EFFECT OF WHEY AND CASEIN ON POST-EXERCISE PROTEIN SYNTHESIS

EFFECT OF WHEY AND CASEIN PROTEINS ON MUSCLE PROTEIN SYNTHESIS AFTER RESISTANCE EXERCISE

By

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ABSTRACT

Protein digestibility, a function of the source of amino acids consumed, can differentially affect postprandial protein anabolism at rest. We investigated the effect of ingesting whey and casein proteins, in isolation and in combination, after an acute bout of unilateral resistance exercise on muscle protein synthesis in eight healthy resistance trained men (24.4 ± 4.8 yr; 177.4 ± 4.2 cm; 85.5 ± 14.8 kg; means ± SD). On three occasions, participants performed a unilateral bout of resistance exercise following which they consumed a drink containing whey, whey and casein (1:1), or casein protein. Each drink provided 10 g of essential amino acids. Mixed muscle protein fractional synthetic rate (FSR) was determined by pulse-tracer injections of L-[ring-$^{2}H_5$]phenylalanine and L-[^{15}N]phenylalanine 120-180 min after protein ingestion. The pattern of amino acid appearance in the blood after consuming the protein drinks was not different. Consequently, while consumption of the protein drinks stimulated a larger increase in FSR in the exercised leg compared to the rested leg ($p < 0.05$), there were no differences between the drinks. Thus, while the source of amino acids may affect protein turnover at rest, this effect is not apparent after resistance exercise. Therefore, we conclude that the ingestion of whey and casein proteins, in isolation or combination, stimulates mixed muscle protein synthesis to similar degrees after an acute bout of resistance exercise.

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Thank you to everyone who has assisted me in completing this thesis – you know who you are.

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CHAPTER 1:
REGULATION OF SKELETAL MUSCLE PROTEIN TURNOVER BY FEEDING AND RESISTANCE EXERCISE

1.1 INTRODUCTION

Skeletal muscle is a dynamic tissue capable of adapting to changes in the functional demands placed upon it. It is well known that chronic loading, in the form of resistance exercise, can induce muscle fibre hypertrophy. Conversely, a lack of stimulation (unloading) resulting from conditions such as inactivity or immobilization inevitably leads to muscle atrophy. The accretion or reduction of muscle mass is ultimately determined by the net balance (i.e., algebraic difference) between the processes of muscle protein synthesis and muscle protein breakdown (Figure 1). Muscle proteins are synthesized from amino acids derived from either exogenous (e.g., dietary protein) or endogenous sources (e.g., reutilization of amino acids arising from protein breakdown and/or *de novo* synthesis of non-essential amino acids). Free amino acids have several fates including export into the venous circulation, re-incorporation into muscle proteins, transamination, deamination, and in certain instances oxidation.

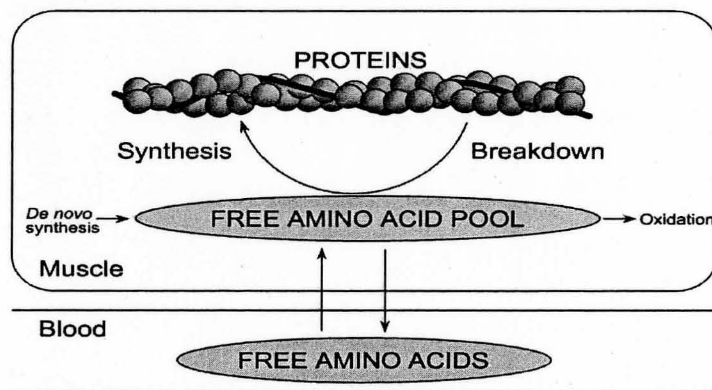

Figure 1-1. Schematic of skeletal muscle protein turnover (Rasmussen & Phillips, 2003).

Feeding of amino acids and resistance exercise have been shown to be potent independent stimulators of muscle protein synthesis that act through separate, but ultimately convergent, cellular signaling pathways (Bolster *et al.*, 2004). Moreover, combining feeding and resistance exercise has been shown to be an additive stimulus for protein anabolism (Biolo *et al.*, 1997; Tipton *et al.*, 1999). When the availability of amino acids is increased after resistance exercise, by either consumption or infusion, the result is a net positive muscle protein balance which suggests that protein is the most important macronutrient for muscle mass gain. As far as other macronutrients are concerned there is little reason to suspect any role of an acute provision of fat, as a source of energy, in modulating protein metabolism (Svanberg *et al.*, 1999). Post-exercise consumption of carbohydrates improves net muscle protein balance (likely due to increased circulating levels of insulin), however, this balance does not become positive (Borsheim *et al.*, 2004).

This review will examine the current understanding of how feeding and resistance exercise interact to regulate protein turnover and skeletal muscle mass. The focus will be on studies which have utilized a human model of resistance exercise to study protein turnover, with particular emphasis on the importance of amino acid availability in stimulating protein anabolism. Finally, the mechanisms underlying the control of protein synthesis will also be briefly discussed.

1.2 REGULATION OF PROTEIN TURNOVER

1.2.1 Effect of Amino Acids on Protein Turnover

The most important variable regulating protein turnover at rest appears to be amino acid availability, which is dependent on arterial amino acid concentration and blood flow. Net protein balance is negative at rest in the fasted-state as protein breakdown exceeds protein synthesis (Phillips *et al.*, 1997). When amino acid availability is increased through intravenous amino acid infusion, however, rates of protein synthesis increase markedly, with little change in protein breakdown (Biolo *et al.*, 1997). The result is a reversal of protein balance such that protein synthesis exceeds breakdown and net balance becomes positive.

The importance of amino acids in the regulation of muscle protein synthesis is also apparent when amino acid availability is reduced. Following a 40% hemodialysis-induced reduction in the concentration of plasma amino acids, muscle protein synthesis was measured in overnight fasted swine using phenylalanine and leucine tracers and was found to be reduced by 44% and 50%, respectively. Hence, while increased amino acid availability can stimulate protein synthesis, these data clearly demonstrate that a fall in extracellular amino acid availability can bring about a reduction in muscle protein synthesis (Kobayashi *et al.*, 2003). Interestingly, when amino acids were re-infused into the animals to restore plasma concentrations to within 94% of pre-hemodialysis levels, protein synthesis was stimulated and net leucine balance returned to basal values.

The time course of the protein synthetic response to an increase in amino acid availability has also been characterized. Changes in mixed muscle as well as myofibrillar, mitochondrial, and sacroplasmic protein synthesis were examined in response to a ~1.7 fold square-wave increase in plasma amino acid concentration for 6 h (Bohé *et al.*, 2001). The results of this study revealed a latency period of 30-60 min before rates of mixed muscle protein synthesis rapidly increased to a zenith and subsequently returned to basal between 2-4 h after the start of the infusion (Figure 2). Most striking was the fact that in the face of a persistent hyperaminoacidemia (amino acid infusion continued for an additional 4 h), rates of muscle protein synthesis remained at basal levels, suggesting that the protein synthetic response becomes refractory to a continuous supply of amino acids after 120 min of infusion.

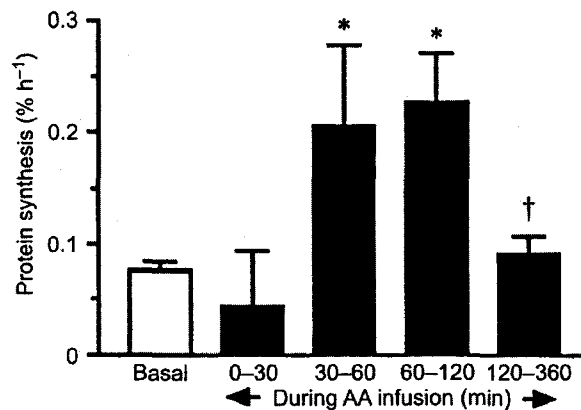

Figure 1-2. Protein synthetic response to a 6 h square-wave infusion of amino acids (Bohé *et al.*, 2001).

In summary, the primary effect of amino acids on protein turnover at rest is a marked stimulation of protein synthesis. The stimulation of protein synthesis is, however, a transient one. Nevertheless, net protein balance becomes positive and the ensuing fed-state gains in muscle protein are roughly equivalent to the preceding fasted-state losses; consequently, diurnal variation in skeletal muscle mass is negligible.

1.2.2 Effect of Resistance Exercise on Protein Turnover

One of the most conspicuous adaptations associated with resistance exercise training is an increase in muscle fibre cross-sectional area, termed hypertrophy. This anabolic response occurs when, over time, rates of protein synthesis exceed those of protein breakdown. Indeed, an acute bout of resistance exercise has been shown to increase muscle protein synthesis (Biolo *et al.*, 1995b; Chesley *et al.*, 1992; MacDougall *et al.*, 1995; Phillips *et al.*, 1997), and to a lesser degree, muscle protein breakdown (Biolo *et al.*, 1995b; Phillips *et al.*, 1997). Changes in protein synthesis on the order of ~100% have been observed in untrained individuals ~3 h after exercise (Biolo *et al.*, 1995b; Phillips *et al.*, 1997). The response in trained subjects ~4 h after exercise is somewhat lower with reported increases of ~50% (Chesley *et al.*, 1992; MacDougall *et al.*, 1995). While these results might suggest that the protein synthetic response to a bout of resistance exercise is lower in trained individuals, to truly understand the nature of the response one must examine its time course.

There have been few studies in the literature in which the acute response of muscle protein synthesis to resistance exercise has been examined over at least 24 h. One

investigation observed that protein synthesis rose rapidly after exercise, reaching a peak elevation at 24 h, which then declined to basal levels by 36 h (MacDougall *et al.*, 1995). A subsequent study reported a similar rise and fall of protein synthesis; however, protein synthesis reached its peak earlier and remained elevated for 48 h (Phillips *et al.*, 1997). The difference in the response may, as previously mentioned, be the result of the training status of the participants in these studies.

In the postabsorptive state, resistance exercise stimulates a moderate increases in muscle protein breakdown on the order of 30-50% ~3 h following exercise (Biolo *et al.*, 1995b; Phillips *et al.*, 1997). The magnitude of the resistance exercise-induced rise in protein breakdown is, however, considerably smaller than that of resistance exercise on protein synthesis. This increase in protein degradation may be reflective of protein remodeling in response to exercise induced damage to muscle fibres; however, our understanding of the regulation of protein breakdown in response to exercise is poor when compared to that of protein synthesis. Future research should focus on expanding our understanding of how resistance exercise and feeding affect protein degradation.

1.2.3 Interaction of Amino Acids and Resistance Exercise

Although resistance exercise stimulates protein synthesis to a greater degree than protein breakdown, in the absence of an increase in amino acid availability protein balance remains negative and there is a net loss of skeletal muscle protein. Amino acid infusion following resistance exercise improves net balance by stimulating a large increase in protein synthesis, while also attenuating the resistance exercise induced

increase in protein breakdown (Biolo *et al.*, 1997). Such an improvement in net balance has also been observed when amino acids are consumed orally in repeated small boluses following resistance exercise (Tipton *et al.*, 1999). In summary, when resistance exercise is accompanied by an increased availability of amino acids, the result is a synergistic stimulation of protein synthesis, greater than that achieved by either stimulus alone (Figure 3). Moreover, this increase in protein synthesis occurs concomitantly with minimal increases in protein breakdown, thus resulting in a net positive protein balance which over time will lead to muscle mass accretion.

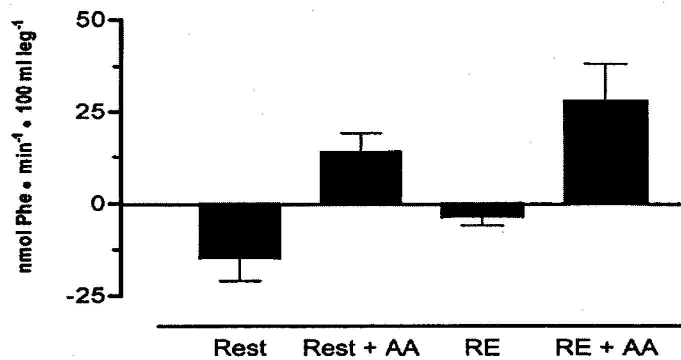

Figure 1-3. Independent and combined effects of amino acids (AA) and resistance exercise (RE) on protein synthesis (Phillips, 2004).

1.3 AMINO ACID AVAILABILITY

1.3.1 Effect of Amino Acid Type and Amount

Amino acids are classified as either essential (EAA) or non-essential (NEAA) on the basis of whether the body can synthesize them. To examine whether EAA and NEAA equally stimulate muscle protein anabolism, Tipton and co-workers (1999) provided subjects with either 40 g of EAA or 40 g of mixed EAA (21.4 g) and NEAA (18.6 g) after resistance exercise. Net muscle protein balance was measured across the exercised muscle and the resulting positive net protein balance achieved when consuming the amino acids after resistance exercise was found to be similar between both conditions. There are two possible conclusions that can be drawn from these results. First, that the additional 18.6 g of NEAA in the mixed amino acid solution were as effective as the EAA in stimulating protein synthesis. Conversely, it may be that the 21.4 g of EAA in the mixed solution stimulated protein synthesis to maximal levels such that 40 g of EAA had no additional effect. The latter conclusion is supported by a study in which the protein synthetic response to large flooding doses of amino acids was examined and protein synthesis was only found to be stimulated by the EAA (Smith *et al.*, 1998). Moreover, a study in elderly individuals reported that consuming 18 g of EAA or an equivalent amount of EAA plus an additional 22 g of NEAA improved net phenylalanine balance from the basal state because of similar increase in muscle protein synthesis (Volpi *et al.*, 2003). Furthermore, net balance resulting from the consumption of 6 g of EAA (Borsheim *et al.*, 2002) has been shown to be approximately twice as great as that

achieved following the consumption of 3 g EAA and 3 g NEAA (Miller *et al.*, 2003). Collectively, these data suggests that NEAA do not have the same stimulatory effect as EAA on protein synthesis. Instead, it appears that protein synthesis is specifically responsive to EAA, and moreover, that this may occur in a dose-dependent manner.

A dose of EAA between 10-20 g appears to stimulate maximal rates of myofibrillar and sarcoplasmic protein synthesis at rest (Cuthbertson *et al.*, 2004). Bohé and co-workers (2003) have also demonstrated that with increasing rates of amino acid infusion muscle protein synthesis increases accordingly. The most noteworthy finding of this study, however, was that muscle protein synthesis showed a curvilinear relationship with extracellular (i.e., blood) amino acid concentration, but little discernable pattern with respect to intramuscular amino acid content (Figure 4). This novel finding suggests that the response of muscle protein synthesis to amino acids is modulated at the level of extracellular, and not intracellular, changes in amino acid availability (Bohé *et al.*, 2003).

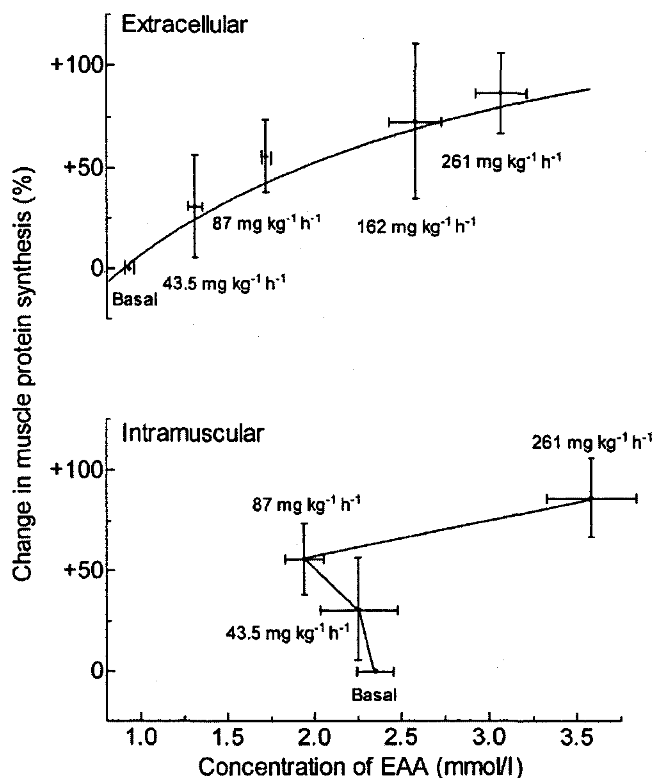

Figure 1-4. Relationship between changes in muscle protein synthesis and extracellular and intramuscular amino acid concentrations (Bohé *et al.*, 2003).

1.3.2 Effect of Amino Acid Source

Recent research has demonstrated that whole proteins (Elliot *et al.*, 2006; Tipton *et al.*, 2004) (e.g., fluid milk and isolated whey and casein proteins) as well as crystalline amino acids (Biolo *et al.*, 1997; Borsheim *et al.*, 2002; Tipton *et al.*, 1999) can promote protein accretion (i.e., a positive net balance) after resistance exercise. Additional research focusing on whole proteins has suggested that the source of amino acids, that is the type of protein and not simply its amino acid composition, can differentially modulate the protein anabolic response (Boirie *et al.*, 1997; Dangin *et al.*, 2001; Dangin *et al.*,

2002; Dangin *et al.*, 2003). It has been suggested that animal proteins, such as bovine milk, are better delivered to peripheral tissues (e.g., skeletal muscle) than plant-derived proteins such as soy (Fouillet *et al.*, 2002). Milk protein has also been shown to promote better whole-body nitrogen retention (Fouillet *et al.*, 2002). The difference in the metabolism of milk and soy proteins has been attributed to their digestion kinetics whereby soy is digested more rapidly than milk (Bos *et al.*, 2003). Milk contains two protein fractions, whey and casein, which are often referred to as "fast" and "slow" proteins respectively (Boirie *et al.*, 1997). Soy contains only one type of protein which acts in a fast manner analogous to whey. The concept of fast and slow proteins can be likened to the classification of carbohydrates using the glycemic index (Jenkins *et al.*, 2002), with proteins that result in a rapid and pronounced hyperaminoacidemia being labeled fast proteins (i.e., high glycemic index carbohydrates in terms of the blood glucose response) and those with a more gradual and lower hyperaminoacidemia being labeled slow proteins (Figure 5).

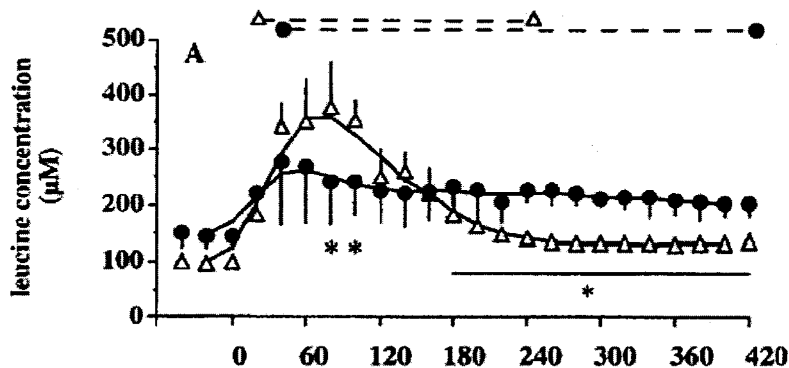

Figure 1-5. Plasma concentrations of leucine after labeled whey (open triangle) and casein (closed circle) meals (Boirie *et al.*, 1997).

Whey protein, which is quickly absorbed from the gastrointestinal tract, results in a pronounced hyperaminoacidemia that stimulates whole-body protein synthesis (Boirie *et al.*, 1997; Dangin *et al.*, 2003; Dangin *et al.*, 2001). Casein, which is more slowly absorbed, has a modest effect on protein synthesis but appears to have a prolonged inhibitory effect on whole-body protein breakdown (Boirie *et al.*, 1997; Dangin *et al.*, 2003; Dangin *et al.*, 2001). When the effects on protein synthesis and breakdown are examined at rest, casein has been shown to improve whole-body leucine balance to a greater degree than whey (Boirie *et al.*, 1997; Dangin *et al.*, 2001; Dangin *et al.*, 2003).

A recent study examined whether the acute differences in protein balance effected by whey and casein protein at rest are manifested after resistance exercise (Tipton *et al.*, 2004). Net leucine and phenylalanine balance were measured across the leg over 4 h following protein ingestion. In contrast to previous whole-body data (Boirie *et al.*, 1997; Dangin *et al.*, 2001; Dangin *et al.*, 2003), leucine balance across the exercised muscle was actually greater with the consumption of whey protein compared to casein. To explain the discordant results with respect to previous studies of whole-body protein metabolism (Boirie *et al.*, 1997; Dangin *et al.*, 2001), it was suggested that other tissues may contribute to the greater net effect (i.e., greater leucine balance seen due to a suppression of proteolysis) seen with casein consumption at the whole-body level (Tipton *et al.*, 2004). Furthermore, resistance exercise itself may have augmented the anabolic response to consuming these proteins (Tipton *et al.*, 2004). Further study is required to

determine how whey and casein may differentially promote muscle protein anabolism after resistance exercise, both acutely and after training.

The pattern of protein consumption may simulate the effects of slow and fast proteins, which are the result of the duration and magnitude of amino acid elevation in the blood associated with digestion (Dangin *et al.*, 2002). For example, the ingestion of repeated small boluses of whey, to mimic a slowly absorbed protein, may inhibit protein breakdown to a degree even greater than that elicited by a single bolus of an equivalent amount of casein (Dangin *et al.*, 2001). One study has found that consuming a protein containing drink both 1 and 2 h after exercise stimulates protein synthesis, in spite of the fact that plasma amino acid levels remained elevated above baseline at the time of the second drink (Borsheim *et al.*, 2002). It is unclear, however, to what degree such a pulse pattern of feeding would continue to stimulate protein synthesis.

1.3.3 Effect of Timing of Ingestion

In addition to the type, amount, and source of amino acids consumed, the timing of when increases in amino acid availability occur may affect protein anabolism. A study by Esmarck and colleagues (2001) showed that delaying provision of a protein, carbohydrate, and fat containing drink by 2 h post-exercise resulted in significantly lower strength gains in elderly individuals. Moreover, participants who did not receive any protein for 2 h after exercise surprisingly showed no gains in muscle cross-sectional area after 12 weeks of resistance training. These findings suggest that, in older individuals, an immediate post-exercise increase in amino acid availability is imperative for adequate

stimulation of muscle protein synthesis to support muscle fibre growth. It has previously been shown that the protein synthetic response to feeding is slower in the elderly (Welle *et al.*, 1994; Cuthbertson *et al.*, 2004). As such, the importance of timing in the elderly may be more related to the rate of amino acid absorption. Whey protein has been shown to be superior for supporting anabolism compared to the more slowly digested casein in elderly individuals (Dangin et al., 2003). Thus, the inclusion of carbohydrates and fat in the post-exercise drink by Esmarck and co-workers (2001) likely delayed absorption of the protein (Jian et al., 1986), making it act more like casein, a slow protein.

Conversely, when young men consume 6 g EAA + 35 g CHO either 1 or 3 h after a bout of resistance exercise, protein synthesis and net balance are increased, with no differences seen between the ingestion times (Rasmussen et al., 2000). These findings suggest that an immediate increase in amino acid availability after resistance exercise is not necessary to stimulate protein synthesis in young men. Instead, it appears that a period of at least 3 h exists during which a protein and carbohydrate supplement may be consumed after a bout of resistance exercise to enhance protein anabolism.

One study has reported that individuals consuming a protein and carbohydrate solution pre-exercise show greater improvements in net protein balance than those doing so post-exercise (Tipton *et al.*, 2001). The conclusion of these investigators is that pre-exercise amino acid consumption combined with an exercise-induced increase in blood flow results in greater amino acid delivery and availability to the muscle. Unfortunately, in this study the group receiving amino acids and carbohydrates before exercise showed a

significantly greater blood flow both during and after exercise. Since net balance is calculated as the product of blood flow and the arterial-venous difference in amino acid concentration, it appears that the differences observed in pre- versus post-exercise amino acid and carbohydrate consumption are solely due to the differences in measured blood flow. The results of this study must therefore be interpreted with caution as the pre-exercise feeding induced increases in blood flow currently remains unexplained and has yet to be reproduced. Indeed, a subsequent study showed no difference in muscle protein balance when whey protein was ingested immediately before or after resistance exercise . (Tipton *et al.*, 2006). While this may indicated a difference between the consumption of crystalline amino acids and whole proteins (Tipton *et al.*, 2006), rates of leg blood flow after pre-exercise protein ingestion in this study were similar to those previously reported following post-exercise amino acid consumption (Tipton *et al.*, 2001). This observation lends further credence to the suggestion that the improved net balance previously reported (Tipton *et al.*, 2001) may have been an artifact of the greater blood flow observed with pre-exercise protein consumption. Perhaps of greater relevance, it has been shown that long-term resistance training with protein supplementation prior to or following exercise in young and elderly men does not affect gains in strength, muscle thickness (a proxy marker of muscle mass), or lean tissue mass (Candow *et al.*, 2006).

1.4 THE ROLE OF INSULIN

A common misconception is that consumption of carbohydrates is required to elicit an increase in insulin secretion, when in fact amino acids can also stimulate insulin

release, albeit to a lesser extent (Bohé *et al.*, 2003). Direct examination of the effect of insulin on protein turnover is difficult because systemic increases in plasma insulin can lead to a reduction in whole-body amino acid availability which can limit protein synthesis (Biolo *et al.*, 1995a). However, some investigators have overcome this problem by infusing insulin into the femoral artery so as to promote a local hyperinsulinemia, without disturbing systemic amino acid availability.

Early studies showed that physiological increases in systemic insulin at rest decreased the rate of appearance of tracer amino acids across a limb, promoting anabolism primarily through the inhibition of protein breakdown (Gelfand & Barrett, 1987). However, rate of appearance has been shown to be a poor marker of protein breakdown (Biolo *et al.*, 1995a). More recently, increases in insulin with adequate availability of amino acids has been found to stimulate protein synthesis, while exerting no effect on protein breakdown at rest (Biolo *et al.*, 1999). In contrast, there appears to be little additional effect of insulin on protein synthesis after resistance exercise (Biolo *et al.*, 1999). This may be due to a reduced availability of intracellular amino acids since insulin attenuates the resistance exercise induced increase in protein breakdown (Biolo *et al.*, 1999). Moreover, maximal stimulation of protein synthesis in rats after resistance exercise has been shown to occur at low insulin concentrations (~8-15 μIU/mL) (Fedele *et al.*, 2000), suggesting that the role of the hormone is permissive for protein synthesis rather than modulatory (Rennie *et al.*, 2002). In other words, higher concentrations of

insulin would not further stimulate protein synthesis above rates achieved by resistance exercise or an increase in amino acid availability (Figure 6).

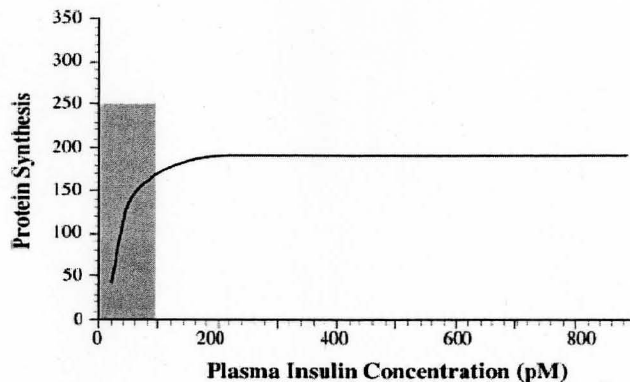

Figure 1-6. Relationship between plasma insulin concentration and protein synthesis in rat gastrocnemius muscle (Kimball *et al.*, 2002).

Although the protein synthetic response to insulin has been well characterized, a dose-response curve of how muscle protein breakdown responds to various concentrations of insulin has not been established. It is possible that the relationship is linear such that protein breakdown may be inhibited to a greater degree at higher concentrations. This is in contrast to protein synthesis which is maximally stimulated at a relatively low concentration of insulin. A greater inhibition of protein breakdown by insulin would, even in the face of unchanging rates of protein synthesis, further improve net protein balance.

1.4.1 Effect of Carbohydrates

Few studies have attempted to examine the effect of carbohydrate consumption on protein turnover after resistance exercise. This is perhaps due to the challenge of attributing any observed changes in protein metabolism to carbohydrates *per se* and not to the insulin response that accompanies carbohydrate ingestion. One investigation, examining the effect of consuming a carbohydrate (CHO) or placebo solution 1 h after a bout of single leg resistance exercise found no significant difference in muscle FSR (despite a 36% increase in the CHO condition) when comparing the single exercised leg and the non-exercised control (Roy *et al.*, 1997). This increase was not statistically significant, likely the result of a type II statistical error as the study only included eight participants. Excretion of urinary 3-methylhistidine (a marker of whole-body myofibrillar protein breakdown, 3-MH), was, however, significantly lower after CHO consumption (Roy *et al.*, 1997). Which tissue contributed to the reduction in 3-MH excretion cannot be determined at the whole-body level and it may have been that more labile proteins in gut tissues were responding to the elevation in insulin with reduced myofibrillar proteolysis, and not necessarily skeletal muscle.

A more recent study used stable isotope tracer methodology to make estimates of both protein synthesis and protein breakdown after resistance exercise and CHO consumption. Protein turnover was measured over a period of 4 h post-exercise after subjects consumed 100 g of maltodextrin or a placebo 1 h after resistance exercise (Borsheim *et al.*, 2004). Protein synthesis estimated from the rate of disappearance of the tracer as well as measured by the muscle FSR was unchanged. However, the rate of

amino acid appearance in the blood decreased following CHO but not placebo consumption. The result was an improvement in net balance which reached statistical significance 3 h after ingestion of the CHO solution; however, balance never became positive.

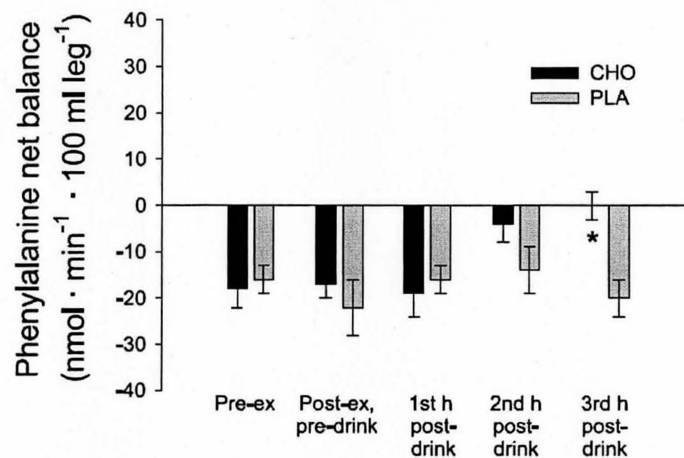

Figure 1-7. Net muscle phenylalanine balance following exercise and the consumption of a carbohydrate (CHO) or a placebo (PLA) solution (Borsheim *et al.*, 2004).

From these two studies it appears that the primary effect of CHO on protein metabolism after resistance exercise is an attenuation of protein breakdown likely mediated by an increase in circulating levels of insulin. Although not significant, the FSR data from Roy *et al.* (1997) showed a trend towards an increase in protein synthesis with CHO consumption while Borsheim *et al.* (2004) reported no effect. The differences in the reported protein synthetic response in these studies can be attributed to that fact that subjects were fed (Roy *et al.*, 1997) in one study but fasted (Borsheim *et al.*, 2004) in the other prior to the respective experiments.

There has been some suggestion that the consumption of CHO in combination with protein after resistance exercise may further enhance lean mass accretion. For example, when comparing the consumption of 6 g EAA + 35 g CHO after a bout of resistance exercise (Rasmussen *et al.*, 2000) protein synthesis and net protein balance appear to be increased to a greater degree than previously reported with amino acid consumption alone (Tipton *et al.*, 1999). This observation was confirmed in a subsequent investigation where subjects performed resistance exercise and then consumed crystalline amino acids, CHO, or a combination of the two macronutrients (Miller *et al.*, 2003). The results from this study suggest that the anabolic effect of amino acids and CHO consumed together is roughly equal to the sum of their independent effects. While the caloric content of each post-exercise drink was not controlled for, the difference in total energy was small (140 kcal) and it was concluded by the investigators that this small amount of extra energy could not adequately explain the results observed.

The combined ingestion of amino acids and CHO has been suggested to inhibit the absorption of each other (Vinardell, 1990). When we consider that protein digestibility is an independent factor regulating postprandial protein anabolism (Dangin *et al.*, 2001), it should not be surprising that the addition of CHO to a source of amino acids would differentially affect protein metabolism (Miller *et al.*, 2003; Rasmussen *et al.*, 2000; Tipton *et al.*, 1999). However, while acute differences in protein balance suggest that the combined ingestion of protein and CHO after exercise stimulates the greatest anabolic response, it is unclear whether these differences would translate into

significantly greater lean mass gains with resistance training; such long-term studies remain to be conducted.

1.5 TRANSLATIONAL CONTROL OF PROTEIN SYNTHESIS

The rapid increases in muscle protein synthesis following resistance exercise and an increase in amino acid availability suggest that these changes are mediated through post-transcriptional mechanisms (Chesley *et al.*, 1992). While a detailed examination of the control of transcription is beyond the scope of this review (see Bolster *et al.*, 2004; Kimball & Jefferson, 2006; Blomstrand *et al.*, 2006; Jefferson & Kimball, 2001; Kimball *et al.*, 2002), the key regulatory steps involved in the signaling pathways that respond to resistance exercise and feeding will be briefly discussed for completeness. Resistance exercise and feeding appear to stimulate muscle protein synthesis through separate but convergent signaling pathways, with maximal stimulation of translation initiation and protein synthesis requiring basal levels of insulin (Figure 1-8).

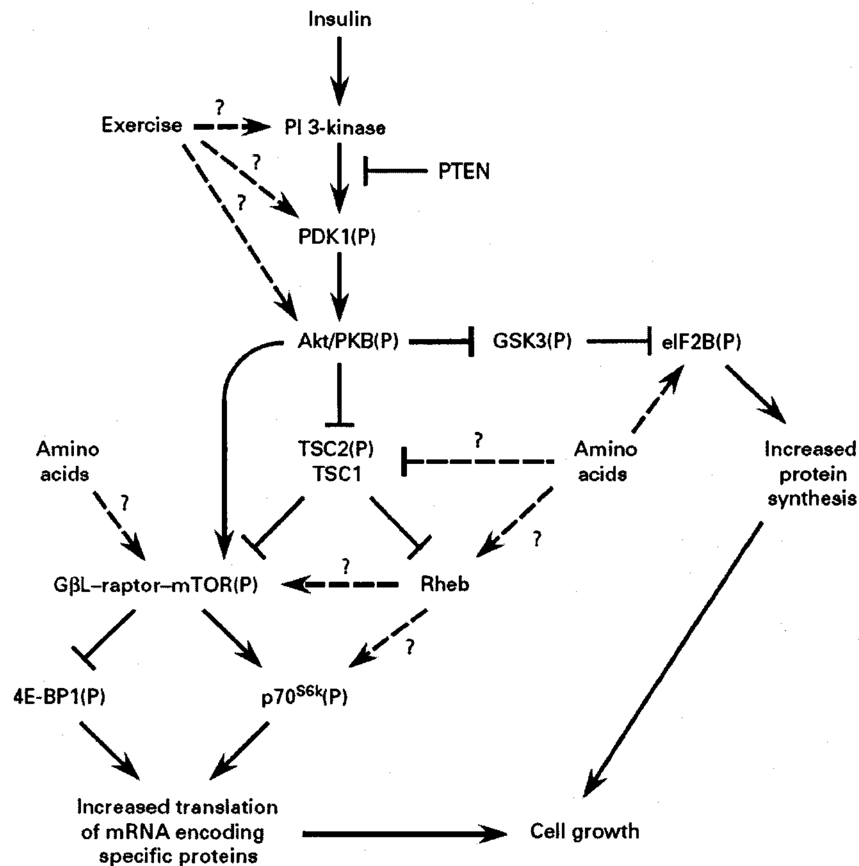

Figure 1-8. Signaling pathway for the regulation of protein synthesis by resistance exercise, amino acids, and insulin (Bolster *et al.*, 2004).

There are three distinct stages of mRNA translation: initiation, elongation, and termination. With respect to the regulation of muscle protein synthesis, peptide-chain initiation has been identified as the primary locus of control (Kimball *et al.*, 2002). Translation initiation is mediated by several regulatory proteins referred to as eukaryotic initiation factors (eIFs). The first step of translation involves the recruitment of a methionyl-tRNA (Met-tRNA) residue to the small 40S ribosomal subunit to form the 43S pre-initiation complex. The eukaryotic initiation factor 2 (eIF2) assists in the binding of

the charged Met-tRNA to the 40S subunit by forming a ternary complex with it and GTP. Once the complex has formed, the Met-tRNA residue is released following hydrolysis of the GTP molecule to GDP. This reaction occurs through eukaryotic initiation factor 2B (eIF2B) mediated guanine nucleotide exchange activity (Kimball *et al.*, 2002). When amino acid availability was reduced by hemodialysis , Kobayashi and co-workers (2003) reported decreases in eIF2B activity and marked repression of protein synthesis. It appears, therefore, that a certain level of eIF2B activity is permissive for protein synthesis when amino acid availability is adequate. Work in rodents has identified that the activity of eIF2B is also increased after resistance exercise (Farrell *et al.*, 2000) as well as in response to insulin (Farrell *et al.*, 1999).

The next step in initiation involves the formation of the eIF4F complex which binds mRNA to the pre-initiation complex (Kimball *et al.*, 2002). The formation of the eIF4F complex requires the recruitment of three initiation factors, eIF4E, eIF4A, and eIF4G, each serving a highly specific function in the process of binding target mRNA to the ribosome. Prior to export into the cytosol mRNA transcripts are protected against degradation by post-transcriptionally capping of the 5' terminal with a 7-methylguanosine residue. This 5' cap is recognized by eIF4E, while eIF4A functions to unwind the secondary structure of the 5'-untranslated region of the transcript. Finally, eIF4G acts as a scaffold protein to support the binding of the eIF4f complex to the 43S pre-initiation complex.

The formation of the eIF4F complex is believed to be the rate-limiting step in translation initiation. The regulation of this process occurs primarily thorough the association of eIF4E with the eIF4E binding protein 4E-BP1. Binding of 4E-BP1 to eIF4E prevents it from associating with eIF4G and consequently impedes formation of the eIF4F complex. Whether 4E-BP1 binds to eIF4E depends on its phosphorylation state, whereby hyperphosphorylation causes it to dissociate from eIF4E. Amino acids have been shown to cause 4E-BP1 phosphorylation through both a mammalian target of rapamycin (mTOR) dependent (Anthony *et al.*, 2000) and independent mechanism (Vary *et al.*, 1999). Stimulation of protein synthesis by amino acids through the mTOR independent pathway, however, appears to be dependent on physiological concentrations of plasma insulin (Anthony *et al.*, 2002b), suggesting some degree of integrated control between these two stimuli.

The 4E-BP1 is one of several downstream targets of the mammalian target of rapamyacin (mTOR), a protein kinase that plays a central role in regulating the translational control of muscle protein synthesis. Another target of mTOR is the 70-kd ribosomal protein S6 kinase (p70S6K) (Anthony *et al.*, 2002a). Phosphorylation of $p70^{s6k}$ has been shown to be correlated with increases in skeletal muscle mass after resistance exercise (Baar & Esser, 1999). Indeed, phosphorylation of $p70^{s6k}$ is responsible for the hyperphosphorylation and activation of ribosomal protein S6 (rpS6). Phosphorylation of rpS6 has been associated with increased translation of specific

mRNAs which code for proteins involved in mRNA translation (Kimball *et al.*, 2002), increasing the translational capacity of the cell.

1.6 SUMMARY

Resistance exercise and feeding independently stimulate muscle protein synthesis. When combined, the result is a synergistic stimulation of muscle protein anabolism with amino acid availability playing a particularly important role in this response. Amino acids stimulate protein synthesis and inhibit protein breakdown following resistance exercise, resulting in a net positive protein balance. This can be achieved with essential amino acids alone as non-essential amino acids do not appear to be required to stimulate protein synthesis.

The stimulation of protein synthesis by amino acids is transient but appears responsive to a pulse pattern of feeding, while refractory to continuous infusion. Whole proteins also stimulate protein synthesis as they provide an adequate source of essential amino acids; however, the type of protein consumed appears to differentially affect the protein turnover response. The timing of protein ingestion does not appear to be a crucial factor in young men, but may be considerably more important in older individuals in whom the protein synthetic response to feeding is diminished.

Carbohydrates primarily affect protein turnover via an insulin-mediated reduction in protein breakdown. However, increases in protein synthesis following carbohydrate consumption do occur in the presence of adequate amino acid availability. Some evidence suggests that the combination of carbohydrates and amino acids results in the

greatest increase in net balance. It is likely that this improved net balance occurs as a result of interactions between the macronutrients which modulate the rate at which the amino acids are absorbed into the bloodstream.

Finally, acute regulation of protein synthesis occurs primarily at the level of mRNA translation. Amino acids, insulin, and resistance exercise have all been shown to stimulate protein synthesis through integrated cellular signaling pathways. Amino acid availability plays a key role in activating protein kinases which in turn phosphorylate downstream regulators of the pathway that ultimately up-regulate mRNA translation and the cellular capacity for protein synthesis.

1.7 FUTURE RESEARCH DIRECTIONS

Considerable advances have been made in the past 10-15 years with respect to our understanding of how skeletal muscle mass is regulated. Amino acid availability appears to be one of the most important factors determining changes in protein turnover both at rest and after resistance exercise. Thus, a principal goal of future research must be establishing a dose response curve in order to determine the optimal amount of essential amino acids required to maximally stimulate muscle protein synthesis. A dose response curve characterizing the effect of insulin on muscle protein breakdown must also be constructed.

Research focusing on how whole proteins (e.g., whey and casein protein) stimulate muscle protein synthesis has found a role for protein digestibility in determining the anabolic response. Additional research should compare how optimal combinations of

these proteins, as well as combined ingestion of protein and other macronutrients, can maximize increases in muscle protein synthesis after exercise. Moreover, further study of how different patterns of feeding (i.e., pulse vs. bolus) affect muscle protein accretion is warranted.

Finally, little research had been conducted in humans investigating the cellular signaling pathways which stimulate muscle protein synthesis. Specifically, it remains unclear how the stimuli of resistance exercise and amino acid availability are sensed and in turn result in increased protein synthesis. Finally, while protein synthesis appears to be the more responsive variable, more research examining the regulation of protein breakdown is necessary to provide a comprehensive understanding of the regulation of protein turnover.

1.8 RATIONAL FOR RESEARCH

An increase in amino acid availability is critical for maximizing protein accretion following an acute bout of resistance exercise (Biolo *et al.*, 1997; Tipton *et al.*, 1999). While the type (Tipton *et al.*, 1999; Volpi *et al.*, 2003) and amount (Bohé *et al.*, 2003)of amino acids provided are important considerations, the source of amino acids can also affect the anabolic response (Fouillet *et al.*, 2002; Bos *et al.*, 2003). Such differences have been attributed, at least in part, to the pattern of amino acid appearance in the blood associated with the digestion of whole proteins (Bos *et al.*, 2003; Boirie *et al.*, 1997; Dangin *et al.*, 2001; Dangin *et al.*, 2002; Dangin *et al.*, 2003). Milk contains two protein fractions, whey and casein. Whey protein is soluble, quickly absorbed, and when

ingested stimulates a large but transient increase in protein synthesis (Boirie *et al.*, 1997; Dangin *et al.*, 2001; Dangin *et al.*, 2003). Consumption of casein protein, which is absorbed more slowly, inhibits protein breakdown while exerting a smaller influence on protein synthesis (Boirie *et al.*, 1997; Dangin *et al.*, 2001; Dangin *et al.*, 2003). The consumption of whey or casein protein has been shown to improve net whole-body protein balance at rest (Boirie *et al.*, 1997; Dangin *et al.*, 2001; Dangin *et al.*, 2003) and net muscle protein balance after resistance exercise (Tipton *et al.*, 2004). However, no study to date has directly examined the effect of consuming whey and casein proteins on muscle protein synthesis following an acute bout of resistance exercise. Moreover, it remains unclear how a combination of whey and casein protein may influence the anabolic response. The purpose of this study, therefore, was to characterize the response of muscle protein synthesis to consuming whey and casein protein, in isolation and in combination, after a unilateral bout of resistance exercise. Employing a unilateral model of exercise, allows us to compare the effect of consuming these proteins at rest and after resistance exercise within a given individual.

1.8.1 Hypotheses

We propose that consuming whey and casein protein after an acute bout of unilateral resistance exercise will stimulate muscle protein synthesis to a greater extent in the exercised leg versus the rested leg. Moreover, we expect that whey protein will stimulate the largest increase in muscle protein synthesis, while casein will yield the

smallest increase. Finally, we propose that the combination of whey and casein proteins will stimulate muscle protein synthesis to an intermediate degree.

CHAPTER 2:
EFFECT OF WHEY AND CASEIN PROTEINS ON MUSCLE PROTEIN SYNTHESIS AFTER RESISTANCE EXERCISE

2.1 INTRODUCTION

In the non-diseased state, skeletal muscle mass is acutely regulated by feeding-induced fluctuations in muscle protein synthesis, and to a lesser extent, muscle protein breakdown. Muscle proteins are synthesized from amino acids derived from either exogenous (e.g., dietary protein) or endogenous (e.g., protein breakdown and/or *de novo* synthesis of non-essential amino acids) sources. The products of protein breakdown (i.e., free amino acids) have several fates including export from the cell into the venous circulation, re-incorporation into muscle protein, and in certain cases, oxidation. Hence, the gain or loss of skeletal muscle mass is ultimately determined by the net algebraic difference between muscle protein synthesis and muscle protein breakdown.

Resistance exercise and an increase in the systemic concentration of circulating amino acids (e.g., via protein/amino acid ingestion/infusion) are potent independent stimulators of protein synthesis that act through separate, but convergent, cellular signaling pathways (Bolster *et al.*, 2004; Kimball *et al.*, 2002). Moreover, the synergistic effect of combining resistance exercise followed by an increase in amino acid availability has been shown to result in the greatest stimulus for muscle protein anabolism (Biolo *et al.*, 1997; Tipton *et al.*, 1999). The exact nature of how resistance exercise and feeding signal a rise in muscle protein synthesis is not currently known. Insight in this area may provide clues as to how resistance exercise and amino acid consumption can be manipulated to maximize muscle protein accretion, which may be beneficial for persons with reduced muscle mass due to disease or aging.

It has been demonstrated that whole proteins (e.g., milk, whey, and casein) (Elliot *et al.*, 2006; Tipton *et al.*, 2004) and crystalline amino acids (Biolo *et al.*, 1997; Borsheim *et al.*, 2002; Tipton *et al.*, 1999) can support protein anabolism after resistance exercise. Additional research focusing on whole proteins has suggested that the source of amino acids, that is the type of protein and not simply its amino acid composition, can differentially modulate the protein anabolic response (Boirie *et al.*, 1997; Dangin *et al.*, 2001; Dangin *et al.*, 2002; Dangin *et al.*, 2003). It has been suggested that milk promotes better whole-body nitrogen retention and is better delivered to peripheral tissues (which include skeletal muscle) than soy protein (Fouillet *et al.*, 2002; Bos *et al.*, 2003). The difference in the metabolism of milk and soy proteins has been attributed to their digestion kinetics, wherein soy is digested more rapidly than milk (Bos *et al.*, 2003). Milk contains two protein fractions, whey and casein, which have been characterized based on their rate of digestion as "fast" and "slow" proteins respectively (Boirie *et al.*, 1997). Soy, on the other hand, contains only one type of protein, which acts in a fast manner similar to whey.

The concept of fast and slow proteins is analogous to high and low glycemic index foods. Whey protein, which is absorbed quickly, results in a pronounced hyperaminoacidemia and stimulates a rapid but transient rise in whole-body protein protein synthesis and leucine oxidation (Boirie *et al.*, 1997; Dangin *et al.*, 2003; Dangin *et al.*, 2001). Casein, which is absorbed more slowly, has a modest effect on protein synthesis but a prolonged inhibitory effect on protein breakdown (Boirie *et al.*, 1997;

Dangin *et al.*, 2003; Dangin *et al.*, 2001; Dangin *et al.*, 2002). When the aggregate effect on protein turnover is examined at rest, casein improves whole-body leucine balance to a greater degree than whey (Boirie *et al.*, 1997; Dangin *et al.*, 2001; Dangin *et al.*, 2003). Thus, protein digestion rate appears to be an independent factor regulating protein anabolism (Dangin *et al.*, 2001).

One study has noted improved net muscle protein balance after resistance exercise and the ingestion of whey or casein proteins (Tipton *et al.*, 2004). However, the manner by which whey and casein proteins equally improve net muscle protein balance (i.e., changes in protein synthesis, breakdown, or both) remains unknown. At present no study has directly examined changes in muscle protein synthesis following the consumption of whey and casein proteins after resistance exercise. Moreover, it remains unclear how a combination of whey and casein protein may influence the anabolic response. The purpose of this study, therefore, was to characterize the response of muscle protein synthesis to the consumption of whey and casein proteins after resistance exercise. We employed a unilateral model of exercise, allowing us to compare the effect of consuming these proteins both at rest and after resistance exercise within a given individual. Our hypothesis was that the consumption of whey and casein proteins after resistance exercise would differentially stimulate muscle protein synthesis, with whey resulting in the largest increase, casein the smallest increase, and the combination of whey and casein yielding an intermediate degree of stimulation.

2.2 METHODS

2.2.1 Subjects

Eight healthy men who regularly engaged in whole-body resistance training (2-3 $d \cdot wk^{-1}$) volunteered to take part in the study (24.4 ± 4.8 yr; 177.4 ± 4.2 cm; 85.5 ± 14.8 kg; means ± SD). Subjects were informed of the purpose of the study, experimental procedures to be used, and potential risks. Written consent was obtained from all subjects prior to commencing the study. This study was approved by the McMaster University and Hamilton Health Sciences Research Ethics Board.

2.2.2 Experimental protocol

The protocol was designed to examine the effect of consuming whey and casein protein on mixed muscle protein fractional synthetic rate (FSR) after an acute bout of resistance exercise. At least one week before their first experimental trial, subjects participated in a familiarization session to become acquainted with the testing procedures and training equipment to be used. During the familiarization session, each subject's 10-12 repetition maximum (RM) was determined for the seated leg press and knee extension exercises (Universal Gym Equipment, West Point, MS). Subjects performed both exercises unilaterally such that the contralateral leg could serve as a non-exercised control. For the 2 d prior to each experimental trial, subjects were asked to refrain from performing any resistance exercise with their legs. In addition, subjects consumed pre-packaged diets on those 2 d designed to meet daily caloric (Harris-Benedict Equation) and protein requirements (1.2-1.4 $g \cdot kg^{-1}$).

Subjects arrived at the laboratory on the morning of each experimental trial after an overnight fast in the postabsorptive state. At this time a resting 'background' blood sample was obtained from an antecubital arm vein. Subjects then performed a bout of intense unilateral resistance exercise consisting of four sets each of leg press and knee extension exercises at a workload equivalent to previously determined 10-12 RM, with 2 min of passive rest between sets. The exercise took approximately 30 min to complete.

After the exercise bout, subjects had a 20-guage catheter inserted into a dorsal hand vein, which was kept patent with a 0.9% saline drip; a second venous blood sample was taken at this time. Subjects then consumed a 250 ml drink containing either whey protein (21.2 g), whey (10.6 g) and casein (11.0 g) protein, or casein (21.9 g) protein dissolved in water with small amounts of sucralose (Splenda®; 1 g) and vanilla extract (2 mL) to increase palatability (Table 2-1). The amount of protein in each drink was selected to provide 10 g of essential amino acids (Cuthbertson *et al.*, 2004). Whey protein hydrolysate and micellar casein was obtained from the American Casein Company (Burlington, NJ).

Table 2-1. Amino Acid Content of Protein Drinks (g).

	Whey	Whey-Casein	Casein
Alanine	1.1	0.8	0.6
Arginine	0.6	0.7	0.8
Apspartic acid	2.2	1.8	1.4
Cystine	0.4	0.2	0.1
Glutamic acid	3.7	4.1	4.5
Glycine	0.5	0.5	0.5
Histidine	0.4	0.5	0.6
Isoleucine	1.4	1.3	1.2
Leucine	2.3	2.1	1.8
Lysine	2.0	1.8	1.6
Methionine	0.5	0.5	0.6
Pheylalanine	0.7	0.8	1.0
Proline	1.4	1.8	2.2
Serine	1.1	1.2	1.2
Threonine	1.1	1.0	1.0
Tryptophan	0.3	0.3	0.2
Tyrosine	0.7	1.0	1.2
Valine	1.0	1.2	1.4

Pulse tracer injections of L-[*ring*-$^2H_5$]phenylalanine (35 $\mu mol \cdot kg^{-1}$) and L-[^{15}N]phenylalanine (35 $\mu mol \cdot kg^{-1}$) were administered through a 0.2 μm filter into the antecubital vein catheter at 120- and 150-min after consumption of the drink respectively to measure mixed muscle FSR over the final hour of each experimental trial. "Arterialized" blood samples were taken from the dorsal hand vein catheter by warming the hand with a heating blanket at 30-, 60-, 90-, 120-, 125-, 130-, 135-, 140-, 150-, 155-, 160-, 170-, and 180-min after consumption of the protein drink. The experimental

protocol is illustrated in Figure 2-1. L-[*ring*-$^2H_5$]phenylalanine (98% enriched) and L-[^{15}N]phenylalanine (98% enriched) were purchased from Cambridge Isotope Laboratories (Woburn, MA).

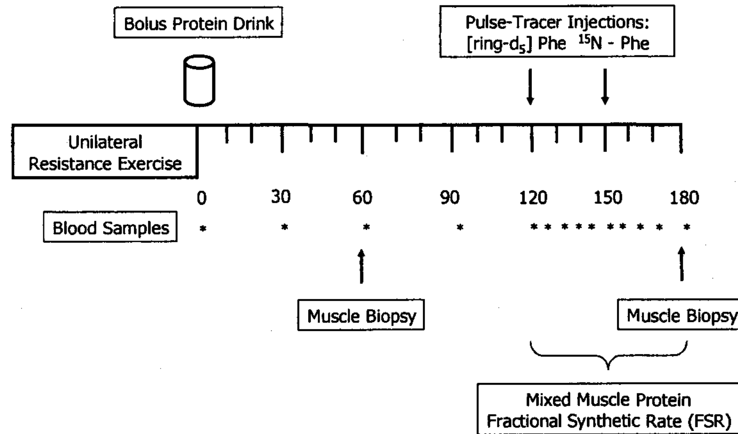

Figure 2-1. Experimental protocol.

2.2.3 Muscle needle biopsy

A percutaneous needle biopsy was taken from the *vastus lateralis* muscle of both the exercised and non-exercised legs 60- and 180-min following the consumption of the protein drink. On the first experimental trial, biopsies were only obtained at 180-min as no subject had previously been infused with any of the phenylalanine tracers being used, thus allowing the baseline enrichment of the muscle to be inferred from the arterial blood samples. Muscle tissue samples were obtained under local anaesthesia (2% lidocaine) using a 5 mm Bergström biopsy needle modified for manual suction. A new incision was made for each biopsy. Upon excision, tissue samples were immediately dissected free of

visible fat and connective tissue and snap frozen in liquid N_2. All samples were stored at -80°C until analysis.

2.2.4 Blood analyses

Blood samples were collected into evacuated containers (Vacutainer®) containing lithium heparin. Samples were extracted in percholoric acid (PCA) by adding 100 µl of whole blood to 500 µl of ice cold 0.6 M PCA, vortexing the samples, and allowing them to sit on ice for 10 min. After being centrifuged at 15 000 rpm for 2 min at 4°C 250 µl of ice cold 1.25 M potassium bicarbonate ($KHCO_3$) was added and the reaction allowed to proceeded for 10 min on ice. Following another centrifugation at 15 000 rpm for 2 min at 4°C, the supernatant was removed and stored at -20°C until analysis. An aliquot of the whole-blood PCA extract (100 µl) was dried on a Speed-Vac rotary evaporator (Thermo Savant, Holbrook, NY) for determination of blood isotope enrichments via gas-chromatography-mass spectrometry (GC-MS). The remaining whole blood was centrifuged at 4500 rpm for 10 min at 4°C to separate the plasma. Plasma was removed and stored at -20°C until further analysis. Blood glucose concentration was determined on the PCA extract via an enzymatic assay adapted for fluorometry (Passoneau & Lowry O.H., 1993), while plasma insulin concentration was determined using standard radio-immunoassay kits (Diagnostic Products, Los Angeles, CA).

Whole-blood amino acid concentrations were determined by High-Performance Liquid Chromatography (HPLC). PCA extracts were derivatized prior to injection using an AccQ-Fluor™ reagent kit (Waters, Milford, MA) by heating for 10 min at 55°C.

Samples and amino acids standards (Sigma, St. Louis, MO) were run on a 2690 HPLC separation module through a Water Nova-Pak C_{18}, 4-μm column (Waters, Milford, MA). Amino acids were detected using a scanning fluorescence detector (Waters 474, Milford, MA) at an excitation wavelength of 250 nm and an emission wavelength of 395 nm. Amino acid peak areas were integrated, compared with known standards, and analyzed using the Waters Millennium®32 software package (Milford, MA). This method achieved separation of 18 of the 20 physiological amino acids, with the exception of cysteine and tryptophan, which were not included in the analysis.

2.2.5 Muscle analyses

Frozen muscle samples (~20 mg) were weighed, allowed to thaw, and then precipitated with ice cold acetonitrile to extract the intracellular free amino acids. After acetonitrile (100 μl per mg) was added to each muscle sample, the samples were manually homogenized, vortexed for 10 min, and then centrifuged at 15 000 rpm for 2 min at 4°C. The supernatant was collected and the procedure repeated. The pooled supernatant was then dried under N_2 gas prior to analysis of the muscle intracellular free (MIF) amino acid enrichments. The remaining muscle pellets were washed twice with dH_2O, once with absolute ethanol, and then freeze dried overnight. The dried pellets were subsequently weighted and hydrolyzed with 6N HCl (400 μl per mg) for 24 h at 110°C. Prior to analysis, 300 μl of each bound protein hydrolysate was passed over a C18 reverse phase chromatography spin column (Harvard Apparatus, Holliston, MA) then dried under N_2 gas.

All amino acid enrichments were determined by making the tert-butyldimethyl silyl (tBDMS) derivative of phenylalanine (50ul MTBSTFA + 1% TBDMCS (Pierce Chemical, Rockford, IL) + 50ul anhydrous acetonitrile, heated for 15 min at 100°C). The isotopic enrichments were determined on a Hewlett-Packard 5980/5989B GC-MS; ions were selectively monitored at mass-to-charge (*m/z*) ratios of 234, 235, 239, and 240 for phenylalanine enrichment in the blood and intracellular muscle extracts, and 237, 238, 239, and 240 in the bound muscle hydrolysate. Isotopic enrichments were expressed as tracer-to-tracee ratio for the enrichment decay in the blood and MIF pool. Bound muscle protein enrichments were determined using the standard curve method (Patterson *et al.*, 1997). Enrichments were corrected for the contribution of the abundance of isotopomers of lower weight to the apparent enrichment of isotopomers with larger weight (Rosenblatt *et al.*, 1992).

2.2.6 Calculations

Mixed muscle FSR was calculated from the determination of the rate of tracer incorporation into muscle protein and using the MIF phenylalanine enrichment as a precursor, according to the equation

$$\text{FSR } (\%\cdot h^{-1}) = \frac{(Em_1 - Em_0)}{[Ef \cdot (t_1 - t_0)]} \cdot 100$$

where Em_0 is the enrichment of the protein-bound isotope tracer from the first biopsy, Em_1 is the enrichment of the protein-bound isotope tracer from the second biopsy, Ef is

the mean intracellular tracer enrichment during the time period for determination of protein incorporation, and $(t_1 - t_0)$ is the incorporation time.

2.2.7 Statistical analyses

All data were analyzed using a two-factor repeated measures analysis of variance (ANOVA). When significant differences were indicated, a Tukey post-hoc procedure was used to identify pairwise differences. All statistical analyses were performed using SigmaStat 3.10.0 (www.systat.com, Systat Software, Inc, Point Richmond, CA) and significance was accepted at $P < 0.05$. All data are presented as means ± SE.

2.3 RESULTS

2.3.1 Blood glucose

There was no change in blood glucose concentration over time during any of the experimental trials (Figure 2-2).

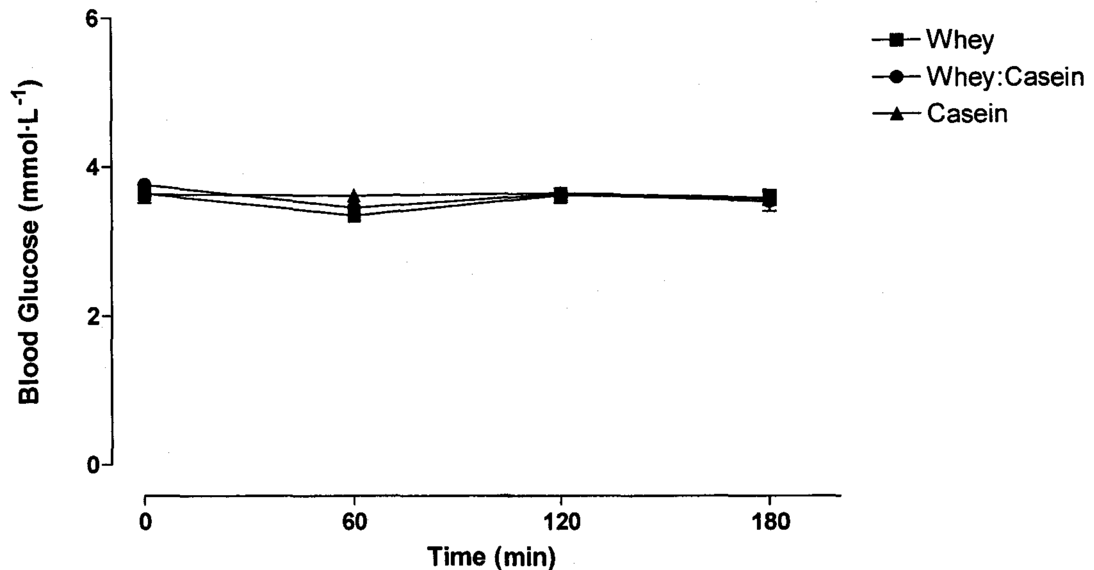

Figure 2-2. Blood glucose concentration at rest and for 3 h after ingestion of whey, whey-casein, or casein protein at rest and after resistance exercise. All values are means ± SE; N=8.

2.3.2 Plasma insulin

Due to a freezer malfunction all plasma samples thawed and remained at room temperature for at least 24 but no more than 48 h. Consequently, the insulin concentrations we report are lower than those typically observed. Ingestion of the protein drinks resulted in a transient rise in insulin concentration (Figure 2-3). Plasma insulin concentration increased 60 min, but returned to baseline by 120 min ($p < 0.05$). There were no differences in the plasma insulin response between protein conditions.

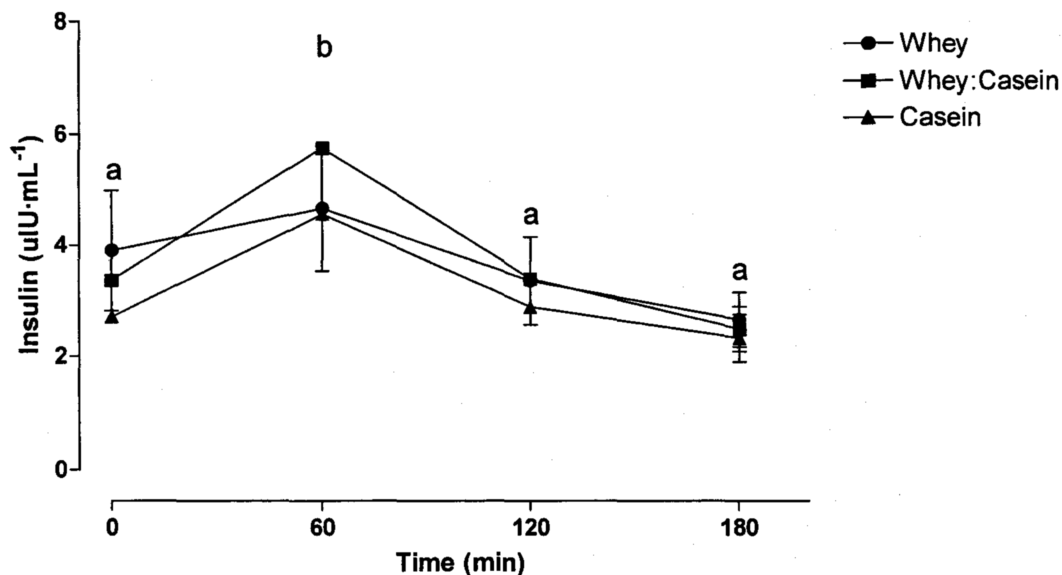

Figure 2-3. Plasma insulin concentration at rest and for 3 h after ingestion of whey, whey-casein, or casein protein at rest and after resistance exercise. Means with different letters are significantly different ($p < 0.05$). All values are means ± SE; N=8.

2.3.3 Amino acid concentrations

The time course for changes in the concentration of essential and branched-chain amino acids in the blood are summarized in Figure 2-4A and Figure 2-4B. The concentration of essential and branched-chain amino acids rose at 30 min and returned to baseline at 120 min ($p < 0.05$) but there were no differences between trials. The concentration of the amino acid leucine also increased at 30 min and returned to baseline at 120 min ($p < 0.05$), with no differences between trials (Figure 2-4C).

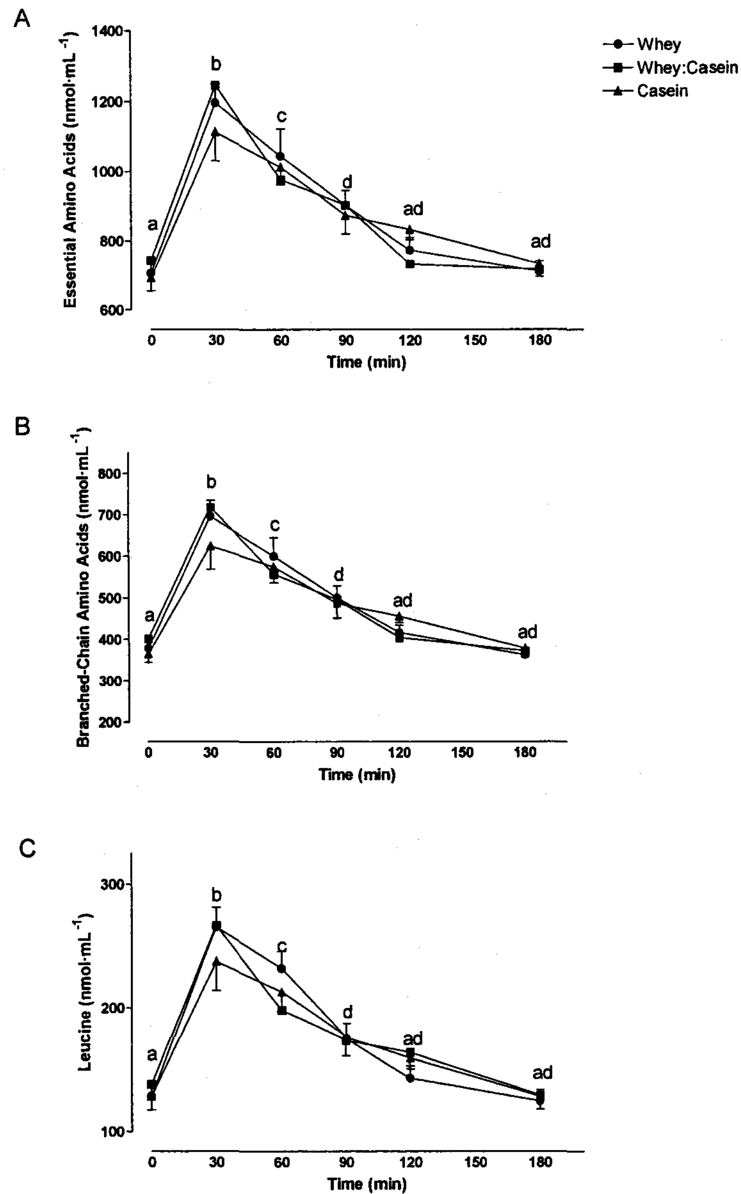

Figure 2-4. Blood concentration of A) essential amino acids, B) branched-chain amino acids, and C) leucine at rest and for 3 h after ingestion of whey, whey-casein, or casein protein at rest and after resistance exercise. Some error bars have been omitted for clarity. Means with different letters are significantly different ($p < 0.05$). All values are means ± SE; N=8

2.3.4 Mixed muscle protein synthesis

Mixed muscle protein synthesis was higher in the resistance exercised leg when compared to the rest leg ($p < 0.05$), but there was no difference between feeding conditions (Figure 2-5).

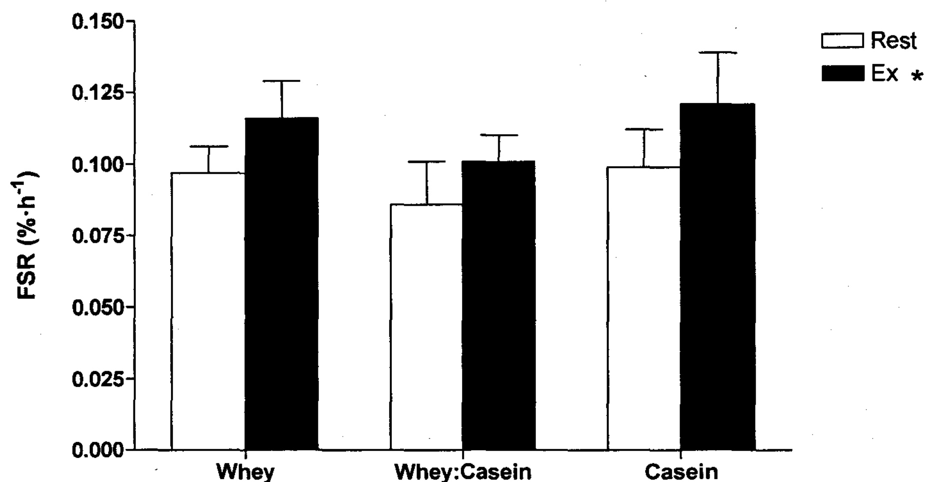

Figure 2-5. Mixed muscle protein fractional synthetic rate (FSR) after ingestion of whey, whey-casein, or casein protein at rest and after resistance exercise (Ex). * main effect for exercise vs. rest ($p < 0.05$). All values are means ± SE; N=8.

2.4 DISCUSSION

The principle finding of this study was that the consumption of whey and casein proteins, either in isolation or combination, stimulated mixed muscle protein synthesis to a greater degree after resistance exercise compared to rest. However, since no differences existed between the protein feeding conditions at rest or after resistance

exercise, these results suggest that the stimulatory effect of feeding on muscle protein synthesis is not dependent on the source of amino acids consumed.

The results of this study are contrary to our hypothesis that whey and casein proteins would stimulate muscle protein synthesis to different degrees. Based on previous literature identifying protein digestibility as an independent factor regulating whole-body protein anabolism (Dangin *et al.*, 2001), we expected that the pattern of appearance of amino acids in the systemic circulation following consumption of whey and casein would result in a differential stimulation on muscle protein synthesis. While we did observe a greater effect of feeding and resistance exercise on muscle protein synthesis than feeding at rest, there was no difference in FSR when comparing the consumption of whey, whey-casein, or casein.

One other study recently examined the effect of consuming whey and casein proteins on muscle anabolism after resistance exercise (Tipton *et al.*, 2004). Whey and casein proteins both improved net muscle protein balance to a similar extent, despite a marked difference in the digestibility of the proteins as seen by the time course of leucine appearance in the blood. Muscle protein balance has also been shown to improve following the ingestion of whey protein either before or after an acute bout of resistance exercise (Tipton *et al.*, 2006). While these studies did not specifically measure muscle protein synthesis, when taken together with the FSR results of the present study, it appears that whey and casein protein both stimulate muscle protein synthesis after resistance exercise, promoting a net positive muscle protein balance.

Previous studies have noted distinct differences in whole-body protein metabolism following the consumption of whey and casein proteins at rest. Indeed, several studies have shown that casein promotes a higher whole-body leucine balance than whey when consumed at rest (Boirie *et al.*, 1997; Dangin *et al.*, 2001; Dangin *et al.*, 2003). In the present study, muscle protein synthesis rates were similar at rest regardless of the protein consumed. The reason for the discordance between these results is likely that skeletal muscle only accounts for ~25-30% of whole-body protein turnover (Nair *et al.*, 1988). Moreover, the rate of protein turnover in skeletal muscle tissues is considerably lower than in splanchnic or hepatic tissues (Deutz *et al.*, 1999). Thus, small changes in protein turnover within non-muscle tissues are likely to manifest themselves as larger changes in whole-body protein balance. Notwithstanding, it may be that small differences in muscle protein synthesis do exist between whey and casein protein intake after resistance exercise. However, these differences may not be currently detectable after only 1 or 3 h following consumption; indeed, the greater net leucine balance after casein consumption that has been previously reported (Boirie *et al.*, 1997; Dangin *et al.*, 2001; Dangin *et al.*, 2003) was measured over a 7 h postprandial period. Moreover, any small differences that might exist could be masked by the massive anabolic stimulus of resistance exercise itself.

The blood amino acid response we observed failed to show the marked differences between the proteins that have previously been reported (Tipton *et al.*, 2004; Boirie *et al.*, 1997; Dangin *et al.*, 2001; Dangin *et al.*, 2003). However, the lack of

difference in the blood amino acid concentration in the present study may underlie the similar rates of muscle protein synthesis we observed when comparing the three protein drinks. It is not immediately clear why we failed to observe differences in the pattern by which the amino acids from each protein appeared in the blood. It is possible that consuming the drink shortly after the bout of resistance exercise may have resulted in a differential response than when the drink was consumed 60 min after exercise (Tipton *et al.*, 2004) or at rest (Boirie *et al.*, 1997; Dangin *et al.*, 2001; Dangin *et al.*, 2003). Indeed, the concentration of amino acids in the blood reached a zenith at 30 min in the present study, which is ~30 min sooner than that previously reported after resistance exercise or at rest. The peak concentration of leucine reported in the present study following whey consumption was also lower than that observed in other studies after ingestion of an equivalent amount of protein (Tipton *et al.*, 2004). However, given the different methods used to determine the amino acid concentrations, the true nature of this observation is not clear. Another possibility is a difference in post-exercise hyperemia to unilateral exercise in the present study versus those in which a bilateral exercise routine was employed (Tipton *et al.*, 2004); such differences may have altered blood flow patterns to the gut and hence amino acid digestion. There is considerable variability in the response to the consumption of whey protein before (Tipton *et al.*, 2006) and after resistance exercise (Tipton *et al.*, 2004; Tipton *et al.*, 2006). Thus, it is possible that the subjects in the present study simply did not respond to the consumption of whey protein in the same manner as participants in prior investigations. Whether training status may

have affected the rate of protein digestion is unknown. However, it is worth noting that the participants in the present study were resistance trained as compared to untrained in the study by Tipton et al. (2004).

To date, no study has examined the effect of whole protein consumption on muscle protein breakdown. It is known that an acute bout of resistance exercise increases muscle protein breakdown, albeit to a much smaller extent than synthesis (Biolo *et al.*, 1995b; Phillips *et al.*, 1997). However, feeding of amino acids has been shown to attenuate the post-resistance exercise induced increase in muscle protein breakdown (Biolo *et al.*, 1997; Tipton *et al.*, 1999). Since it has previously been shown that net muscle protein balance is similar after consumption of whey and casein protein in isolation (Tipton *et al.*, 2004), it is likely that in the present study muscle protein breakdown was inhibited to similar degrees in all trials since the FSR values did not differ between whey, whey-casein, and casein protein ingestion.

Amino acids are known to have insulinotropic properties (Floyd, Jr. *et al.*, 1966b; Floyd, Jr. *et al.*, 1966a). Recently, milk has been shown to elevate the postprandial insulin response (Ostman *et al.*, 2001; Liljeberg & Bjorck, 2001). Specifically, the whey protein fraction of milk has been identified as a greater insulin secretagogue than casein, although the exact mechanism remains unknown (Nilsson *et al.*, 2004). In the present study, ingestion of whey and casein proteins stimulated a rise in plasma insulin at 60 min post consumption. The similar insulin response to consuming the proteins may be secondary to the comparable amino acid response observed in the blood. Indeed, the rate

of postprandial amino acid appearance in the blood has been suggested as one mechanism by which whey more efficiently stimulates insulin secretion (Nilsson *et al.*, 2004). While its regulatory function has been a topic of much debate, recent evidence suggests that insulin plays a permissive, rather than modulatory, role in regulating muscle protein synthesis (Rennie *et al.*, 2002).

In summary, we report that the consumption of whey and casein protein stimulates muscle protein synthesis to a greater extent after resistance exercise when compared to rest. However, there was no difference in the anabolic nature of these proteins when consumed individually or in combination. Thus, our results suggest that the type of protein consumed after resistance exercise is not a major factor in determining post-exercise anabolism in young healthy men. It appears, therefore, that providing an increase in amino acid availability is the most important consideration to support maximal protein accretion after resistance exercise.

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APPENDIX A
SUBJECT CHARACTERISTICS

SUBJECT CHARACTERISTICS

	Age (yr)	Height (cm)	Weight (kg)	Dominant Leg	Exercised Leg
S1	35	177.5	89.2	Right	Dominant
S2	25	180.5	72.5	Left	Non-Dominant
S3	21	181	84.2	Right	Non-Dominant
S4	22	173	73	Right	Dominant
S5	27	174	79.5	Right	Non-Dominant
S6	23	184	102.5	Right	Non-Dominant
S7	21	172	71.3	Right	Dominant
S8	21	177	111.4	Right	Dominant
Mean	24.4	177.4	85.5		
SD	4.8	4.2	14.8		
SE	1.7	1.5	5.2		

APPENDIX B

BLOOD GLUCOSE RAW DATA AND ANOVA TABLES

BLOOD GLUCOSE CONCENTRATION (mmol·L^{-1})

Whey

	Pre	60	120	180
S1	3.96	3.36	3.58	3.52
S2	3.52	3.42	3.78	3.97
S3	3.64	3.48	3.58	3.55
S4	3.71	3.36	3.80	3.47
S5	3.16	3.27	3.39	3.35
S6	4.10	3.13	3.94	3.85
S7	3.16	2.98	3.06	3.14
S8	3.84	3.74	3.79	3.73
Mean	3.64	3.34	3.62	3.57
SD	0.34	0.23	0.28	0.27
SE	0.12	0.08	0.10	0.10

Whey-Casein

	Pre	60	120	180
S1	3.98	3.81	3.61	3.62
S2	3.38	3.39	3.47	3.10
S3	3.69	3.45	3.81	3.79
S4	3.44	3.31	3.51	3.43
S5	4.05	3.17	3.28	3.24
S6	4.06	3.71	4.34	4.03
S7	3.38	3.03	3.26	3.14
S8	4.09	3.71	3.83	3.95
Mean	3.76	3.45	3.64	3.54
SD	0.32	0.28	0.35	0.36
SE	0.11	0.10	0.13	0.13

Casein

	Pre	60	120	180
S1	4.06	3.90	3.55	3.27
S2	3.55	3.77	4.61	4.09
S3	3.67	4.18	3.67	3.85
S4	3.27	3.45	3.46	3.54
S5	3.65	3.03	3.33	3.39
S6	3.76	3.77	3.78	3.78
S7	3.16	3.21	3.16	3.33
S8	3.91	3.59	3.61	3.47
Mean	3.63	3.61	3.65	3.59
SD	0.30	0.37	0.44	0.29
SE	0.11	0.13	0.15	0.10

BLOOD GLUCOSE ANOVA RESULTS

Source of Variation	DF	SS	MS	F	P
subject	7	4.484	0.641		
condition	2	0.102	0.0509	0.484	0.626
condition x subject	14	1.471	0.105		
time	3	0.588	0.196	3.147	0.047
time x subject	21	1.308	0.0623		
condition x time	6	0.296	0.0493	1.280	0.287
Residual	42	1.616	0.0385		
Total	95	9.864	0.104		

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **time**

Comparison	Diff of Means	p	q	P	P<0.050
0.000 vs. 60.000	0.207	4	4.065	0.042	Yes
0.000 vs. 180.000	0.108	4	2.118	0.456	No
0.000 vs. 120.000	0.0413	4	0.810	0.939	No
120.000 vs. 60.000	0.166	4	3.255	0.130	No
120.000 vs. 180.000	0.0667	4	1.309	0.792	No
180.000 vs. 60.000	0.0992	4	1.947	0.527	No

APPENDIX C

PLASMA INSULIN RAW DATA AND ANOVA TABLES

PLASMA INSULIN CONCENTRATION ($\mu IU \cdot mL^{-1}$)

Whey

	PRE	60	120	180
S1	2.17	3.60	1.87	1.45
S2	1.14	2.45	1.50	1.50
S3	3.11	3.47	2.25	2.16
S4	2.33	2.08	2.57	1.34
S5	2.49	3.95	2.18	2.29
S6	7.74	8.13	7.18	4.34
S7	2.59	2.59	2.62	3.15
S8	9.63	11.00	6.67	5.00
Mean	3.90	4.66	3.35	2.65
SD	3.05	3.19	2.24	1.39
SE	1.08	1.13	0.79	0.49

Whey-Casein

	PRE	60	120	180
S1	3.13	4.42	2.73	1.42
S2	1.49	2.48	3.13	1.68
S3	3.22	5.75	3.23	3.14
S4	2.87	3.49	2.30	2.15
S5	1.79	2.25	2.61	2.32
S6	3.86	11.71	3.08	2.09
S7	3.37	4.78	3.26	2.08
S8	7.24	11.09	6.82	5.00
Mean	3.37	5.75	3.40	2.49
SD	1.76	3.68	1.43	1.13
SE	0.62	1.30	0.50	0.40

Casein

	PRE	60	120	180
S1	2.93	4.70	2.20	1.92
S2	1.45	2.34	1.35	0.98
S3	2.71	1.82	1.93	1.69
S4	2.23	2.92	1.75	1.60
S5	1.84	2.02	2.78	1.57
S6	5.57	3.86	4.11	2.91
S7	1.92	2.87	3.30	3.28
S8	3.04	15.86	5.66	4.65
Mean	2.71	4.55	2.88	2.32
SD	1.29	4.67	1.43	1.20
SE	0.45	1.65	0.51	0.43

PLASMA INSULIN ANOVA RESULTS

Source of Variation	DF	SS	MS	F	P
subject	7	331.747	47.392		
condition	2	7.315	3.658	2.472	0.120
condition x subject	14	20.714	1.480		
time	3	80.188	26.729	6.781	0.002
time x subject	21	82.778	3.942		
condition x time	6	7.090	1.182	0.639	0.698
Residual	42	77.633	1.848		
Total	95	607.466	6.394		

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **time**

Comparison	Diff of Means	p	q	P	P<0.050
60.000 vs. 180.000	2.497	4	6.161	0.002	Yes
60.000 vs. 120.000	1.773	4	4.375	0.026	Yes
60.000 vs. 0.000	1.657	4	4.089	0.040	Yes
0.000 vs. 180.000	0.840	4	2.072	0.475	No
0.000 vs. 120.000	0.116	4	0.286	0.997	No
120.000 vs. 180.000	0.724	4	1.786	0.596	No

APPENDIX D

BLOOD AMINO ACID RAW DATA AND ANOVA TABLES

ESSENTIAL AMINO ACID CONCENTRATION (nmol·mL^{-1})

Whey

	0	**30**	**60**	**90**	**120**	**180**
S1	787	1438	1046	846	787	824
S2	781	1177	1109	845	807	804
S3	707	1246	1113	926	886	730
S4	699	959	1297	1054	873	767
S5	655	1339	1261	1022	784	712
S6	570	1143	870	751	647	623
S7	670	1039	1063	1012	677	600
S8	422	721	534	418	387	314
Mean	661	1133	1036	859	731	672
SD	119	227	242	207	162	165
SE	42	80	86	73	57	58

Whey-Casein

	0	**30**	**60**	**90**	**120**	**180**
S1	848	1562	1223	903	837	684
S2	757	1321	1041	906	695	614
S3	799	1397	976	993	994	714
S4	754	926	1010	952	889	811
S5	834	1202	979	918	896	813
S6	670	1175	868	869	886	731
S7	633	1136	720	867	677	661
S8	659	1250	991	816	926	718
Mean	744	1246	976	903	850	718
SD	82	189	143	55	111	69
SE	29	67	51	19	39	24

Casein

	0	**30**	**60**	**90**	**120**	**180**
S1	838	1581	1175	1030	819	702
S2	757	997	996	727	880	879
S3	802	880	857	914	838	615
S4	616	869	857	857	910	829
S5	603	1144	990	811	811	743
S6	668	1049	1254	832	707	757
S7	535	1087	909	683	801	561
S8	725	1297	1060	1132	898	783
Mean	693	1113	1012	873	833	734
SD	105	235	145	149	65	106
SE	37	83	51	53	23	37

ESSENTIAL AMINO ACID CONCENTRATION ANOVA RESULTS

Source of Variation	DF	SS	MS	F	P
Subject	7	438115.630	62587.947		
Condition	2	22388.549	11194.275	0.544	0.592
Condition x Subject	14	288263.328	20590.238		
Time	5	4072733.225	814546.645	45.053	<0.001
Time x Subject	35	632789.882	18079.711		
Condition x Time	10	148625.652	14862.565	1.622	0.118
Residual	70	641495.752	9164.225		
Total	143	6244412.017	43667.217		

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Time**

Comparison	Diff of Means	p	q	P	P<0.050
30.000 vs. 0.000	470.075	6	17.127	<0.001	Yes
30.000 vs. 180.000	463.473	6	16.886	<0.001	Yes
30.000 vs. 120.000	366.133	6	13.340	<0.001	Yes
30.000 vs. 90.000	291.907	6	10.635	<0.001	Yes
30.000 vs. 60.000	158.492	6	5.775	0.003	Yes
60.000 vs. 0.000	311.583	6	11.352	<0.001	Yes
60.000 vs. 180.000	304.981	6	11.112	<0.001	Yes
60.000 vs. 120.000	207.640	6	7.565	<0.001	Yes
60.000 vs. 90.000	133.415	6	4.861	0.018	Yes
90.000 vs. 0.000	178.168	6	6.491	<0.001	Yes
90.000 vs. 180.000	171.566	6	6.251	0.001	Yes
90.000 vs. 120.000	74.226	6	2.704	0.412	No
120.000 vs. 0.000	103.943	6	3.787	0.106	No
120.000 vs. 180.000	97.340	6	3.547	0.150	No
180.000 vs. 0.000	6.602	6	0.241	1.000	No

BRANCHED-CHAIN AMINO ACID CONCENTRATION (nmol·mL^{-1})

Whey

	0	30	60	90	120	180
S1	423	874	616	478	437	433
S2	428	675	626	454	427	402
S3	378	709	613	490	470	359
S4	352	506	731	602	483	404
S5	354	798	743	557	409	362
S6	299	657	483	395	331	307
S7	367	628	638	604	382	315
S8	422	721	534	418	387	314
Mean	378	696	623	500	416	362
SD	45	111	88	80	50	48
SE	16	39	31	28	18	17

Whey-Casein

	0	30	60	90	120	180
S1	474	950	747	521	481	359
S2	434	771	584	501	378	324
S3	433	786	557	534	543	366
S4	396	506	571	529	488	428
S5	441	686	549	493	489	453
S6	359	683	475	478	490	362
S7	335	657	400	480	364	344
S8	336	709	573	421	522	344
Mean	401	718	557	495	469	372
SD	53	127	99	36	64	45
SE	19	45	35	13	23	16

Casein

	0	30	60	90	120	180
S1	452	940	720	603	474	384
S2	408	531	536	381	466	449
S3	416	464	461	516	443	308
S4	330	470	456	476	523	457
S5	306	619	536	425	424	369
S6	346	597	745	450	379	379
S7	279	629	537	389	444	282
S8	379	743	604	651	489	396
Mean	365	624	574	486	455	378
SD	59	157	109	98	44	61
SE	21	56	38	35	15	22

BRANCHED-CHAIN AMINO ACID CONCENTRATION ANOVA RESULTS

Source of Variation	DF	SS	MS	F	P
Subject	7	187257.534	26751.076		
Condition	2	11804.508	5902.254	0.649	0.537
Condition x Subject	14	127277.700	9091.264		
Time	5	1746393.948	349278.790	41.951	<0.001
Time x Subject	35	291407.929	8325.941		
Condition x Time	10	65207.076	6520.708	1.721	0.093
Residual	70	265262.988	3789.471		
Total	143	2694611.683	18843.438		

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Time**

Comparison	Diff of Means	p	q	P	P<0.050
30.000 vs. 180.000	308.634	6	16.570	<0.001	Yes
30.000 vs. 0.000	298.288	6	16.015	<0.001	Yes
30.000 vs. 120.000	232.710	6	12.494	<0.001	Yes
30.000 vs. 90.000	185.912	6	9.982	<0.001	Yes
30.000 vs. 60.000	94.655	6	5.082	0.012	Yes
60.000 vs. 180.000	213.979	6	11.488	<0.001	Yes
60.000 vs. 0.000	203.633	6	10.933	<0.001	Yes
60.000 vs. 120.000	138.055	6	7.412	<0.001	Yes
60.000 vs. 90.000	91.257	6	4.900	0.017	Yes
90.000 vs. 180.000	122.722	6	6.589	<0.001	Yes
90.000 vs. 0.000	112.375	6	6.033	0.002	Yes
90.000 vs. 120.000	46.797	6	2.513	0.493	No
120.000 vs. 180.000	75.924	6	4.076	0.068	No
120.000 vs. 0.000	65.578	6	3.521	0.155	No
0.000 vs. 180.000	10.346	6	0.555	0.999	No

LEUCINE CONCENTRATION (nmol·mL^{-1})

Whey

	0	**30**	**60**	**90**	**120**	**180**
S1	423	874	616	478	437	433
S2	428	675	626	454	427	402
S3	378	709	613	490	470	359
S4	352	506	731	602	483	404
S5	354	798	743	557	409	362
S6	299	657	483	395	331	307
S7	367	628	638	604	382	315
S8	422	721	534	418	387	314
Mean	378	696	623	500	416	362
SD	45	111	88	80	50	48
SE	16	39	31	28	18	17

Whey-Casein

	0	**30**	**60**	**90**	**120**	**180**
S1	474	950	747	521	481	359
S2	434	771	584	501	378	324
S3	433	786	557	534	543	366
S4	396	506	571	529	488	428
S5	441	686	549	493	489	453
S6	359	683	475	478	490	362
S7	335	657	400	480	364	344
S8	336	709	573	421	522	344
Mean	401	718	557	495	469	372
SD	53	127	99	36	64	45
SE	19	45	35	13	23	16

Casein

	0	**30**	**60**	**90**	**120**	**180**
S1	452	940	720	603	474	384
S2	408	531	536	381	466	449
S3	416	464	461	516	443	308
S4	330	470	456	476	523	457
S5	306	619	536	425	424	369
S6	346	597	745	450	379	379
S7	279	629	537	389	444	282
S8	379	743	604	651	489	396
Mean	365	624	574	486	455	378
SD	59	157	109	98	44	61
SE	21	56	38	35	15	22

LEUCINE CONCENTRATION ANOVA RESULTS

Source of Variation	DF	SS	MS	F	P
Subject	7	51937.294	7419.613		
Condition	2	622.092	311.046	0.220	0.805
Condition x Subject	14	19758.960	1411.354		
Time	5	304797.983	60959.597	39.776	<0.001
Time x Subject	35	53640.173	1532.576		
Condition x Time	10	11054.333	1105.433	1.636	0.114
Residual	70	47299.372	675.705		
Total	143	489110.208	3420.351		

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Time**

Comparison	Diff of Means	p	q	P	P<0.050
30.000 vs. 180.000	129.201	6	16.168	<0.001	Yes
30.000 vs. 0.000	124.950	6	15.636	<0.001	Yes
30.000 vs. 120.000	101.217	6	12.666	<0.001	Yes
30.000 vs. 90.000	81.880	6	10.246	<0.001	Yes
30.000 vs. 60.000	42.516	6	5.320	0.008	Yes
60.000 vs. 180.000	86.685	6	10.848	<0.001	Yes
60.000 vs. 0.000	82.434	6	10.316	<0.001	Yes
60.000 vs. 120.000	58.701	6	7.346	<0.001	Yes
60.000 vs. 90.000	39.364	6	4.926	0.016	Yes
90.000 vs. 180.000	47.321	6	5.922	0.002	Yes
90.000 vs. 0.000	43.070	6	5.390	0.007	Yes
90.000 vs. 120.000	19.337	6	2.420	0.534	No
120.000 vs. 180.000	27.984	6	3.502	0.159	No
120.000 vs. 0.000	23.733	6	2.970	0.311	No
0.000 vs. 180.000	4.251	6	0.532	0.999	No

APPENDIX E

MIXED MUSCLE FRACTIONAL SYNTHETIC RATE (FSR) RAW DATA AND ANOVA TABLES

MIXED MUSCLE FRACTIONAL SYNTHETIC RATE (FSR; %·h^{-1})

	Whey		Whey-Casein		Casein	
	Rest	**Ex**	**Rest**	**Ex**	**Rest**	**Ex**
S1	0.093%	0.123%	0.090%	0.107%	0.083%	0.203%
S2	0.082%	0.113%	0.097%	0.109%	0.094%	0.112%
S3	0.093%	0.148%	0.184%	0.192%	0.066%	0.108%
S4	0.095%	0.162%	0.073%	0.074%	0.113%	0.175%
S5	0.066%	0.056%	0.053%	0.122%	0.045%	0.105%
S6	0.083%	0.076%	0.061%	0.089%	0.170%	0.077%
S7	0.123%	0.107%	0.065%	0.116%	0.112%	0.142%
S8	0.087%	0.142%	0.068%	0.060%	0.105%	0.049%
Mean	0.090%	0.116%	0.086%	0.109%	0.099%	0.121%
SD	0.016%	0.036%	0.042%	0.040%	0.037%	0.050%
SE	0.006%	0.013%	0.015%	0.014%	0.013%	0.018%

MIXED MUSCLE FRACTIONAL SYNTHETIC RATE ANOVA RESULTS

Source of Variation	DF	SS	MS	F	P
subject	7	0.00000148	0.000000212		
condition	2	0.000000125	0.0000000625	0.352	0.709
condition x subject	14	0.00000249	0.000000178		
leg	1	0.000000664	0.000000664	6.361	0.040
leg x subject	7	0.000000730	0.000000104		
condition x leg	2	0.00000000266	0.00000000133	0.0122	0.988
Residual	14	0.00000152	0.000000109		
Total	47	0.00000702	0.000000149		

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **leg**

Comparison	Diff of Means	p	q	P	P<0.050
Ex vs. Rest	0.000235	2	3.567	0.040	Yes

APPENDIX F
GC-MS ANALYSIS PROTOCOL

GC-MS ANALYSIS OF STABLE PHENYLALANINE ISOTOPES

PART A: Extraction of Intracellular Free Amino Acids

Reagents:

- 100% HPLC grade acetonitrile (ACN)

Procedure:

1. Weight 20 mg of frozen wet muscle into an eppendorph tube.
2. Add 100 μL ice cold ACN to muscle sample to precipitate protein (5 μL ACN/mg muscle).
3. Manually homogenize the muscle sample in the acetonitrile using polypropylene pestle.
4. Vortex sample for 10 min.
5. Centrifuge sample for 5 min @ 15 000 g.
6. Transfer supernatant to polypropylene GC-MS sample tube.
7. Repeat steps 2-6.
8. Wash (i.e., vortex then centrifuge) remaining muscle pellet with dH_2O twice, then absolute ethanol once.
9. Dry supernatant (intracellular free amino acid extract) on heating block (100°C) under a gentle stream of nitrogen and store lyophilized until analysis.

PART B: Hydrolysis of Bound Muscle Protein

Reagents:

- 6N HCl

Procedure:

1. Freeze dry muscle pellet overnight.
2. Weight dried muscle pellet into a 4 mL screw top glass vial.
3. Hydrolyze muscle pellet in 6N HCl (400 μL per mg dry muscle) for 24 h at 110°C.

PART C: Preparation of Bound Muscle Hydrolysate for Analysis

Reagents:

- 100% ACN
- 5% ACN w/ 0.5% trifluoroacetic acid (TFA)
- 0.5% TFA in dH_2O
- 70% ACN w/ 0.5 % TFA
- 20% ACN w/ 2% TFA

Stock	ACN	TFA	dH_2O	Final Volume
5% ACN w/ 0.5% TFA	1000 µL	100 µL	18.9 mL	20 mL
0.5% TFA in dH_2O		100 µL	19.9 mL	20 mL
70% ACN w/ 0.5 % TFA	14 mL	100 µL	5.9 mL	20 mL
20% ACN w/ 2% TFA	4 mL	400 µL	15.6 mL	20 mL

Sample Preparation:

1. Add 300 µL of bound muscle hydrolysate to 100 µL 20% ACN w/ 2% TFA reagent in an eppendorph tube.

Procedure:

1. Condition C18 spin columns with 200 µL 100% ACN and spin at 150 g for 1 min.
2. Repeat step 1.
3. Equilibrate C18 spin columns with 200 µL 5% ACN w/ 0.5% TFA and spin.
4. Repeat step 3.
5. Load sample mixture onto columns and spin.
6. Wash C18 spin columns with 200 µL 0.5% TFA in dH_2O and spin.
7. Repeat step 6.
8. Elute C18 spin columns with 100 µL 70% ACN w/ 0.5% TFA and spin.
9. Repeat step 8.
10. Dry eluant on heating block (100°C) under a gentle stream of nitrogen and store lyophilized until analysis

PART D: GC-MS Analysis

Reagents:

- MTBSTFA + 1% TBDMCS (Pierce Chemical, Rockford, IL)
- Anhydrous acetonitrile

Sample Derivatization:

1. Mix 50ul MTBSTFA + 1% TBDMCS + 50ul anhydrous acetonitrile.
2. Heat for 15 min at 100°C.

GC-MS Conditions:

- 1 μL splitless injection
- Heater: 200°C
- Purge flow to split vent: 100 mL/min @ 0.5 min
- Initial flow: 1.2 mL/min
- Post flow: 2.0 mL/min

Column:

- Agilent 19091S-433: HP-5MS, 0.25mm x 30 m x 0.24 μm

Oven Settings:

	Ramp	Oven Temperature	Hold
Initial		150°C	2 min
Ramp	60°C/min	300°C	2 min
Post		300°C	2 min

MS Settings:

- Mode: Select Ion Monitoring (SIM)
- Solvent delay: 4 min
- Dwell time: 100 msec, high resolution