18.8	42.1	11.5	44.8	15.1
4	25.9	15.4	31.3	7.2	52.5	12.6	38.9	22.1
5	31.3	12.4	37.1	21.2	27.8	26.7	41.1	26.6
6	29.6	20.4	50.2	12.1	34.9	15.5	38.5	30.2
7	28.6	9.2	23.5	16.3	17	8.9	50	19.3
8	38.9	10.7	29	20.2	48.9	17	64.1	20.9
9	12.2	7.5	24.7	13.7	24.9	30.4	32	27
Mean	**27.1**	**10.4**	**30.1**	**14**	**34.5**	**15.9**	**38.4**	**20.2**
SEM	4.2	1.7	3	1.9	4.5	2.7	4.9	2.4

ANOVA - Summary of all Effects; design: (new.sta)
1-SUBSTRAT, 2-ACTIVITY, 3-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	845.21	8	57.55871	14.68431	**0.005004**
2	1	243.8363	8	46.57187	5.2357	**0.051412**
3	1	5466.092	8	118.8988	45.97263	**0.000141**
12	1	3.448593	8	80.46176	0.04286	0.841161
13	1	18.4291	8	82.15154	0.224331	0.648416
23	1	1.090214	8	40.15698	0.027149	0.873213
123	1	0.025853	8	136.1299	0.00019	0.989342

N.2 ACC (P:Actin)

	PRO REST		PRO EX		PRO+CARB REST		PRO+CARB EX	
	60	**180**	**60**	**180**	**60**	**180**	**60**	**180**
1	4.7	3.4	6.4	4.1	6.6	1.9	11.4	4
2	NV	NV	NV	NV	NV	NV	NV	NV
3	7.1	5.9	10.9	2.9	3	3.4	10.5	2.2
4	NV	NV	NV	NV	NV	NV	NV	NV
5	2.6	2.9	12.2	2.2	2.7	3.2	8.8	4.5
6	8.9	4.9	13.9	12.7	3.3	9.3	9.4	5.9
7	8.3	13.1	4.5	2.9	7	11.7	16.6	9.9
8	16.1	10.3	12.7	24.8	15.9	11.3	21.5	16.3
9	15.8	14.1	18.9	12.9	11.2	32.2	30.9	21.2
Mean	**9.1**	**7.8**	**11.4**	**8.9**	**7.1**	**10.4**	**15.6**	**9.1**
SEM	1.7	1.5	1.6	2.8	1.6	3.5	2.7	2.4

ANOVA - Summary of all Effects; design: (new.sta)
1-SUBSTRAT, 2-ACTIVITY, 3-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	22.7294	6	30.69073	0.740595	0.422521
2	1	97.84673	6	9.858306	9.925309	**0.019804**
3	1	40.41911	6	8.898762	4.542105	0.077079
12	1	12.46693	6	11.96429	1.042011	0.346736
13	1	0.297336	6	17.00362	0.017487	0.89912
23	1	103.7978	6	31.74056	3.270196	0.120547
123	1	64.92031	6	15.32236	4.236967	0.085246

N.3 ERK (P:Actin)

	PRO REST		PRO EX		PRO+CARB REST		PRO+CARB EX	
	60	**180**	**60**	**180**	**60**	**180**	**60**	**180**
1	19.3	16.4	21.8	4.3	44.8	6.3	22.6	8.1
2	4.8	7.2	17.9	12.2	17	14.1	4.6	4.9
3	35.5	4.6	36.4	9.4	13.7	22.9	23.4	6.8
4	16.1	45.2	94.4	10.9	45.1	5.2	49.2	9.6
5	30.8	10.7	11.7	11.1	14.6	16.2	16.1	21.1
6	11.2	40.9	50.4	71.1	41.5	52.8	33.2	39
7	42.7	22.9	58.9	31.2	16.1	46.1	73.2	13.8
8	66.1	18.5	36.2	53.1	98.4	55.5	36.8	55
9	49.8	56.2	95.6	64.8	13.2	57.4	91.5	66.7
Mean	**36**	**28.4**	**54.8**	**35.9**	**34.7**	**36.6**	**46.2**	**30.3**
SEM	6.4	6.4	10.4	9	10.4	7.1	9.1	7.9

ANOVA - Summary of all Effects; design: (new.sta)
1-SUBSTRAT, 2-ACTIVITY, 3-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	15.82048	8	165.8418	0.095395	0.765318
2	1	486.825	8	438.3703	1.110534	0.322751
3	1	1823.763	8	419.1834	4.350751	0.070472
12	1	543.5837	8	208.2668	2.610035	0.144852
13	1	42.51153	8	125.5181	0.338688	0.576615
23	1	550.9086	8	719.8029	0.76536	0.407153
123	1	0.208071	8	362.6739	0.000574	0.981477

N.4 4EBP1 (P:Actin)

	PRO REST		PRO EX		PRO+CARB REST		PRO+CARB EX	
	60	**180**	**60**	**180**	**60**	**180**	**60**	**180**
1	23.7	13.3	40	12.8	25.8	16.4	26.6	24.5
2	25.2	14.8	21.7	14.5	22.3	26.9	11.1	24.3
3	35	15.9	22.3	20.2	21.2	18.9	16.2	15.4
4	19.7	11.6	20.5	10.4	21.7	13.4	27.5	9.2
5	14.8	23.7	17.9	31.6	33.2	48.1	32.1	30.6
6	22.2	27.4	36	18.3	27.2	24.9	31.3	19.2
7	19.2	19	12.7	15.9	13.9	15.5	23.7	18.6
8	37.3	20.7	16.4	35.4	13.9	19.4	23.9	17.6
9	15.6	12.6	30.3	10.5	23.5	27.7	26.2	25.6
Mean	**23.4**	**18.7**	**22.3**	**20.3**	**22.1**	**24**	**25.8**	**19.5**
SEM	3	1.9	2.7	3.3	2.3	3.9	1.8	2.3

ANOVA - Summary of all Effects; design: (new.sta)
1-SUBSTRAT, 2-ACTIVITY, 3-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	47.1784	8	90.77097	0.519752	0.491475
2	1	0.369196	8	22.5276	0.016389	0.901294
3	1	222.7398	8	87.72974	2.538932	0.149736
12	1	9.349319	8	28.84261	0.32415	0.584751
13	1	82.02843	8	39.2641	2.089146	0.186356
23	1	18.16277	8	44.01656	0.412635	0.538604
123	1	31.75957	8	59.0353	0.537976	0.48421

N.5 p70S6K (P:Actin)

	PRO REST		PRO EX		PRO+CARB REST		PRO+CARB EX	
	60	**180**	**60**	**180**	**60**	**180**	**60**	**180**
1	37.6	14.9	22.7	11.8	41.1	24	14.1	35.5
2	31.9	31.8	24.2	19.2	52.6	36.4	16.4	18.1
3	49.9	27.8	86.9	53.8	45.6	26.6	63	39.8
4	31.2	29.5	79.2	34.2	26	37.1	44.2	30
5	44.3	38.4	47	58.4	29.5	43.3	49.3	71.1
6	74.2	35.9	28.4	31	63.1	50.2	56.7	24.8
7	56.2	40.7	15	22.6	38.8	16.7	28.7	46
8	66.7	35.6	26.5	42	69.8	50.1	54	33
9	54.7	39.4	43.8	19.5	40.6	48.7	38.5	23.1
Mean	**53.9**	**35.3**	**46.7**	**37.3**	**44.8**	**38.9**	**47.8**	**38.2**
SEM	4.7	1.6	9.1	4.9	5.5	4.3	3.9	5.5

ANOVA - Summary of all Effects; design: (new.sta)
1-SUBSTRAT, 2-ACTIVITY, 3-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	5.281118	8	97.91994	0.053933	0.822186
2	1	229.6501	8	608.2376	0.377566	0.555977
3	1	1715.41	8	206.2066	8.318892	**0.020378**
12	1	5.882648	8	172.5463	0.034093	0.858104
13	1	187.3683	8	74.30132	2.521736	0.150949
23	1	144.2459	8	228.9622	0.629999	0.450255
123	1	22.92582	8	122.4976	0.187153	0.676722

N.6 eEF2 (P:Actin)

	PRO REST		PRO EX		PRO+CARB REST		PRO+CARB EX	
	60	**180**	**60**	**180**	**60**	**180**	**60**	**180**
1	28.2	26.5	44.7	26.8	49.3	26.2	35.6	33.3
2	33.2	27.6	33.6	32	34.8	42.5	17	32.8
3	49.7	39.6	52	48.2	49.8	46.9	31.1	37.4
4	27.2	29.9	40.3	23.3	42.1	24.9	38.4	23
5	44.7	34.3	20	34	33.1	58.3	20	40.1
6	48.6	51.1	39.8	27.6	37.3	49	47.6	40.7
7	38.7	35.7	26.2	34.9	22.3	27.3	41.7	33.4
8	54.6	46.5	40.4	64.5	51.7	46.8	54.3	51.2
9	44.6	30.5	54	31.8	40	59.8	49.3	38.1
Mean	**44**	**38.2**	**38.9**	**37.8**	**39.5**	**44.7**	**40.3**	**37.7**
SEM	3	2.7	4.1	4.7	3.3	4.6	3.9	2.8

ANOVA - Summary of all Effects; design: (new.sta)
1-SUBSTRAT, 2-ACTIVITY, 3-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	23.94817	8	31.29879	0.765147	0.407216
2	1	123.4878	8	66.85458	1.847111	0.211192
3	1	49.18238	8	116.4081	0.4225	0.533908
12	1	49.78407	8	95.69743	0.520224	0.491285
13	1	116.6707	8	82.78596	1.409305	0.269224
23	1	0.514401	8	76.34111	0.006738	0.936595
123	1	29.56579	8	71.48076	0.413619	0.538132

Appendix O: Communication of Research – Abstracts

O.1 Ontario Exercise Physiology (OEP) Conference 2009

Mixed muscle protein synthesis is not augmented by protein and carbohydrate co-ingestion at rest or following resistance exercise

Aaron W. Staples, Scott S. Sherriffs, Nicholas A. Burd, Daniel WD. West, Daniel R. Moore, Jason E. Tang, Maureen J. MacDonald, Steven K. Baker, and Stuart M. Phillips.

McMaster University

PURPOSE: To determine whether an endogenous increase in plasma insulin can augment mixed muscle protein synthesis (MPS) when a bolus dose of whey protein (25g), previously shown to be maximally effective for stimulation of MPS, is consumed at rest and following resistance exercise. **METHODS:** Nine male participants (23 ± 1.9 y, 24.2 ± 2.1 kg/m^2; mean±SD) completed two trials in a double-blind randomized cross-over design. Trials consisted of unilateral knee extension exercise and the post-exercise consumption of a drink that contained 25g of whey protein (PRO) or 25g of whey protein plus 50g of maltodextrin (CHO). **RESULTS:** The area under the glucose and insulin curves were 17.5 and 5 times greater ($P < 0.05$), respectively, for CHO than PRO. There was no difference in mixed MPS between CHO and PRO ($P = 0.99$, n=5) in the resting leg. Exercise increased mixed MPS by 36.8% and 36.2% in PRO and CHO respectively, with no significant differences between the groups ($P = 0.99$, n=5). **CONCLUSIONS:** These data suggest that carbohydrate consumption, and the consequent increase in plasma insulin, does not further augment the synthesis of muscle proteins at rest or following resistance exercise, when a maximally effective dose of protein is consumed.

Supported by NSERC.

Delivered: January 24th, 2009; Ontario Exercise Physiology (OEP) Conference 2009; Barrie, ON.

O.2 American College of Sports Medicine (ACSM) Annual Meeting 2009

Muscle protein synthesis is not augmented by protein-carbohydrate co-ingestion at rest or following resistance exercise

Aaron W. Staples, Scott S. Sherriffs, Nicholas A. Burd, Daniel WD. West, Daniel R. Moore, Jason E. Tang, Maureen J. MacDonald, Steven K. Baker, and Stuart M. Phillips

Kinesiology and Neurology, McMaster University, Hamilton ON Canada

PURPOSE: To determine whether increases in plasma insulin induced by carbohydrate ingestion would augment mixed muscle protein synthesis (MPS) when a bolus dose of whey protein, previously shown to be maximally effective for stimulation of MPS, is consumed at rest and following resistance exercise. **METHODS:** Nine male participants (23 ± 1.9 y, 24.2 ± 2.1 kg/m^2; mean±SD) completed two trials in a double-blind randomized cross-over design. Trials consisted of unilateral knee extension exercise and the post-exercise consumption of a drink that contained 25g of whey protein (PRO) or 25g of whey protein plus 50g of maltodextrin (CHO). Participants completed 4 sets of unilateral resistance exercise performed at 80% maximal effort (8-12 repetitions to failure). **RESULTS:** There was a main effect for drink composition (CHO > PRO) for plasma glucose concentration ($P < 0.05$) and plasma insulin concentration

($P < 0.05$). The area under the glucose and insulin curves were 17.5 and 5 times greater ($P < 0.05$), respectively, for CHO than PRO. There was no difference in mixed MPS between CHO and PRO ($P = 0.99$) in the resting leg. Exercise increased mixed MPS by 36.8% and 36.2% in the PRO and CHO groups respectively, with no significant differences between the groups ($P = 0.99$). **CONCLUSIONS:** These data suggest that when a maximally effective bolus dose (25g) of protein is consumed, carbohydrate consumption, and the consequent increase in plasma insulin, does not further augment the synthesis of mixed muscle proteins at rest or following resistance exercise. These findings support the conclusion that insulin does not stimulate mixed MPS beyond protein ingestion, and the accompanying hyperaminoacidemia, alone.

Supported by NSERC.

Delivered: May 29th, 2009; American College of Sports Medicine (ACSM) Annual Meeting; Seattle, WA.

O.3 International Biochemistry of Exercise Conference (IBEC) 2009

Carbohydrates and hyperinsulinemia do not augment signaling or muscle protein synthesis when a saturating dose of protein is consumed

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PURPOSE: To investigate the potential for carbohydrate to augment muscle protein synthesis (MPS) at rest and following resistance exercise (RE) when co-ingested with a saturating dose of protein. **METHODS:** Nine male participants (age=23y, BMI=24.2kg/m^2) performed two trials consisting of a bout of unilateral knee extension RE. Following RE, participants consumed a drink that contained 25g of whey protein (PRO) or 25g of whey protein plus 50g of maltodextrin (CHO). The drinks were administered in a double-blind, randomized, cross-over fashion between trials. **RESULTS:** The area under the insulin curve was 5 times greater for CHO than PRO ($P < 0.05$). Exercise increased mixed MPS ($P < 0.05$), but there was no difference between CHO and PRO in the resting (REST) or the exercised legs (EX). Phosphorylation of Akt was greater for CHO than PRO ($P < 0.05$) and was greater for EX than REST ($P = 0.05$). ACC phosphorylation was greater for EX than REST ($P < 0.05$), whereas ERK 1/2, p70S6K, 4EBP-1, and eEF2 phosphorylation did not differ in any condition. **CONCLUSIONS:** When a bolus dose of whey protein, previously shown to be saturating for stimulation of MPS following RE, is consumed at rest and following RE, the co-ingestion of carbohydrate (50g) and the subsequent increase in plasma insulin does not further stimulate mixed MPS. These data also demonstrate that insulin and exercise independently act to phosphorylate upstream proteins such as Akt and AMPK; however, there is no differential phosphorylation of the downstream proteins that regulate translation initiation and elongation. Thus, the magnitude of phosphorylation of the initiation and elongation factors reported in this study did not account for the elevated rate of MPS following RE.

Supported by NSERC

Delivered: June 1st, 2009; International Biochemistry of Exercise Conference (IBEC) 2009; Guelph, ON.