EFFECTS OF ACUTE CREATINE SUPPLEMENTATION ON RESTING MUSCLE PROTEIN FRACTIONAL SYNTHETIC RATE

By

Gianni Parise, B.Kin.

A Thesis Submitted to

the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (1999) (Human Biodynamics)	McMaster University Hamilton, Ontario
TITLE:	Effects of Acute Creatine Supplementation on Resting Muscle Protein Fractional Synthetic Rate
AUTHOR:	Gianni Parise, B.Kin. (McMaster University)
SUPERVISOR:	Dr. Mark Tarnopolsky, MD, PhD,FRCP(C)
SUPERVISORY COMMITTEE:	Dr. Duncan MacDougall, Ph.D. Dr. Stuart Phillips, Ph.D.
NUMBER OF PAGES:	viii, 134

ABSTRACT

Background & Rationale: During high intensity muscle contractions phosphocreatine is enzymatically degraded by creatine kinase (CK) to form creatine and the free energy which is released used to phosphorylate ADP to ATP. Creatine is then rephosphorylated during periods of relative ATP abundance (ie. rest) by CK back to phosphocreatine. Recognition of the importance of the phosphocreatine system to energy transduction has led many to believe that creatine monohydrate supplementation, which may lead to increases in phosphocreatine (PCr), may be beneficial during high intensity exercise. Several studies have demonstrated that creatine monohydrate supplementation for as few as three days can result in significant performance gains during exercise such as sprinting, or a weight lifting program. A common observation during these studies is a 1-2 kg increase in lean body mass (LBM). Although most researchers have speculated that this increase in LBM is due to water retention, some *in vitro* work has demonstrated that creatine may stimulate protein synthesis. The purpose of this investigation was to examine whether a loading dose of creatine (20g/d x 7d) would have any affect on mixed muscle protein synthesis (MPS) in resting human skeletal muscle.

Methods: A total of 22 young healthy subjects (n = 11 male, n = 11 female) were included in the study. On the day of measurement, subjects were provided with a meat free pre-packaged diet that was based upon individual diet records. Measurements of mixed muscle protein fractional synthetic rate (FSR) were completed over a 14 h resting period using a primed constant infusion of L[1-^{13}C]leucine and muscle biopsies of

quadriceps femoris at isotopic plateau. Subjects were then randomly assigned to either a creatine (20g/d x 7d) or a placebo (isoenergetic glucose polymer) group. Following 7 days of supplementation, subjects reported to the lab under the same conditions as in the pre-trial, and resting mixed muscle protein FSR was again determined.

Results: There were no significant between group differences in the baseline subject characteristics. No significant difference in FSR was observed with regards to condition (Pl: pre – 0.63 ± 0.02 %/h; pst – 0.71 ± 0.016 %/h; Cr: pre – 0.56 ± 0.02 %/h; pst – 0.58 ± 0.023 %/h) (creatine supplementation), time, or gender (Males: pre – 0.06 ± 0.02 %/h; pst – 0.068 ± 0.023 %/h; Females: pre – 0.057 ± 0.02 %/h; pst – 0.058 ± 0.015 %/h) Creatine supplementation resulted in a 13.1% increase in total creatine, however, no significant increases in PCr or free Cr were observed. Similarly, no significant increases for fat free mass (FFM), or total mass were observed.

Conclusion: It is concluded that creatine monohydrate supplementation for 7 d at 20g/d significantly increases muscle total creatine concentration, however, does not significantly affect muscle protein FSR in males or females.

Acknowledgements

This thesis would not be complete without the acknowledgment of several friends and mentors. Firstly, a special thanks to my parents, whom without their constant encouragement, and pride in my work, the completion of this thesis would not have been possible. Also a special thanks to Kristen Lougheed who made the final year of this Master's degree truly enjoyable and memorable.

I would like to express my gratitude to Dr. Mark Tarnopolsky for his tutorage over the last four years. His ability to be not only a exceptional mentor, but also a friend helped make this experience very positive. Thank you, and I look forward to continued work with you.

An additional thank you to my committee. To Dr. Duncan MacDougall for his constant advice and willingness to share his time and knowledge. To Dr. Stuart Phillips who has become a great friend and mentor. Your encouragement during this thesis, and reminding me to "keep the ball rolling" has been invaluable. Your friendship does not go without appreciation.

To Dr. Kevin Yarasheski, who helped avoid my arrest at U.S customs, and ensured completion of the data analysis for this project. His expertise on the GC-C-IRMS was priceless, and his concern with regards to my nutritional status during my stay in St. Louis was comforting!

In, addition I would like to thank my fellow grad students. Special thanks to David Ditor, who has kept me laughing over the last two years. Thanks to Kevin Gagne for all the grief he has given me since starting grad school. Special thank you to Tim Welsh who spent endless hours playing nintendo during the overnight infusions, and who consumed the better part of my time during a memorable summer.

Finally, a special thanks to my co-investigators on this project, Sasa Mihic, and Dan MacLennan. It was truly a pleasure to work with you both, and the numerous hours spent working on this project will not soon be forgotten. Thanks for the memories..

TABLE OF CONTENTS

BACKGROUND AND STATEMENT OF PURPOSE

1.1. Introduction

In recent years, creatine (Cr) has become a popular supplement among the athletic community. Many believe that an increase in muscle Cr concentration can act as an ergogenic aid, with its greatest benefits most apparent in high intensity activity. Although research has yielded conflicting results, there is a general consensus that Cr may have some benefit during repeated bouts of high intensity exercise (Juhn and Tarnopolsky, 1998). A second observation that has consistently been demonstrated following creatine supplementation is an increase in lean body mass (LBM), with studies reporting an increase of 1-2kg following one week of creatine supplementation. This increase in LBM has been speculated to be due to an increase in body water retention (Hultman *et al.* 1996), however *in vitro* work (Ingwall, 1972, 1974, 1975, 1976) has shown that when cultured muscle cells are incubated in a creatine-rich medium there is an increase in protein synthesis. The phenomenon of increased protein synthesis (Ingwall, 1976) has yet to be duplicated in a human model. Body water retention and protein synthesis may be mechanistically linked in that cellular hydration status has been shown

to effect protein synthesis (Millar et al. 1997), degradation (Mortimore et al. 1988) and nitrogen balance (Haussinger et al. 1993).

Creatine is composed of arginine and glycine and requires methionine for its synthesis. It comprises 0.3-0.5% of total muscle weight (Linder, 1991), and is one of eight naturally occurring compounds derived from guanadine. When phosphorylated, it functions in the maintenance of cellular ATP homeostasis (Ellington *et al.* 1989).

Chevreul first described Cr in 1835 after discovering that Cr existed in different meat extracts, and suggested that it may also be a substance found in humans. Twelve years later, Liebig discovered that, although Cr could be extracted from many different kinds of muscle, it could not be extracted from other organs which he investigated, suggesting that it was a compound exclusive to muscle tissue. Almost a century later, in 1926, Chanutin observed that a large portion of Cr ingested was not excreted in urine, and thus logically must be retained by the body, demonstrating that it was not just a metabolic by-product. Finally in 1929, following many years of debate on the role of Cr during muscular contraction, Schlossman and Tiegs discovered that Cr concentration increased during muscular contraction. During this same time period, Fiske and Subbarow made the discovery of phosphocreatine (PCr; Needham, 1971). These two discoveries, allowed for a connection to be made between Cr and PCr and they were quickly implicated as playing key roles in bioenergetics.

The importance of the temporal role of PCr to muscular contraction was first described in 1965 by Davies. Kammermeier first described the spatial role of PCr to muscular contraction in 1987, and stated that PCr has the ability to maintain high

intracellular ATP/ADP ratios. The role of PCr as a temporal energy buffer is well established as being important in high intensity short duration exercise.

1.2. Creatine Synthesis

Although Cr is exclusively synthesized by the liver and pancreas in humans (Walker, 1979), 85% of the body's Cr pool is located in skeletal muscle. Thus, the transport of Cr from the liver and pancreas to the muscles is essential. Walker (1979) stated that because little Cr is present in the major sites of synthesis, there must be a distinct separation of biosynthesis from utilization. Synthesis of Cr involves three amino acids and two enzymes. Arginine and glycine are bound by the rate limiting enzyme amidinotranferase , and then N-methyltransferase catalyzes the transfer of a methyl group (CH_3) from methionine (S-adenosylmethionine) to form the dipeptide, creatine (Fig. 1; Walker, 1979). It has also been shown that Cr can be obtained from a typical meat containing diet (Harris *et al.* 1996). However, oral ingestion of Cr will depress biosynthesis of the compound, and conversely when oral supplementation has ceased biosynthesis activity increases (Walker, 1979).

1.3. Creatine Transport

Creatine transport into the muscle cell has been shown to be Na^+ and Cl^- dependant (Guimbal *et al.* 1993). In a somewhat analogous process, sub-maximal exercise has been reported to increase Cr uptake due to increased Na^+/K^+ ATPase pump activity (Harris *et al.* 1992). More specifically, subjects supplementing with either 20 or

30g/day, for a period of 21 days, and performing one-legged cycle ergometry one hour per day, had a significant increase in total resting muscle Cr, as opposed to the non exercised group (Harris *et al.* 1992). Beyond the basic necessity of Na^+ for Cr transport, it has also been found that carbohydrate may augment creatine retention during Cr feedings (Green *et al.* 1996). Green and colleagues (1996) found that when carbohydrate was ingested in conjunction with Cr, whole body Cr retention appeared to be markedly increased. This increased Cr retention is likely due to an increased insulin response following carbohydrate ingestion. Work, *in vitro,* by Haugland and Chang (1975) has shown that following insulin injection into rat muscle both the rate of transport, as well as total Cr uptake, was enhanced. Work, *in vivo,* has shown similar findings with a decreased Cr uptake in irradiated rat skeletal muscle (Koszalka and Andrew, 1972).

Free Cr itself has also been implicated in modulating its own uptake in both rodent and human myocytes. Creatine transport, for example was higher (70%) in the Cr starved as compared to the fed condition (Loike *et al.* 1987). When myoblasts were exposed to a medium containing Cr, there was a down regulation of the Cr transporters, however, this was partially reversed when creatine-fed cells were incubated in a medium containing no creatine (Loike *et al.* 1987). In essence, the results reported by Loike and associates (1987) showed that creatine may induce the expression of a protein or other compound that may down-regulate the creatine transporters.

Guerrero-Ontiveros and Walliman (1998) measured the content of creatine transporters following chronic creatine supplementation in rats. Rats that were fed creatine for 3-6 months showed a down-regulation of both creatine transporter isoforms.

Furthermore, rats that were fed with the creatine analogue 3-GPA (known to decrease muscle creatine concentrations), showed that the expression of both creatine transporter isoforms remain similar or are slightly increased compared to control rats. Thus, it would appear that Cr transport and uptake into muscle is influenced by several factors including Na^+ and Cl^-, insulin, carbohydrate, free Cr, and sub-maximal exercise.

1.4. The Role of Creatine in Muscle

In normal human skeletal muscle, the concentration of total Cr ranges from 100 to 160 mmol/kg dry matter (dm) with an average of about 125 mmol/kg dm. A resting muscle contains approximately 60% of its Cr content in the form of PCr (Greenhaff *et al.* 1994). However an inverse relationship between Cr and PCr has been shown to exist, such that as PCr is utilized for ATP synthesis, free Cr concentrations increase. Conversely, as Cr is rephosphorylated a stoichiometric change occurs between Cr and PCr. Hultman and associates (1991) as well as Katz and associates (1986) have demonstrated that the availability of PCr is one of the major limitations to muscle performance during high intensity, short duration exercise. This is due to the idea that the depletion of PCr reduces the availability of free energy for the resynthesis of ATP, such that ATP cannot be restored at the rapid rate required to sustain muscular contraction. Also the subject of much debate in the recent past, is whether or not free Cr has a role in regulating mitochondrial ATP production during steady state contraction (Walliman *et al.* 1992). It has been hypothesized that Cr, phosphorylated in the mitochondria, diffuses to the myofibrils where it can be utilized for muscular contraction (Bessman, 1985). As

ADP increases, the enzyme creatine kinase is stimulated and catalyzes the resynthesis of ATP (Fig. 2). The resulting Cr, from the breakdown of PCr, is rephosphorylated by the mitochondria. In other words it appears that the PCr energy system allows for the transport of high energy phosphates from the mitochondria to sites of ATP utilization in the muscle (Fig. 3).

Human (Hultman *et al.* 1991), and animal studies (Meyer, 1986), have shown that Cr/PCr availability is necessary for muscle function during sustained short duration maximal exercise. Hultman *et al.* (1991) has suggested that during high intensity activity, a cascade of events, consisting of a depletion of PCr and a consequent depression in ATP resynthesis, may be responsible for the onset of fatigue . This relationship was established in a study in which subjects were required to maintain 30 seconds of near maximal isometric contraction (Hultman *et al.* 1991). When measuring ATP derived from phosphocreatine hydrolysis and glycolysis, it was discovered that in the final 10 seconds of maximal contraction in a human mixed-fibre skeletal muscle, only 2% and 40% respectively, of their peak rates of ATP production were physiologically attainable. The low rates of ATP production from the two energy systems coincided with a decline in force production and power output. This evidence provides a strong argument that a decline of PCr may be one of the determining factors in the onset of fatigue during high intensity activity (Hultman *et al.* 1991).

1.5. Creatine Supplementation

Early isotope dilution studies using labeled ^{15}N-Cr and creatinine excretion measurements showed that the body Cr pool was influenced by dietary intake (Crim *et al.* 1976). Crim and colleagues (1976), made three specific conclusions based on data from the administration of di-^{15}N-creatine. First, the body creatine pool can be influenced by dietary creatine; second, that the administration of precursor amino acids can increase the rate of creatine synthesis; and third, that creatinine output is a constant fraction of the body's Cr pool which can change independently of lean body mass. These results (Crim *et al.* 1976) were the first reports examining the effect of Cr supplementation, and its metabolism.

Harris and associates (1992) have reported that the retention of Cr in muscle was greatest during an initial period of supplementation (20g/d). In fact Cr uptake into muscle was greatest during the first two days of supplementation. It was also noted that the excretion of Cr in urine was 40, 61, and 68% of the administered dose (20g/d) over a three day period. Although these results (Harris *et al.* 1992) are interesting, it must be noted that these reports are based only on three subjects. In the same study Harris (1992) also noted that 20% or more of the Cr absorbed was measured as PCr, suggesting that an actual expansion of an anaerobic energy system might be possible.

Earlier reports by the group of Crim and colleagues (1976) showed similar results to those discussed above. Subjects were monitored in four different conditions, which took place over a 90 day period. While supplementing creatine at 10g/d, it was found that creatinine excretion was significantly elevated. Creatinine excretion decreased

immediately after discontinuing the Cr feeding. When subjects supplemented the amino acids - glycine, arginine and alanine small but significant increases in creatinine excretion occurred. The results of this study indicate that the body's Cr pool is not only significantly influenced by dietary Cr intake, but also by the intake of amino acid precursors to creatine.

Findings reported by Odland *et al.* (1997) are similar to those of Harris and colleagues (1992) in that muscle free Cr levels were increased by Cr supplementation, however, in contradiction with Harris, muscle PCr levels were not. Odland *et al.* (1997) suggested that PCr can only be increased by Cr supplementation if initially low PCr levels are present. Similarly Harris *et al.* (1992) suggested that those with lower base-line creatine were more likely to benefit from creatine supplementation. In the study by Harris, it should be noted that two of the subjects were vegetarians, which may have skewed the results to favor an increase in PCr. It has been shown that vegetarians, who have low initial PCr levels, tend to have lower Cr and creatinine concentrations in serum and a decreased output in urine (Delanghe *et al.* 1989).

The creatine loading phenomenon can be paralleled with other substrate loading protocols that have been proven to work. It has been well established and accepted that increased intramuscular glycogen storage can occur through high carbohydrate ingestion, and can subsequently result in improved performance during sub-maximal exercise (Bessman, 1985). Many studies have shown that the duration at which a sub-maximal workload can be sustained is highly dependent on the level of glycogen storage in the working muscles (Hultman and Sjoholm, 1983). In a similar manner to PCr depletion,

and with similar consequences, as glycogen depletes, work can only be continued at reduced intensities. Based on these findings, many endurance athletes and coaches have adopted a carbohydrate-rich diet several days prior to competition. This has been shown to significantly increase glycogen content in resting muscles (Bergstrom and Hultman, 1966). Therefore, in past years, substrate loading has been shown to act as an ergogenic aid, at least with respect to carbohydrates. These results would justify further investigation into whether increased dietary Cr intake and subsequent PCr accumulation has ergogenic effects in high intensity anaerobic activity.

Greenhaff and colleagues (1994) demonstrated that ingestion of 20g Cr/day (4 doses of 5g) for five days produced a 20% increase in muscle creatine concentration. Consequently supplementation with 20g/day for 3-5 d has become the usual dose. Supplementation with 20 or 30g per day resulted in similar significant increases in the total creatine content of the quadriceps femoris muscle illustrating that 20g/day is sufficient to achieve maximal intramuscular levels (Harris *et al.* 1992).

Although there is evidence that muscles can undergo Cr loading, it is important to note that human muscle has been shown to have an upper limit of Cr storage of about 150-160mmol/kg dry matter. Once this upper limit is achieved, it cannot be exceeded even with Cr supplementation. Therefore, supplementation among those with already high Cr levels might be redundant, whereas those with the lowest Cr levels tend to acquire the greatest gains in muscle Cr concentration from ingestion of Cr (Greenhaff *et al.* 1994; Harris *et al.* 1992).

Hultman and colleagues (1996) have demonstrated that intramuscular Cr content can be significantly increased with a 6 d loading protocol. Following ingestion of creatine (20g/d) for 6 d muscle creatine concentration increased by 20%. This elevated muscle Cr concentration was maintained when supplementation was continued for 30 days at 2g/d. When subjects discontinued creatine supplementation of 2g/d, total Cr concentration decreased back to pre-supplementation values within thirty days. During this period of no supplementation urinary creatinine was also increased. Another group of subjects was administered a Cr supplement of 3g/d for 28 d, without a loading phase (20g/d x 6 d). A similar 20% increase in muscle total creatine concentration was observed. These results suggest that it is possible to rapidly Cr load the muscle (20g/d x 6 d), however, creatine ingestion of 3g/d is, in the long term, as effective at raising muscle Cr tissue levels as the higher doses. The general consensus concerning Cr loading, is that anything more than 20g/d is excessive during a loading phase, and the increase which accompanies supplementation can be sustained with lower doses (2-3g/d) following a loading protocol (Hultman *et al.* 1996).

1.6. Creatine and Physical Performance

In the search for athletic dominance, athletes have turned to many legal and illegal methods to attain ergogenic effects. This has prompted the investigation of Cr supplementation as a possible ergogenic aid. Cr has been one of the more popular and controversial substances to enter the world of sport in recent years. Although some studies have found no effect of creatine supplementation on performance (Snow *et al.*

1998; Mujika *et al.* 1996; Burke *et al.* 1996, Redondo *et al.* 1994), there appears to be a general consensus that creatine supplementation may improve performance during high intensity exercise (Juhn and Tarnopolsky, 1998). Birch and colleagues (1994) have reported significant performance improvements following creatine supplementation during 3-30s bouts of maximal isokinetic cycling with 4 minutes of passive recovery between bouts. Following Cr supplementation (20g/d x 5 d), the Cr group demonstrated an 8% increase in peak power output (PPO) during bout 1, but no improvement during the second and third bouts. Following Cr ingestion mean power output (MPO) increased by 6% during bouts 1 and 2 but not 3, and work output was increased in bouts 1 and 2 but not 3. It has been suggested by Birch et al. (1994) that the ergogenic effect of Cr ingestion was achieved by Cr supplementation causing a higher muscle ATP turnover rate during contraction. The lack of an effect in the third bout, was postulated to be due to the fact that fatigue at that stage during the test, may have been caused by factors other than substrate availability (Hultman *et al.* 1990).

A similar study by Earnest and associates (1994) showed slightly different results. It was required that subjects Cr load for 14 days before being re-tested using bicycle Wingate tests as a performance measure. The results showed increases in work output of 13%, 18%, and 18%, respectively for the three tests. The placebo trial showed no increases. The study group also examined weightlifting capabilities after 28 days of creatine loading. The bench press performance increased 6% for 1RM. Similarly, Balsom and colleagues (1993) demonstrated that there was a significant increase in total

lifting volume in a Cr supplemented group by lifting 26% more repetitions than the placebo group.

Others have also reported significant increases in high intensity performance following acute creatine supplementation protocols using different study designs (Bosco et al. 1997; Prevost et al. 1997; Schneider et al. 1997; Grindstaff et al. 1997). For example, Bosco and colleagues (1997) reported performance improvements in running and jumping exercises, and Prevost and associates (1997) reported improvements during high intensity cycle ergometry, as did Schneider and colleagues (1997). Grindstaff and colleagues (1997) demonstrated that swim time performances were improved with short-term creatine supplementation, as did Peyrebrune and colleagues (1998), despite the findings of Mujika and colleagues (1994), who reported performance detriments during swimming following creatine supplementation.

It should be noted, however, that most studies examining a single maximum effort show no metabolic benefit of Cr supplementation (Cooke *et al.* 1995; Dawson *et al.* 1995; Odland *et al.* 1994; Snow *et al.* 1998). In contrast, most studies examining repeated bouts do show some metabolic benefit (Birch *et al.* 1994; Earnest *et al.* 1995; Kreider *et al.* 1998; Prevost *et al.* 1997).

Although several acute Cr ingestion studies examined performance, only two studies, to my knowledge have examined the effect of exercise training with Cr supplementation. Vandenberghe and colleagues (1997) provided evidence that weight training with creatine can result in strength gains above that of weight training without creatine. A design utilizing 10 weeks of resistance training, 3 d/week, with a loading

dose of creatine (20g/d x 4), followed by a maintenance dose (5 g/d) was utilized for the study. After 4 days of loading, PCr concentration increased by 6%, and was maintained over the ten-week study. Maximal strength increased 25%, maximal intermittent exercise capacity increased 17%, and lean body mass increased 60% more during creatine supplementation. Interestingly, during 10 weeks of detraining while continuing low dose supplementation of Cr (5 g/d) all of the variables remained elevated in the Cr group. Following cessation of the Cr, muscle PCr concentration returned to normal resting values within four weeks. These results suggest that long term creatine supplementation can enhance muscle strength, and thus may play a role in increasing lean body mass indirectly by increasing the training volume that one is able to perform.

Similarly Kreider and colleagues (1998), have demonstrated that creatine supplementation can promote greater gains of fat free mass, lifting volume, and improved sprint performance during a weight training regimen. Twenty-five varsity athletes supplemented creatine for 28 d during a resistance/agility-training regimen. Total body weight increased significantly in the creatine group while total body water, as assessed by BIA (biolelectrical impedence) did not change. Also, fat free mass as determined by DEXA significantly increased in the creatine group. Furthermore, significant gains were observed in bench press lifting volume, and the sum of bench press, squat, and power clean lifting volume, as well as the total work completed in the first five of twelve 6 s sprints on a cycle ergometer. In sum these results suggest that creatine supplementation may have a significant effect on body composition and performance.

The studies of Vandenbergh and colleagues (1997), and Kreider and colleagues (1998), suggest that over the course of training, those supplementing Cr gained more muscle mass than the placebo groups. This increase in muscle mass may be explained by a direct effect of creatine upon muscle protein fractional synthetic rate (FSR) and/or an increase in the number of contractions performed over the course of training which may lead to an increase in muscle protein FSR, since exercise has been shown to stimulate FSR (Chesley et al. 1992; Yarasheski et al. 1992; Phillips *et al.* 1997).

1.7. Creatine Supplementation and Body Composition

The majority of studies involving Cr supplementation report significant increases in body mass following Cr ingestion (Balsom *et al.* 1994, Greenhaf *et al.* 1994, Stroud *et al.* 1994). It has been suggested by Ingwall (1976), and Bessman and Savabi (1988) that Cr increases the rate of muscle protein synthesis. Therefore, it has been speculated that the increase in body weight might (especially in the long-term) be partially explained by increased rates of protein synthesis, and thus muscle protein accretion. Ingwall (1976) measured the effect of increased creatine content on the rates of both muscle specific and total protein synthesis in both skeletal muscle cells, and fetal mouse heart cells (Ingwall, 1976). The results demonstrated that creatine led to higher rates of both myosin heavy chain and actin protein synthesis. Interestingly, the rate of total protein synthesis was not increased (Ingwall, 1976). The proposed mechanism for these increased rates of myofibrillar protein synthesis was based on the fact that creatine is an end-product of muscular contraction, and thus may be the chemical signal coupling increased muscular

activity and increased muscular mass (Ingwall, 1972). The rationale for this hypothesis is that approximately 60% of all creatine is in the form of phosphocreatine, a high energy phosphate. With exercise, a stoichiometric change has been observed such that the majority of PCr is hydrolyzed resulting in increased levels of free Cr (Edwards *et al.* 1972). This theory would implicate Cr as a transcription regulatory factor. Previous work by Ingwall (1972, 1974, 1975, 1976) indicated that creatine stimulated incorporation of radio-labeled leucine into myosin heavy chain proteins. The findings of Ingwall (1972, 1974, 1975, 1976) do not conclusively prove that Cr is a transcription factor as it may act on a number of loci, such as improving translational events, or increasing transcritptional efficiency. Also baseline endogenous Cr concentration in the cultured muscle cells are extremely low, and supplementing there cells with creatine will have a significant effect. Conversely, the increase in creatine in supplemented humans appears minimal, and thus any effect might be more difficult to establish. Thus, if Cr acts to increase protein synthesis, the site of action is yet to be determined.

Young and Denome (1984), conducted a similar *in vitro* study which examined the effect of creatine on the contents of myosin heavy chain and myosin heavy chain mRNA in steady state chicken muscle cell cultures. The incorporation of [^{3}H]leucine into myosin heavy chain was found to have been stimulated 30-40% by optimum creatine concentrations (0.2 mM). Despite the increase in incorporation of [^{3}H]leucine, only a 15% increase in myosin heavy chain mRNA occurred. This may suggest that creatine can induce both a transcriptional and translational response in embryonic muscle tissue. Furthermore, there appeared to be no stimulation of radio-labelled leucine into rapidly

differentiating muscle cells, suggesting that creatine can enhance the quantity of myosin heavy chain in steady state embryonic muscle cell culture, but probably does not mediate regulation of myosin content in adult skeletal muscle.

Although, most *in vitro* work concerning creatine has been positive, work by Fry and Morales (1980), showed that creatine had no effect on cultured cells. When the extra-cellular creatine concentration was manipulated over a 5700-fold range, intracellular creatine only changed by a factor of 20, indicating that cells can regulate their intracellular creatine concentrations by up or down-regulating transport. This may have implications for those supplementing creatine as down regulation of the creatine transporter would not be beneficial for attaining maximum intracellular concentrations of creatine. Unlike the work of Ingwall (1974), and Young and colleagues (1984), Fry and Morales (1984) demonstrated that neither total protein synthesis, nor myosin heavy chain protein synthesis rates were stimulated in cells bathed in a creatine rich medium.

A more recent study (Oopik *et al.* 1998), reported that Cr may play an integral role in maintaining body mass. Well trained subjects were studied before and after a 3-4% loss in body mass. The results of the study demonstrated that creatine supplementation in comparison with placebo during body mass reduction may help to maintain muscle peak torque. Since decreasing muscle mass has been correlated with decreasing rates of muscle protein synthesis (Ferrando *et al.* 1997), then the maintenance of muscle force, following weight loss, while supplementing creatine may be attributable to the maintenance of muscle mass, or improved muscular energetics. A study by Sipila and colleagues (1981) showed an increase in the diameter of type II muscle fibers

following long term Cr supplementation in patients with gyrate atrophy. Patients were administered a total of 550g of Cr over a 1 year period (a rate far less than 20g/day). In addition Mainwood and colleagues (1985), reported that an increase in body weight may in fact be a result of an elevated rate of protein synthesis. While looking at post-tetanic responses in creatine depleted rat muscle, it was shown that when an analog of creatine was used (ß-guandinopropionate), the muscle total creatine was depleted (20% of resting level), as was the weight of the muscle (ß-GPA - 141.5g, Normal - 150.4g) suggesting that perhaps creatine plays a role in regulating muscle protein synthesis and degradation rates.

In contrast Hultman *et al.* (1996) have shown that urinary volume following creatine supplementation declined 0.6L, suggesting that the rapid increase in body mass may have been attributable to body water retention.

Studies consistently report increases in body weight following creatine supplementation (Sipila *et al.* 1981; Harris *et al.* 1992; Hultman *et al.* 1996; Earnest *et al.* 1994; Birch *et al.* 1994, Redondo *et al.* 1994; Burke *et al.* 1995, Balsom *et al.* 1993). This increase in weight has been demonstrated both acutely (Harris et al. 1992), and following long term creatine supplementation (Vandenberghe *et al.* 1997; Kreider *et al.* 1998). Acutely, the increase in body weight is likely due to water retention as suggested by Harris and colleagues (1992), however, studies investigating chronic creatine supplementation with resistance training may suggest other stimuli for the increase in weight. An increase in muscle cell water over a long period of time may improve nitrogen balance (Haussinger *et al.* 1993), or even increase protein synthetic rate (Millar

et al. 1997). Another possible explanation for the increase in body weight may be an increased muscle protein synthetic rate in response to lifting a greater number of repetitions during weight training (Phillips *et al.* 1997; Roy et al. 1997) over a given period of time. Furthermore, creatine may stimulate muscle protein FSR directly as suggested by Ingwall and colleagues (1976).

2.0 Strategies to Increase Muscle Protein Synthesis

2.1 Introduction

For the patient population and the athlete, there is an interest in strategies designed to increase muscle protein synthesis. Regular resistance exercise has been shown to result in muscle fibre hypertrophy (Hortobagyi *et al.* 1996, McCall *et al.* 1996, Roman *et al.* 1993, Staron *et al.* 1994). It has been suggested by Roman and colleagues (1993), and supported by others (McCall *et al.* 1996, Staron *et al.* 1994), that muscle fiber hypertrophy is due to the addition of new sarcomeres in parallel, as well as myofibrillar splitting (MacDougall and Sale, 1978). Addition of these sarcomeres requires the synthesis of additional contractile proteins. Strategies such as resistance exercise, amino acid supplementation, increasing cell volume, and increasing the circulating concentration of key hormones have been implicated in increasing protein synthesis and preventing protein breakdown. It is possible that creatine monohydrate might have an anabolic effect on protein by: 1) allowing for a greater number of muscular contractions during training, 2) increasing cell volume by increasing water content 3) directly through a transcriptional effect 4) or through an interaction with certain hormones. Findings from

several studies have led support to the idea that creatine can have a significant effect on body composition (Vandenbergh *et al.* 1997; Kreider *et al.* 1998), however, the locus of action of creatine has not been elucidated.

2.2 Exercise and FSR

Based on exercise training studies which have reported greater increases in LBM with Cr supplementation (Vandenbergh *et al.* 1997; Kreider *et al.* 1998), it is conceivable that creatine may allow one to perform more contractions during training, and thus possibly stimulate MPS to higher levels

The stimulus underlying muscle hypertrophy following resistance exercise has not yet been identified. In 1992, Chesley measured biceps muscle protein synthesis following an acute bout of elbow flexor exercise. Utilizing a cross-sectional design, subjects were matched for training and assigned to either a +4 h post-exercise group, or a +24 h post-exercise group. Muscle protein FSR was significantly elevated 4 h post exercise (50% higher than rest), and peaked at 24 h (109% higher than rest). Subsequent work by the same group revealed that muscle protein FSR returned to normal resting levels by 36 h post-exercise (MacDougall *et al.* 1995). Furthermore, to determine whether the increases in FSR are due to transcriptional and/or posttranscriptional events, RNA capacity and activity were measured in both the exercised and non-exercised arms. RNA capacity remained unchanged in the exercised biceps, however, a significantly increased RNA activity in the exercised arm was observed. Thus, the author's concluded that increases in muscle FSR were likely due to post-transcriptional events. However, it

should be noted that rather large increases in specific mRNA's could occur immediately following heavy resistance exercise with no detectable increase in total RNA.

Welle and colleagues (1999) confirmed the theories proposed by Chesley and associates (1992) by showing that protein FSR was increased by approximately 30% following resistance exercise, while total RNA, mRNA, total myosin heavy chain mRNA, and actin mRNA were not significantly different between sedentary and exercised muscles. Thus, the increase in protein synthetic rate must result from post-transcriptional mechanisms. For example, an enhanced rate of recycling of ribosomes after each round of peptide-chain elongation, a more efficient translation initiation, or a more rapid peptide-chain elongation by each ribosome.

Another study investigating the time course of protein synthesis reported that *vastus lateralis* muscle protein synthesis was elevated for up to 48 h following an acute bout of resistance exercise (Phillips *et al.* 1997). In this study the authors investigated mixed muscle FSR, and fractional breakdown rate (FBR) after an isolated bout of eccentric or concentric knee extension exercise. The author's examined the subjects at four time points: rest, 3, 24, and 48 h following the cessation of exercise. Exercise resulted in a significant increase in muscle FSR at all time points: 3 h = 112%, 24 h = 65%, 48 h = 34%, as compared to rest. Similarly muscle FBR was also elevated, but only at 3 h (31%), and 24 h (18%), and returned to normal within 48 h. Muscle net balance was always negative because subjects were tested in the fasted state, however, was less negative at all time points after exercise. These findings differ slightly from those of Chesley and colleagues (1992) in which FSR increased at 4 h postexercise and peaked at

24 h, and returned to normal within 36 h (MacDougall *et al.* 1995). These discrepancies may be accounted for by the training status of the subjects (Phillips *et al.* 1999), and by the differing study designs. The time course reported by Chesley (1992), and MacDougall (1995), was constructed from three independent groups, whereas the study by Phillips and colleagues (1997) utilized was a repeated measures design.

To answer the questions concerning training status, Phillips and co-workers (1999) conducted a study to investigate the effects of resistance training on mixed muscle protein fractional synthetic rate (FSR), and breakdown rates (FBR) in young, healthy males. A cross-sectional design was used to measure the effect of an isolated bout of pleiometric resistance exercise in trained versus the sedentary subjects. The acute response (~ 4 h) of human mixed muscle protein synthesis was significantly elevated in comparison to the non-exercised leg. The untrained group increased FSR by 118%, whereas a resistance exercise trained group increased FSR by only 48%. Although resistance exercise had no apparent effect on muscle FBR at rest, following exercise FBR was unchanged in the trained group, whereas FBR increased 37% in the untrained group. Thus, the findings of a smaller increase vs. the nonexercised leg in muscle protein FSR and FBR after pleiometric exercise in the trained group, suggests that resistance training may reduce muscle protein turnover after exercise. Furthermore, the authors demonstrated a significant correlation between mixed muscle protein FSR and FBR ($r=0.84$). This correlation suggested that perhaps FSR and FBR are related, and that FSR and FBR may be coordinately regulated.

While investigating the effects of training on *vastus lateralis* muscle protein synthesis in both young and elderly men and women, Yarasheski and colleagues (1993) found that, in the initial phase (2wk) of a resistance exercise program, marked increases in quadriceps FSR occurred. The initial FSR in an elderly population was significantly lower (39%) than that of the young population. Following two weeks of resistance exercise training, FSR increased in both groups to similar levels, with no significant difference between the groups. Furthermore, whole body protein breakdown rate did not change. Although these results demonstrate an effect of training on muscle FSR, it should be noted that FSR was measured within 3 h of the last exercise session. Since it has been shown that the acute effect of exercise can last as long as 48 h (Phillips *et al.* 1997), the authors (Yarasheski *et al.* 1993) demonstrated the effect of training on muscle FSR following an acute bout of exercise. Thus, the effect of training on true basal FSR has not yet been established using a longitudinal study design. However, the results provided by Yarasheski and colleagues (1993) demonstrate that skeletal muscle protein synthesizing pathways are activated in both young and elderly subjects alike.

Having established the effect of acute resistance exercise (Phillips *et al.* 1997; Phillips *et al.* 1999; Yarasheski *et al.* 1993), and acute endurance exercise (Carraro et al. 1990) on the rate of muscle protein synthesis, the effect of both endurance and resistance exercise in combination was studied by Tipton and associates (1996). Posterior *deltoid* muscle protein turnover was examined in female swimmers after a combination of resistance and endurance exercise. Subjects were studied on four different occasions: 1) rest; 2) following a swimming workout; 3) following a whole body resistance exercise

workout 4) and a combination of 2 + 3. Whole body protein breakdown, as measured by phenylalanine rate of appearance, was similar in all exercise conditions. Posterior deltoid muscle protein FSR was not statistically different between exercise conditions, however, all exercise conditions increased FSR above rest. Although statistical significance was not achieved between conditions, it is important to note that there were trends in the data. The endurance plus resistance exercise treatment significantly increased FSR by 81% over resting levels, 30% greater than after swimming alone, and 73% greater than after resistance exercise alone. The trends reported by Tipton and colleagues, although non-significant are likely due to the variability inherent in the measurement of MPS in humans. From this study, one can conclude that the combination of swimming and resistance exercise significantly stimulated FSR above the resting value. No increases over rest were seen in whole body protein synthesis rate, and thus appears that the combination of swimming and resistance exercise work-outs provided a greater stimulatory effect of muscle protein synthesis than either swimming or resistance exercise alone.

It is apparent that resistance exercise may be an effective strategy in combating muscle atrophy, and if continued for an extended period, inducing hypertrophy. Muscle atrophy is a typical characteristic observed in astronauts following space flight, due to exposure to a micro-gravity environment. Bed rest appears to simulate a micro-gravity environment and mimics the rest-induced loss resulting from space flight. Previously, bed rest has been shown to decrease skeletal muscle protein synthesis (Ferrando *et al.* 1996), and thus resistance exercise, which is known to increase protein synthesis, may be

a viable countermeasure for those undergoing space flight. In fact, Ferrando et al. showed that bed rest resulted in a 46% decrease in muscle protein FSR, whereas resistance exercise performed every other day over 14 d of bed rest maintained muscle protein synthesis. In fact, a non-significant increase in muscle protein FSR of .028%/h was noted in the resistance exercise group. This study suggests that resistance training may be an effective countermeasure to the reduction in skeletal muscle mass and strength, as a result of space flight. Furthermore, the results suggest that repeated stimulation of muscle protein synthesis may be crucial in maintaining muscular strength (Ferrando *et al.* 1997). In this study, however, measurements were taken 24 h following the last bout of resistance exercise. In light of previous work (Chesley *et al.* 1992; Phillips *et al.* 1997), the non-significant increase in FSR observed following resistance exercise training may simply be in response to an acute bout of exercise.

2.3 Resistance Exercise and Protein Availability

Since amino acids are the building blocks of contractile protein, it has been suggested that increased amino acid availability may increase protein synthesis following exercise (Biolo *et al.* 1995). Biolo and colleagues (1995) showed that protein breakdown was significantly elevated following resistance exercise. At the same time an acceleration of the rate of amino acid transport into muscle cells may have contributed to muscle anabolism, by increasing amino acid availability for protein synthesis. Whether creatine may function in an analogous manner due to it's amino acid (aa) content, is not known.

Biolo and colleagues (1995) found a significant increase in muscle blood flow following an intense lower body resistance exercise protocol (90.4% above resting). Rates of arterial amino acid delivery (flow x [aa]) were increased after exercise by ~80% - 110% depending on the amino acid. Transport rates of leucine, lysine, and alanine significantly increased after exercise by ~60% - 120%, depending on the amino acid. Following exercise, protein synthesis was increased by about 100%, and breakdown increased by about 50%, thus improving net balance. Intracellular appearance of leucine, lysine, and alanine increased after exercise due to an increase in transmembrane transport, as well as an increase in protein breakdown. The authors suggested that an increased availability of intracellular free amino acids may have caused the post-exercise stimulation of protein synthesis. If this hypothesis is true, then acute stimulation of protein synthesis would likely be due to a post-transcriptional mechanism, as suggested by Chesley and associates (1992). In addition, arterial amino acid concentration did not change following exercise, but leg blood flow increased 90% above resting levels. In accordance with this increase in blood flow, amino acid delivery to the muscle increased. Based on these observations the authors suggest that physical exercise may not have a direct effect on membrane transport systems, but its effect may be due to an increase amino acid delivery to the muscle, secondary to an increase in blood flow.

The results reported by Biolo and colleagues (1995) raise the issue, that if amino acid transport plays such an integral part in protein kinetics, then perhaps hyperaminoacidemia would increase protein synthesis above and beyond the effect of exercise alone, while suppressing protein breakdown. Biolo *et al.* (1997), investigated

protein breakdown to restore normal concentrations, however, an increase in the concentration above normal has no further inhibitory effect on protein breakdown.

Another important observation made in this study (Biolo *et al.* 1997), was the strong correlation (r=0.72) between blood flow and protein FSR in muscle. It would appear that variations in blood flow may affect protein metabolism by increasing the transport of amino acids into muscle cells and thus stimulating synthesis by increasing the [aa]. These results reported by Biolo and colleagues (1997) suggest that an increased delivery of amino acids to skeletal muscle after performance of physical exercise can maximize the anabolic effect of nutrition and exercise.

A similar study from the same laboratory (Volpi *et al.* 1998), investigated the effect of exogenous amino acids on muscle protein synthesis and breakdown in the elderly. The age-dependent reduction in muscle mass may be associated with an impairment in muscle protein metabolism, as has been shown in previous studies examining protein synthesis in the elderly (Yarasheski *et al.* 1993). In accordance with Biolo and co-workers (1997), amino acid infusion resulted in a significant increase in FSR (100%) at rest, however, amino acid infusion had no effect on blood flow. Amino acid infusion significantly increased amino acid delivery to the muscle, followed by an increase in amino acid transport. As expected, proteolysis did not change from baseline, indicating that increased intra-cellular amino acid concentrations at rest do not affect protein breakdown. The data presented by Volpi and colleagues (1998), suggested that an increase in amino acid transport enhanced protein synthesis by simply providing more substrate, however, there was no correlation between intracellular amino acid

concentrations and the rate of utilization of aa for synthesis. Thus, the concentration of aa does not seem to be the key factor in up-regulating protein synthesis in the elderly. Furthermore, in contrast to previous work (Biolo *et al.* 1997) no increase in blood flow occurred in association with amino acid infusion. The authors suggest that perhaps there is an age related vascular impairment in the muscle blood flow to amino acids, as has been observed with insulin infusion (Meneilly *et al.* 1995).

Although several studies support the positive effect of amino acids on protein synthesis in both the young and old population (Biolo *et al.* 1995; Biolo *et al.* 1997; Volpi *et al.* 1998), the practicality of these interventions remains an issue, since intravenous infusions were used to increase amino acid delivery. A study by Welle and Thornton (1998) investigated the effect of high protein meals on myofibrillar synthesis after resistance exercise in elderly men and women. Subjects performed knee extension exercise three times within a six day period and FSR measurements were made ~ 23 h after the final bout of exercise. On the day of FSR determination subjects were randomly assigned to three groups. (A) a low dietary protein group (7% protein), (B) an intermediate group (14% protein), and (C) a high protein group (28% protein). Interestingly, insulin concentrations were 70% greater in the group consuming the high protein diet in the final two hours of feeding, which might suggest that a high protein diet acutely increases protein synthesis since insulin has been shown to be a potent stimulator of protein synthesis (Biolo *et al.* 1999). Myofibrillar protein synthesis rates did not differ significantly between groups consuming different protein intakes. The exercised muscle had an elevated rate of synthesis than the sedentary muscle, with an average increase of

27% for all the diet groups combined. Perhaps most interestingly, and in contrast with earlier work (Biolo *et al.* 1997), was the fact that the increase in myofibrillar synthesis was greater in the subjects fed the low protein meals (37%), vs. the normal protein meal (28%), vs. the high protein meal (20%).

An increasing amount of research suggests that increasing protein intake might stimulate myofibrillar protein synthesis. Both *in vitro* (Fulks *et al.* 1975; Li and Jefferson, 1978: Lundholm and Scherston, 1975), and *in vivo* (Bennet *et al.* 1989: Biolo *et al.* 1997; Mosoni *et al.* 1993; Svanberg *et al.* 1996: Watt *et al.* 1992) studies provide support for increasing muscle protein synthesis following an acute rise in amino acid concentration. Similarly, increases in whole body protein synthesis and breakdown are associated with consuming a high protein diet (Pannemans *et al.* 1995: Pannemans et al. 1995). Tarnopolsky et al (1992) showed a higher rate of synthesis in strength trained athletes as compared to sedentary individuals, and many have shown that increasing protein intake results in a greater nitrogen balance in both young and older populations (Campbell *et al.* 1995; Fern *et al.* 1991; Lemon *et al.* 1992).

In contrast to the studies discussed above, the work of Welle and colleagues (1998) does not support an increase in myofibrillar protein synthesis associated with a greater protein intake. One major discrepancy between this study design and the previous designs of others, is the suggestion that the timing of energy intake is integral to the effect on protein synthesis and degradation (Roy *et al.* 1997). Furthermore Biolo and colleagues (1997) showed that amino acid infusion immediately following exercise had a significant effect on mixed muscle protein synthesis. However, there are several

differences in design which separate the studies of Biolo and colleagues (1997), and that of Welle and colleagues (1998). Biolo and associates (1997) examined protein metabolism immediately following the cessation of resistance exercise. Subjects were administered amino acids through intravenous infusion, and mixed muscle protein synthesis was examined in contrast to myofibrillar protein synthesis. Perhaps the discrepancy in results stems from the fact that protein was continuously provided in the study by Welle and colleagues (1998). If, as suggested by Biolo and associates (1997), the synthetic rate is dependent on the concentration of the intracellular amino acid pool, then the subjects in Welle's study may have already been in optimal conditions. In Biolo's study the subjects were in an over-night fasted condition, and therefore, the amino acids infused were necessary to increase FSR. It would appear that the work by Welle et al. (1998) would have benefited from a control group in which no protein was provided. The work by Welle and colleagues (1998) provides a somewhat surprising conclusion, that high protein meals did not increase protein synthesis in the sedentary leg more than the low protein meals. This result is surprising due to the fact that Biolo et al (1997), showed that increased plasma [aa] were well correlated with amino acid transport into muscle.

A recent study by Tipton and associates (1999) provides further support for the work of Biolo and colleagues (1997). From a practical standpoint, one of the limitations of the work by Biolo *et al.* (1997) is the fact that they used intravenous infusions of amino acids. Intravenous infusion is not practical and thus poses the question of whether or not oral amino acid administration would produce the same results. Previous studies

suggest that first-pass splanchnic uptake of ingested amino acids is about 20-90% (Cortiella *et al.* 1988; Matthews *et al.* 1993;). It has also been demonstrated that splanchnic protein breakdown is increased during endurance exercise (Williams *et al.* 1996). Thus, it is unknown whether amino acids from an oral dose would be directly delivered to the muscle tissue, and whether an oral amino acid solution would be effective as a stimulus for protein synthesis. Following intense leg exercise, 40g of mixed amino acids, essential amino acids, or a placebo were ingested in a double blind cross-over fashion. Amino acid ingestion resulted in significant hyperaminoacidemia. Amino acid concentration were dramatically increased by both amino acid oral solutions, and the levels attained were similar to those reported previously during amino acid infusion (Biolo *et al.* 1997). Net muscle protein balance, as determined by arterio-venous differences and corrected with intracellular concentrations of amino acids, was significantly increased from negative during placebo ingestion, to positive during both oral solutions. Under the influence of oral amino acids, blood flow was unchanged and thus it is likely that the increased availability of amino acids, and not blood flow *per se*, is the primary mechanism for increasing protein accretion.

2.4 Insulin and Protein Synthesis

Although insulin has generally been thought of as an anabolic hormone in glucose and glycogen metabolism, the role of insulin in controlling muscle protein synthesis and degradation remains unclear. Animal (Jefferson *et al.* 1977) and human models (Fukagawa *et al.* 1985) have both established an anti-catabolic effect of insulin on muscle

protein, however, insulin's effect on synthesis is less clear. Since the Cr transporter has been shown to be insulin-sensitive, and insulin has been shown to enhance muscle creatine accumulation (Steenge *et al.* 1998), Cr supplementation may help stimulate muscle protein FSR.

It has been suggested that insulin's anabolic effect is at least in part due to an increase in protein synthesis. Gelfand and colleagues (1987) used a forearm model to measure amino acid kinetics in response to a 2 h insulin intra-arterial infusion. The insulin infusion raised insulin levels to high physiologic levels, and subsequently increased glucose disposal four fold following the infusion. Forearm blood flow increased significantly (25%) in response to the insulin. Forearm net balance of both phenylalanine and leucine reversed from a net amino acid loss in the basal state, to a net uptake in the insulin condition. Despite the increase in net uptake of amino acids from blood, the tracer data demonstrated that phenylalanine and leucine rates of disappearance were not significantly changed by the insulin. However, the positive net balance observed could be accounted for by the inhibited phenylalanine and leucine release (42% and 50% respectively) from the forearm. These results do not provide evidence supporting this claim. However, the authors suggested that the protein synthetic effect of insulin may be functional *in vivo* at normal post-absorptive insulin concentrations, and therefore insulin infusion would not would not increase protein synthesis to a higher level. On the other hand, the insulin stimulated inhibition of protein breakdown may require much higher insulin concentrations. In summary, these results provide evidence that inhibition of protein breakdown, rather than stimulation of synthesis, is the major

mechanism whereby acute physiologic elevations in insulin promote net positive muscle protein balance in man.

Bennett and associates (1990), examined the effect of euglycemic hyperinsulinemia on amino acid uptake by the quadriceps during hyperaminoacidemia. All subjects were first tested with amino acid infusion with no insulin, then with insulin and glucose infusion with continued amino acid infusion at a higher rate to maintain the concentration of muscle amino acids. Insulin infusion increased blood flow 60% above the low-insulin state. During the insulin infusion, when extra amino acids were infused, amino acid concentrations increased markedly in the plasma. Net balance of phenylalanine in the leg which was slightly positive during the low insulin period, however, became significantly more positive during the insulin infusion, indicating that leg protein synthesis considerably exceeded protein breakdown. Leucine rate of appearance, an index of muscle protein breakdown, was decreased by 30% by insulin and amino acids, however, only a 9% reduction was noted in phenylalanine rate of appearance. On the other hand, phenylalanine rate of disappearance, an index of muscle protein synthesis, increased 55% during insulin infusion. A small increase was found in the whole body rate of non-oxidative leucine disposal, suggesting that whole body protein synthesis was increased. These results suggest that, under favorable conditions, insulin may have a stimulatory effect on skeletal muscle and whole body protein synthesis and an inhibitory effect on skeletal muscle and whole body protein breakdown. It should be noted, however, that the A-V balance model may not be a good indicator of protein synthesis or breakdown due to the lack of knowledge of what is happening in the

tissue. For example, amino acid recycling, or oxidation may be occurring, and thus the amino acid concentrations reported in the vein may not be accurate.

McNurlan and colleagues (1994), utilized a flooding dose of [$^2H_5$]phenylalanine to investigate the effect of both glucose and insulin infusion on amino acid incorporation into muscle. Six subjects were studied in the post absorptive state at rest, and then following a hyperinsulinemic-euglycemic clamp experiment. Interestingly, the authors reported significant reductions in the plasma concentration of some amino acids, which might be indicative of an increased uptake of amino acids for protein synthesis. However, the rate of protein synthesis did not correlate with the increased concentration of insulin, and protein synthetic rates were not different from the post-absorptive value (post-absorptive: 1.65 ± 0.11%/d and insulin:1.66 ± 0.16%/d). This study therefore confirms that insulin does not stimulate protein synthesis in human muscle tissue, at least when using the flooding dose method. The inability to demonstrate any effect of insulin on muscle protein synthesis is in agreement with studies utilizing tracer methodology (Pacy *et al.* 1989), and some arteriovenous measurements (Gelfand *et al.* 1987), however it is in disagreement with other studies using arteriovenous measurements (Bennet *et al.* 1990, Biolo *et al.* 1999). These discrepancies in the literature are likely due to variations in methods used to measure protein synthesis

In a study investigating the effects of starvation on human muscle protein metabolism and its response to insulin (Fryburg *et al.* 1990), it was reported that insulin showed no anabolic effect, however, exerted a strong anti-catabolic effect. Intra-arterial infusion of insulin was started after a 150 min basal period of labeled amino acid

infusion. The results demonstrated that 60 h of fasting led to increased rates of urinary nitrogen excretion (59%), and whole body leucine oxidation (72%), both of which are indices of whole body protein breakdown. Amino acid release increased 31% from the forearm and was due to an increase in local R_a, which is a measure of proteolysis. No significant changes, however, were observed in Rd (whole body or forearm) after fasting or after insulin administration to the 60 h fasted subjects. Fasting prompted an increase in skeletal muscle proteolysis, and insulin administration was able to suppress proteolysis without stimulating synthesis.

Blood flow measurements in this study (Fryburg *et al.* 1990) revealed that following the 60 h fast, forearm blood flow was elevated by 37%. Biolo and colleagues (1997), showed that there was a strong correlation between blood flow and FSR of muscle proteins in the leg. Fryburg and colleagues (1990), despite having shown an increase in blood flow, showed no increase in muscle protein synthesis in the forearm. The discrepancy in these results suggest that perhaps blood flow is not the dependent factor in controlling protein synthesis, but that the arterial amino acid concentration inside or entering the cell may be the stimulus for protein synthesis.

The effect of glucose supplementation (as a method of increasing insulin) on FSR was measured following resistance exercise (Roy *et al.* 1997). Following glucose consumption, urinary 3-MH excretion, a measure of whole-body proteolysis, was decreased. Also FSR of muscle proteins in the exercised vs the control leg was elevated by 36% in the CHO condition as opposed to 6% in the placebo condition, however, these results did not achieve statistical significance. Although statistical significance was not

achieved these results suggest that consumption of a glucose supplement after resistance exercise increases insulin concentration and therefore may enhance muscle protein balance.

Using stable isotopes and an arterio-venous design, the anabolic effect of insulin on muscle protein metabolism was demonstrated (Biolo *et al.* 1995). Insulin was infused into the femoral artery to increase femoral venous insulin concentrations. The FSR of muscle protein increased by 65% during the insulin infusion, and phenylalanine disappearance to protein synthesis significantly increased (~ 50%). Furthermore, the rates of amino acid appearance into the intra-cellular pool from proteolysis did not significantly change during insulin infusion. Thus, it would appear that the primary effect of insulin on skeletal muscle protein metabolism was to increase the rate of protein synthesis, without affecting protein breakdown.

Other work by Biolo and colleagues (1997), showed that insulin promoted net muscle protein accretion by directly stimulating the rate of protein synthesis, and has no effect on transmembrane transport of amino acids. Local insulin infusion decreased the intra-cellular amino acid concentration. This indicates that insulin-mediated stimulation of protein synthesis was not matched by a concurrent increase in amino acid transport and would suggest that the anabolic effect of insulin occurs by acting directly on some step within the process of protein synthesis. Although these results differ from previously discussed studies (Gelfland *et al.* 1987; Fryburg *et al.* 1990), it may be that the results reported of Biolo *et al.* (1995) present a more accurate account of what is occurring within the muscle. The arterio-venous model utilized in previous studies (Gelfand *et al.*

1987; Fryburg *et al* 1990) does not take into account the amino acid metabolism taking place in the muscle cell, and thus the amount of amino acid recycling cannot be established which may lead to an underestimation of synthesis.

More recently, Biolo and colleagues (1999) have determined that insulin infusion into a femoral artery resulted in a significant increase in protein synthesis at rest. Insulin also increased blood flow significantly at rest, however, did not affect blood flow following exercise. Interestingly, insulin infusion did not alter protein breakdown at rest as others have suggested (Fryburg *et al.* 1990). However, following resistance exercise protein breakdown was suppressed with insulin infusion. Furthermore, a decreased amino acid availability may limit the stimulatory effect of insulin on muscle protein synthesis after exercise.

2.5 Testosterone and Protein Synthesis

Testosterone has been characterized as an anabolic hormone due to its ability to promote nitrogen retention. In fact, Kochakian (1950) demonstrated that testosterone increased nitrogen retention in castrated male animals. Interestingly, Hoberman and colleagues (1948) have demonstrated that testosterone may stimulate creatine synthesis, however, the exact pathway by which testosterone promotes anabolism has not been elucidated, and thus it is difficult to decipher whether or not creatine is directly linked to anabolism.

In hypogonadal men, when testosterone levels are lowest, testosterone replacement has proven to significantly alter muscle protein synthesis (Brodsky *et al,* 1996). A dose of 3mg/kg of testosterone was injected every 2 w over the course of 6 months. The goal of the researchers was to raise testosterone concentrations to normal physiological levels. Body weight increased significantly (~ 7%), and was due, almost exclusively, to an increase in fat free mass (~ 14%), since fat mass decreased (~ 11%). Mixed skeletal muscle FSR was ~ 56% greater following 6 m of testosterone replacement. A similar trend was observed with myosin heavy chain FSR (+46%) ($p>0.05$). These data support the notion that testosterone replacement therapy for 6 months in hypogonadal men produced clinically significant increases in fat free mass, and decreases in fat mass. More specifically, the increased fat free mass is largely accounted for by an increase in the mass of skeletal muscle. An increase in the rate of mixed muscle protein FSR, following testosterone treatment indicates that the mechanism underlying an increase in muscle mass is due to an increase in synthesis of muscle proteins. Furthermore, a trend toward an increase in myosin heavy chain synthesis implies that included in the muscle proteins displaying increased synthesis are those responsible for the contractile function of muscle.

Bhasin and colleagues (1997) investigated the effect of testosterone replacement on fat free mass and muscle size in hypogonadal men. Seven hypogonodal men were treated with 100mg/week for ten weeks with intra-muscular testosterone injections. Serum testosterone levels increased six fold from baseline on day 15, and were maintained in the normal male range for the remainder of the study. All subjects

experienced a significant weight gain in the range of 4.5kg. Fat free mass also increased significantly by 5.0 kg on the average. Muscle cross-sectional area of the arm increased by 12%, as did the legs by 8%. Strength increased in the bench press by 22%, and by 44% in the squat. Interestingly, whole body leucine flux, oxidation, and non-oxidative leucine disposal did not change significantly during testosterone treatment.

These results demonstrate that testosterone has substantial effects on body composition. Replacement doses of testosterone increased body mass, primarily through an increase in fat free mass. Most interestingly, muscle size and strength increased significantly despite the lack of any resistance exercise. Despite significant gains in fat free mass whole body leucine turnover did not change significantly after the 10 week treatment. However, the authors suggest that testosterone may increase nitrogen balance and leucine flux early in the course of treatment, and thus may have been missed as the measurement was made at the end of ten weeks. This is supported by the fact that maximum weight gain had been achieved by 6 weeks of treatment. An alternative explanation for the discrepant results is the possibility that testosterone specifically up-regulates muscle protein synthesis, and since muscle protein synthesis accounts for approximately 25% of whole body protein synthesis, the effect may have been missed.

From the results of Bhasin and colleagues (1997) and Brodsky and colleagues (1996) we can conclude that testosterone replacement to the normal male range is an important factor in maintaining the muscle protein synthesis rate and muscle mass in adult hypogonadal men. However, these studies do not offer any information as to the effect of increased doses of testosterone in normal men

The effect of testosterone on muscle size and strength in normal men (i.e. non-hypogonadal) was also investigated by Bhasin and colleagues (1996). Subjects were randomized to one of four groups: placebo with no exercise, testosterone with no exercise, placebo plus exercise, and testosterone plus exercise. Subjects received 600mg of testosterone enanthate weekly for 10 weeks. Serum concentrations of free and total testosterone increased significantly in both testosterone groups, but not in the placebo groups. Increases in body weight were seen in the testosterone without exercise group (3.5 kg), and those in the testosterone plus exercise group had an even greater increase in body weight (6.1 kg). Fat free mass increased significantly in both testosterone treated groups. Similarly, both testosterone groups increased muscle cross-sectional area, with an 11.8% increase in triceps area in the non-exercise group and a 6.7% increase in the quadriceps. The exercise group increased triceps area by 14.4%, and quadriceps increased by 13.7%. Furthermore, testosterone alone increased the one repetition maximum (1RM) for squatting by 19%, while the testosterone plus exercise group increased their 1RM by 38%.

The results of Bhasin *et al.* (1996) demonstrate that supraphysiological doses of testosterone, especially when combined with strength training, increase fat free mass, muscle size and muscle strength. The combined findings of these studies (Bhasin *et al.* 1996; Bhasin *et al.* 1997; Brodsky *et al.* 1996) discussed, suggest that both replacement and supraphysiological doses of testosterone increase fat free mass and strength. Increasing serum testosterone concentrations in hypogonadal men can lead to increases in

fat free mass of 8-10%. When serum testosterone concentrations are increased above the normal range further increases in fat free mass can be achieved.

Since the above studies are largely descriptive, it is important to examine those studies providing direct evidence of an effect of testosterone on muscle protein. Griggs *et al* (1989) first described the effect of testosterone injection on muscle protein FSR. A replacement dose of 3mg/kg every week for twelve weeks was administered. Mean testosterone concentration increased 3 fold in the testosterone treated group. No significant change in mean muscle fiber diameter was observed. Muscle protein synthesis rate was increased during testosterone administration, with a mean increase of 27%. Leucine oxidation decreased significantly during testosterone administration, however, whole body protein synthesis, as estimated by non-oxidative leucine disposal did not increase significantly. This study (Griggs *et al* 1989) provided the first direct evidence of the effect of testosterone injections on muscle protein synthesis and demonstrated that testosterone enanthate increased muscle protein synthesis by 27%, as estimated by the incorporation of stable isotope into muscle. The authors acknowledge the shortcoming of not having any knowledge on the effect of testosterone on muscle protein breakdown. However, the authors hypothesize that protein degradation must increase to a substantial and almost equal extent as synthesis since a 27% increase in the rate of muscle protein synthesis would otherwise have resulted in a much greater increase in muscle mass over a 12 week period.

It has also been reported that testosterone administration to hypogonadal elderly men increased skeletal muscle strength and protein synthesis (Urban *et al.* 1995).

Testosterone concentration was increased to similar concentrations as those found in young men. Following testosterone administration, work per repetition increased in the hamstrings, and quadriceps muscles. Most importantly, a two fold increase in muscle protein FSR resulted from the testosterone treatment. Testosterone administration increased insulin-like-growth-factor-1 (IGF – 1) concentration in the six elderly subjects, suggesting that the intramuscular IGF – 1 system is in part responsible for the increase in FSR. Furthermore, since testosterone administration increased muscle strength and FSR without the influence of exercise, it is likely that synthesis of new muscle proteins can enhance muscle strength.

Ferrando and colleagues (1998), examined the effect of a single testosterone injection on protein synthesis and degradation. Arteriovenous sampling and muscle biopsies, as well as isotope incorporation and decay were used to measure synthesis and degradation. Total testosterone concentrations increased to about twice the physiological range following a 200mg injection. Five days following the injection, total testosterone dropped to the upper limit of the male normal range. Testosterone injection increased muscle protein FSR by about 109%, whereas FBR remained unchanged, thus, net balance increased significantly. However, there was no evidence of increased tissue amino acid transport during the infusion protocol, despite the finding that leg blood flow increased significantly (57%) after testosterone injection. Furthermore, both whole body appearance of phenylalanine, and leucine decreased following the testosterone injection. These results suggest that an augmented protein synthesis following testosterone administration does not respond in the same manner as insulin. Whereas insulin

increased amino acid transport and subsequently protein synthesis (Biolo *et al.* 1995), it would appear that testosterone can increase protein synthesis without a preceding increase in amino acid transport, suggesting that either intra-cellular amino acid concentration is not limiting for protein synthesis, or that testosterone acts directly to improve translational efficiency, or intra-cellular amino acid recycling efficiency. The ratios between outward transport of amino acids and protein breakdown decrease following the injection suggesting an increased efficiency of amino acid recycling when treated with testosterone. Overall, the results reported by Ferrando and colleagues (1998) suggest that testosterone injection results in an increase in net muscle protein synthesis, however the increase in skeletal muscle protein synthesis is not stimulated by an increased tissue transport of amino acids, but rather an improved efficiency of amino acid recycling. (ie. reincorporation of aa appearing as a result of protein breakdown)

2.6 Growth Hormone and Protein Synthesis

Although the importance of growth hormone (GH) has been established in young, growing children, the effect that GH induces on healthy adults is yet to be understood. The role of IGF-1 on protein metabolism has not been elucidated, however it is believed that GH exerts its "anabolic" effect through IGF-1. A direct link between creatine and GH has been established, such that GH induces an increase in the mRNA for liver transamidinase (rate limiting enzyme for creatine synthesis) (Guthmiller *et al.* 1994).

The possible anabolic effects of GH may be important in treating several diseases associated with muscle wasting. Typically, patients treated with glucocorticoids have

been observed to suffer from muscle wasting, poor tissue healing, and an increased incidence of infections (Horber *et al.* 1985). On the other hand, GH is thought to induce an overall anabolic effect. Therefore, concomitant GH treatment with prednisone was examined to investigate whether or not GH would prevent the protein catabolic effects of prednisone (Horber *et al.* 1985). The study demonstrated that GH therapy resulted in increased protein synthesis (non-oxidative leucine disposal), however GH therapy had no effect on proteolysis (leucine rate of appearance). The authors suggest that the most likely mechanism explaining the increase in protein synthesis is a direct effect of GH or IGF – 1 generated as a result of GH administration. Interestingly, although GH was administered daily, only IGF – 1 concentrations increased significantly in a sustained manner.

Since an effect of GH on whole body protein synthesis has been established, Fryburg and associates (1991), examined the effect of short-term GH administration on skeletal muscle-specific protein synthesis and degradation in the forearm. Growth hormone concentration did not increase significantly in the forearm vein in response to the infusion. Similarly IGF – 1 concentrations did not increase during the course of the infusions. Blood flow increased 34% above basal levels after 3 h of GH infusion, and 90% above basal after 6 h of GH administration. In a similar manner GH suppressed the net release of phenylalanine by 73%, and suppressed the net release of leucine by 100%. When phenylalanine, and leucine kinetics were examined, it was discovered that GH was able to induce a more positive balance in these amino acids across the forearm through a stimulation of rate of disappearance. Phenylalanine disappearance increased 71% above

basal levels, and leucine disappearance increased 37% above basal levels. The rate of appearance of these amino acids did not change significantly over the period of the infusion, indicating that a net positive balance was achieved through an increase in protein synthesis.

Interestingly these anabolic effects occurred without an increase in GH, insulin, or IGF – 1, however, an increase in blood flow did occur over the course of the infusion. The authors suggested that the acceleration in blood flow during the infusion was likely not the stimulus for the increase in protein synthesis. However, blood flow has been well correlated with protein synthesis using both an arterio-venous model of measurement and FSR (Biolo *et al.* 1995; Biolo *et al.* 1997). The lack of an increased rate of appearance may be accounted for by a possible inhibitory effect of GH or IGF - 1 on protein breakdown (Jacob *et al.* 1989).

Furthermore, the authors suggest that GH, or IGF – 1 produced locally in response to GH may have a vasodilatory effect accounting for the increased blood flow. In summary, these results suggest that an increased protein synthetic rate associated with GH, may be due to a mechanism in skeletal muscle other than from that of insulin or IGF – 1, which have been traditionally accepted as anabolic agents.

Yarasheski and associates (1992), investigated the effect of growth hormone and resistance exercise on muscle growth in young men. Sixteen men were randomly assigned to either, a resistance training and GH, or resistance training and placebo group. Subjects trained for 12 weeks and muscle protein FSR was measured. Concentrations of GH increased to levels six times greater in the GH treated group, than in the placebo

group following the last injection. Similarly IGF – 1 values were significantly greater in the GH treated group in comparison to the placebo group. Body weight increased comparably in both groups, however, fat free mass was greater in the GH-treated group. However, results from total body water analysis suggest that the increased fat free mass in the GH group may have been due to a greater fluid retention. Chest and upper arm circumference increased in both groups, and thigh and mid-thigh circumferences were greater in the GH-treated group. The authors suggested that the increased thigh and mid-thigh circumference could be accounted for by the increase in total body water. Whole body protein synthesis and breakdown rates were increased in the GH-treated group. In addition, body protein balance increased more in the GH-treated group. Total urinary nitrogen decreased more in the GH-treated group than in the placebo group providing further support for a greater increase in protein accretion in the GH-treated group. Quadriceps mixed muscle protein FSR was, however, not significantly different between groups. These results indicated that prolonged GH treatment with resistance exercise induced no greater increase in muscle anabolism than resistance exercise alone. The increase in fat free mass and whole body protein synthesis indicated that the growth hormone treated subjects had increased lean body tissue. However, the lack of any difference in muscle FSR, strength, and muscle circumferences would suggest that the added tissue was not skeletal muscle. The authors offered the hypothesis that resistance exercise stimulated muscle protein synthesis to a level to which the addition of another stimulus (GH) does not further enhance muscle protein synthesis, yet allows other proteins to increase their synthetic rate. Furthermore, the authors acknowledge that the

measurements were made after 12 weeks of training, and that it may be possible that changes in body and muscle protein turnover occurred at the beginning of the training program .

In light of these results, Yarasheski and colleagues (1993), examined the role of short term GH treatment in experienced weightlifters. The GH supplementation group displayed elevations(~163%) in serum IGF – 1 concentrations above pre-GH treatment concentrations. In spite of a significant increase in IGF - 1 the fractional rate of muscle proteins in the *vastus lateralis* muscle was not increased in the GH group compared to the placebo group, following two weeks of training. Furthermore, the whole body breakdown rate in the GH group was not significantly different from the placebo group. An important characteristic distinguishing this study from other GH research, is that trained individuals were used as the testing population. Yarasheski and colleagues (1993) suggested that the skeletal muscles of young resistance-trained men respond to the resistance-training stimulus, and thus cannot respond to the potential anabolic effect of GH and IGF- 1. The author's further hypothesized that the primary stimulus for protein synthesis is muscle load, and that supplements may augment, but do not dictate the increase in synthesis.

2.7 Cellular Hydration in the Control of Protein Synthesis

The effect of cellular hydration, and cell volume on protein metabolism has been extensively studied *in vitro*, and less so *in vivo*. An increase in cell volume induced by several different methods has recently been shown to have anabolic and anti-catabolic

effects. In fact, cell swelling (ie. over-hydration) appears to put the cell into an overall anabolic state. Increases in glycogen synthesis (Baquet *et al.* 1990), lipogenesis (Baquet *et al.* 1991), polyamine synthesis (Tohyama *et al.* 1991), protein synthesis (Millar *et al.* 1997), and decreases in glycogenolysis have been observed following cell swelling in liver cells. Several studies have suggested that cell volume expansion may up-regulate protein synthesis (Haussinger *et al.* 1993). Since many studies investigating the effect of creatine supplementation have reported significant weight gains (1-2 kg) in very short periods of time (within 7 d), it is possible that creatine may cause a cell swelling effect in muscle and subsequently up-regulate protein synthesis. However, the increased body water retention due to creatine would result in only a marginal increase in intra-cellular water. For example if the weight gain was entirely water retention, and all of the water is retained in skeletal muscle, then a 1-2kg increase in body weight would result in only about a 4% increase in muscle cell volume.

Most studies investigating the effects of cell swelling have investigated skeletal muscle and liver, mainly due to the observation that these tissues are characterized by relatively rapid protein turnover. Work by Stoll and coworkers (1992) examined the effects of liver cell volume on protein synthesis. Incorporation of leucine into hepatocytes was examined in the presence of amino acids at twice the normal physiological concentrations. When the cells were examined in a hyper-osmotic state, cell volume decreased approximately 50%, and caused a subsequent 70% reduction in protein synthesis in comparison to the iso-osmotic state. This data supports an effect of cell swelling on protein synthesis only when the cell is in a dehydrated state.

In another study examining the effect of cellular hydration on protein synthesis in mammary cells (Millar *et al.* 1997), cell swelling via hypo-osmotic shock resulted in an increased incorporation of radiolabeled leucine and methionine into the cells. The hypo-osmotic state resulted in a 43% increase in protein synthesis above and beyond that during the iso-osmotic condition. No increases in mRNA following hypo-osmotic shock were observed, suggesting that the increase in protein synthesis was of post-transcriptional origin. This remains to be one of the few studies which supports an augmented protein synthesis in response to cellular swelling above normo-hydrated levels.

When hepatocyte volume was increased by exposure to a hypo-osmotic media, no subsequent inhibition of proteolysis occurred, and even a slight stimulation was observed in the absence of amino acids (Meijer *et al.* 1993). More importantly, protein synthesis was 60% higher in the hypo-osmotic condition, even in the absence of added amino acids. No difference was observed when amino acids were added in high concentrations suggesting that cell swelling has a profound impact on synthesis, and amino acid transport is not a necessary factor for protein synthesis.

In contrast to the work of Meijer and colleagues (1993), an increase in amino acid transport is generally observed in response to increasing cell volume (Low *et al.* 1996). However, the mechanism responsible for this effect remains to be elucidated. According to Low and colleagues (1996), cell swelling has been shown to up-regulate the Nm amino acid transport system, which is an insulin dependent glutamine transporter. Interestingly amino acid transporter system A is down regulated in response to cell swelling. It has

previously been established that altered amino acid transport activity in liver is G-protein mediated (Haussinger, 1996). In addition, the anabolic signal as a result of cell swelling has been shown to involve activation of lipid and protein-kinase signaling pathways in muscle (Low *et al.* 1996) and liver (Krause *et al.* 1996). These signaling pathways were further investigated by Low and co-workers (1997), and showed that phophatidylinositol-3-kinase was required to enable muscle cells to exhibit rapid, volume-induced changes in amino acid transport. When cells were exposed to hypo-osmotic shock, there was an 85% increase in glutamine uptake, and a 25% decrease when exposed to hyper-osmotic media, establishing that there was a volume regulatory response. Furthermore, when cells were pre-incubated with wortmannin, an inhibitor of the phophatidylinositol-3-kinase signaling pathway, the rapid increase in glutamine uptake was inhibited in response to hypo-osmotic exposure, and the decrease was inhibited in response to hyper-osmotic exposure. These results suggest that phosphatidylinositol-3-kinase is involved in a permissive role, because wortmannin blocked both the up and down-regulation of glutamine transport by hyper or hypo-osmotically-induced cell volume changes.

The effect of increasing cell volume on metabolism has been generalized as being anabolic. However, recent work by Quillard and colleagues (1998) revealed that phosphoenolpyruvate carboxykinase (PCK) mRNA, a cytosolic enzyme, decreased in response to cellular swelling. The findings of Quillard et al (1998) suggest that not all proteins act in the same direction in response to cell volume alterations, and thus the response of contractile proteins to cell swelling cannot be extrapolated from the response of other proteins.

Haussinger and co-workers (1993), have provided *in vivo* data which suggested that a change in cellular hydration might be the link between muscle glutamine content and protein turnover in skeletal muscle, and whole-body nitrogen balance. When examining the relation between cell water content and whole-body nitrogen balance, an inverse relationship was noted in patients with catabolic disorders. More specifically, burn victims exhibited the highest negative nitrogen balance, and also exhibited the lowest muscle cell water volume. Healthy subjects showed a 0 nitrogen balance, and had the highest muscle cell water volume of 280ml/100g dry matter (dm). Although these results show a positive relationship between cell volume and protein synthesis, Haussinger does not provide a group that is hyperhydrated, and thus did not show that hyperhydration would result in a more positive nitrogen balance.

From the evidence provided, there is little doubt that the state of cellular hydration may be an important factor in protein metabolism. Although protein synthesis and degradation seem to be affected in opposite direction by swelling and shrinking, little work has been conducted involving muscle cells. Further, the work that has been reported is rather inconclusive with regards to the effect of cell swelling over and above normal levels. With regards to the effect of creatine on protein metabolism, it is highly unlikely that the effects of water retention as a result of creatine supplementation would have any effect on protein synthesis. Where most have found an increase of up to 2kg in body weight following supplementation, only a 4% increase in muscle cell volume would result, assuming that all of the water is retained in muscle. If creatine is to exert a protein synthetic effect it is unlikely that it occurs due to increasing cell volume. However,

Oopick and colleagues (1998) reported that subjects supplementing Cr maintained strength following a 3-4% loss in body weight as opposed to the placebo group. Since the loss in body mass induced (4%) by Oopick and colleagues (1998) is very similar to the increase in cell volume one would expect from Cr supplementation then Cr may have a role in maintaining cellular water levels in those who dehydrate during exercise, and thus an anti-catabolic effect during exercise.

2.7 Purpose

Although almost every study examining creatine has reported increases in body weight following acute Cr supplementation (Balsom *et al.* 1994, Greenhaf *et al.* 1994, Stroud *et al.* 1994), none have established the source of the weight gain. It is not known whether this increase in body weight is simply attributed to water retention as suggested by Harris and colleagues (1992) or whether Cr supplementation can stimulate resting muscle protein synthetic rate directly resulting in a gain of muscle protein and, hence, mass. Recent evidence (Vandenbergh *et al.* 1997; Kreider *et al.* 1998) suggests that Cr supplementation in combination with resistance training may increase muscle mass above and beyond that of training alone. However, no direct measure of muscle protein synthetic rate has been utilized to verify the results of Vandenbergh (1997), and Kreider (1998). It may be, as these studies (Vandenbergh *et al.* 1997; Kreider *et al.* 1998) suggest, that Cr interacts with resistance training to increase muscle protein accretion. Measurement of muscle protein synthetic rate following Cr supplementation in

combination with resistance training would be the next logical step in attempting to establish the effect of creatine on human muscle protein metabolism.

The purpose of this investigation was to determine the effect of short term creatine supplementation on resting mixed muscle FSR in males and females. The specific hypothesis to be tested is that creatine supplementation could increase resting muscle FSR above and beyond that of the placebo group, as *in vitro* work has suggested (Ingwall, 1976). A second purpose was to investigate whether or not females respond to Cr supplementation in the same manner as males. Several studies have reported that females show no increase in body weight in response to creatine supplementation (Thompson et al. 1996; Hamilton-Ward et al. 1997; Febbraio et al. 1995; Mihic et al. 1997 (personal communication)). This lack of an increase in body mass may suggest that females do not respond to creatine as do males, and thus may not benefit from any positive effect on protein synthesis that creatine may have.

List of Figures

Figure 1. Creatine Synthesis

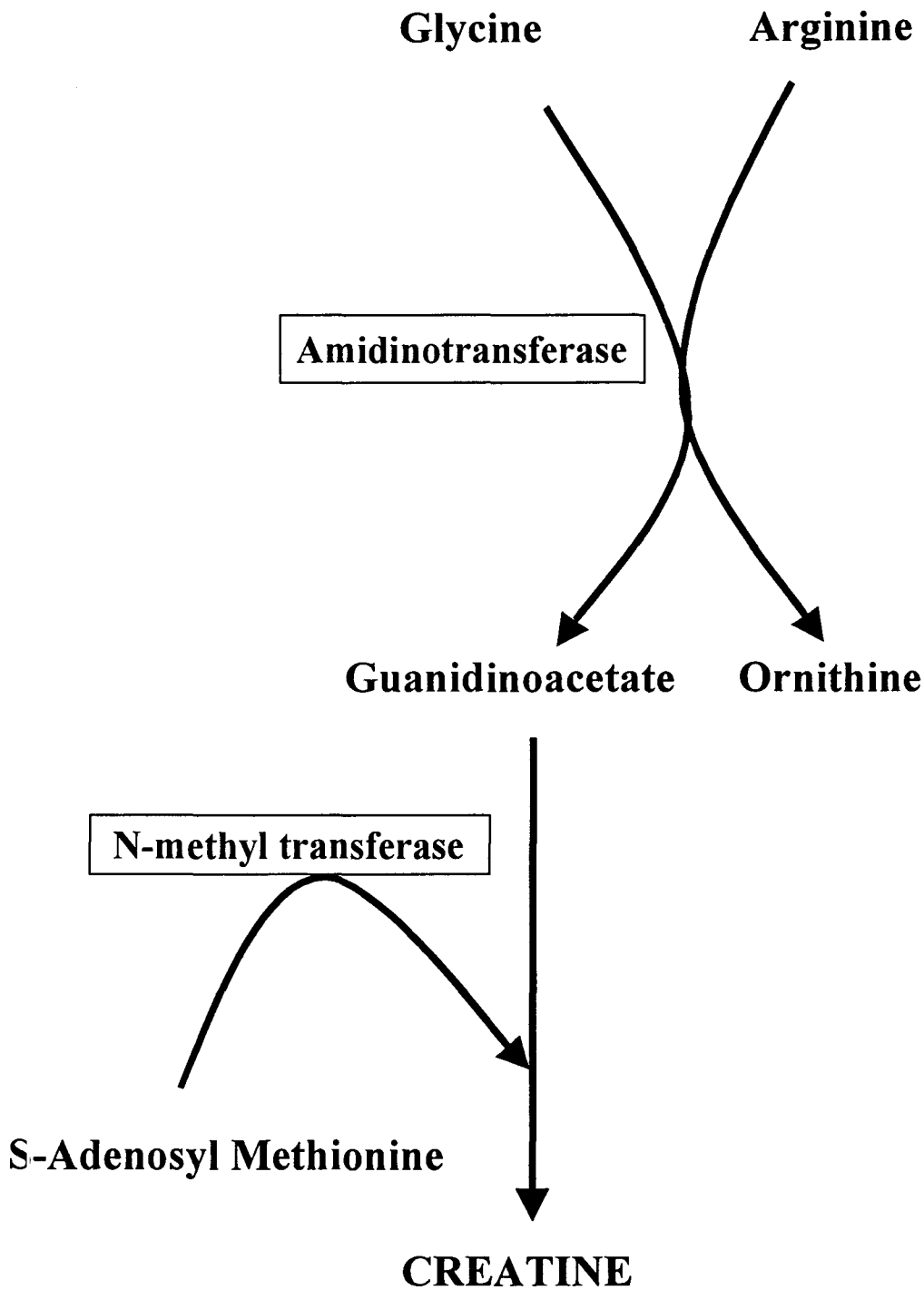


Fig 2. Creatine Kinase Reaction

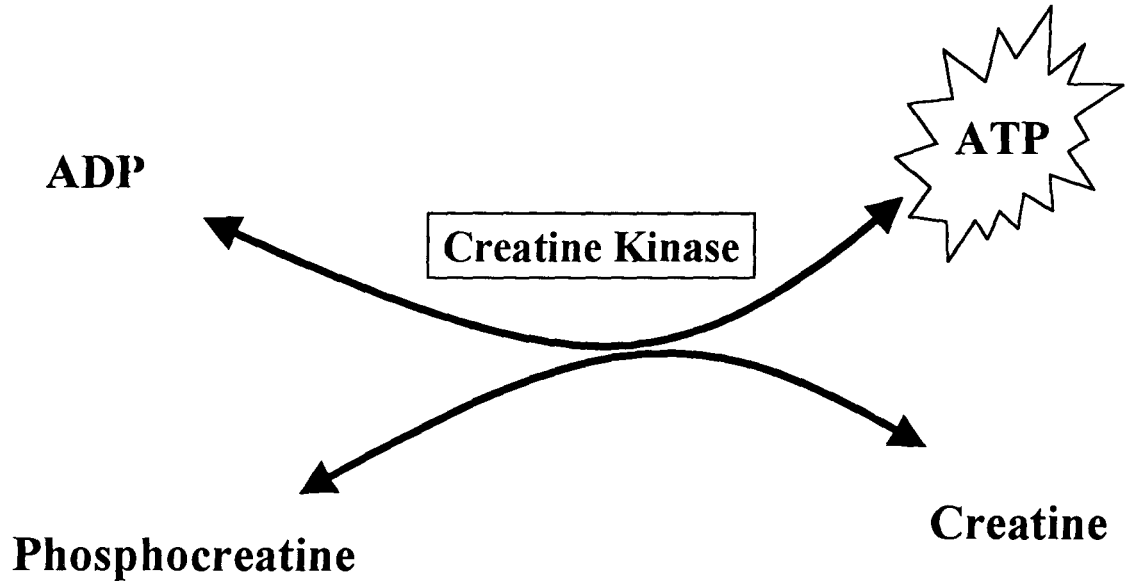
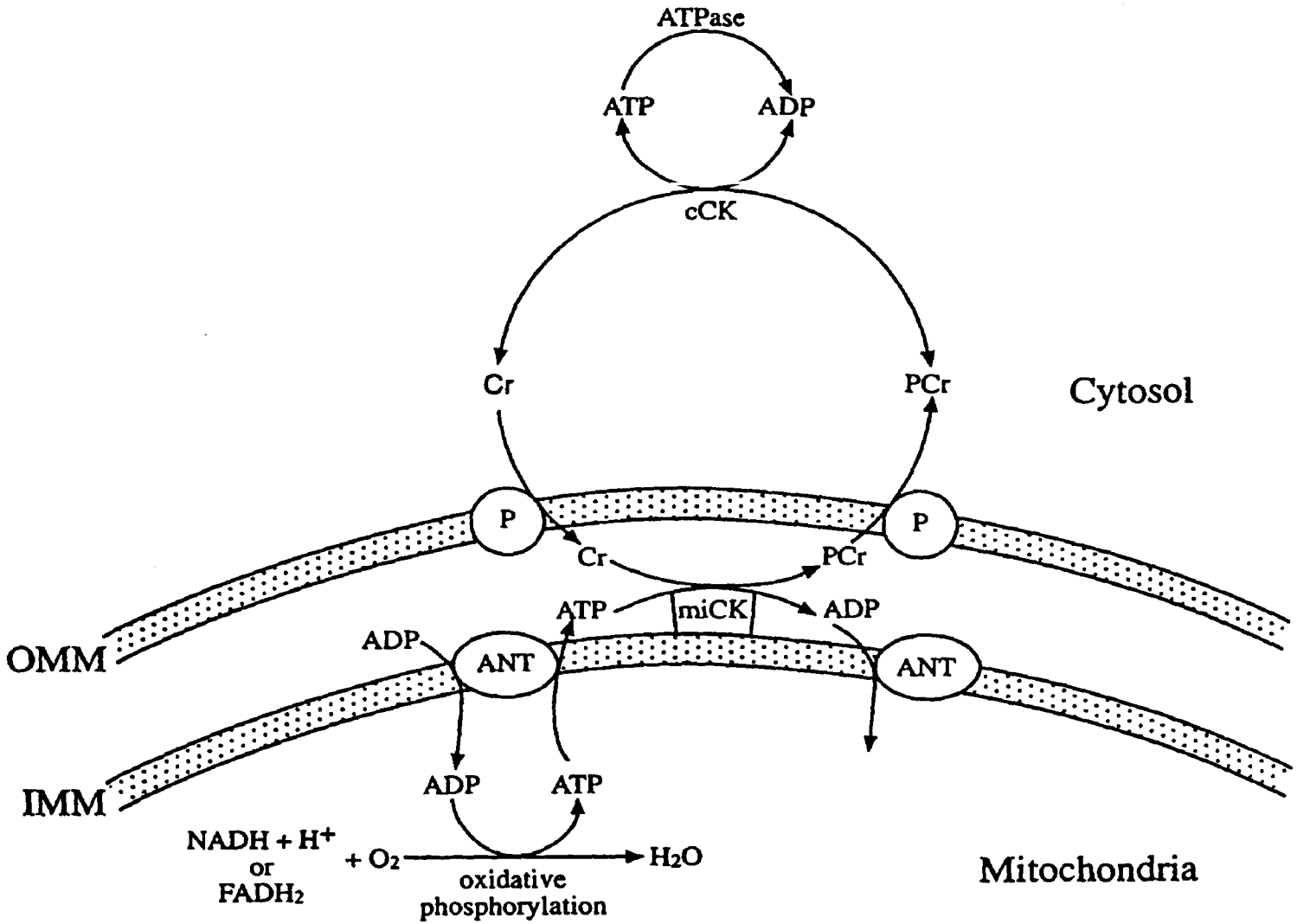


Fig 3. Spatial Characteristic of PCr Energy System



Taken from Juhn and Tarnopolsky, 1998.

3.0 Manuscript

EFFECTS OF ACUTE CREATINE SUPPLEMENTATION ON RESTING MUSCLE PROTEIN FRACTIONAL SYNTHETIC RATE

Gianni Parise B.Kin.
Department of Kinesiology. McMaster University.

Introduction

Creatine is one of eight naturally occurring compounds derived from guanadine, and it has been demonstrated that, when phosphorylated, it functions in the maintenance of cellular ATP homeostasis (1). Creatine is composed of arginine and glycine and requires methionine for its synthesis and comprises ~ 0.3-0.5% of total muscle weight (2).

In recent years, creatine (Cr) has become a popular supplement among the athletic community. Many investigations have shown that creatine loading may be beneficial during high intensity activity, when ATP demand is high (1). A second consistent observation is that, following creatine supplementation, there is an increase in fat free mass (FFM; 2-9). This increase in mass has been demonstrated to occur both acutely (10), and following long term creatine supplementation (11-12). Acutely, the increase in body weight is likely due to water retention as suggested by Harris and colleagues (2), however, studies investigating chronic creatine supplementation combined with resistance training suggest that creatine may promote greater gains in LBM. An increase in water retention over a long period of time may improve nitrogen balance (13), or even increase protein fractional synthetic rate (FSR) (14). Another possible reason for the increase in body weight may be an increased muscle protein synthesis in response to a greater volume of weight-training (15) over a given period of time. Furthermore, creatine

may stimulate muscle protein FSR directly, as suggested by Ingwall and colleagues (18), and Young and Denome (19).

The purpose of this investigation was to examine the effect of acute creatine supplementation, on resting mixed muscle protein FSR. The specific hypothesis tested was that creatine supplementation would increase resting muscle FSR above and beyond that of the placebo group, as *in vitro* work has suggested (18-19). A secondary purpose was to investigate whether or not females respond in the same manner as males to creatine supplementation, as it has been shown in some studies that females do not show an increase in body mass significantly following supplementation (27-30).

Methodology

Subjects

24 healthy males and females volunteered to participate in the study (Table 4). All were kinesiology students, and had not used creatine as a supplement for at least six months prior to the study. For the analysis of FSR a sub-group of 14 (male-n=7, female-n=7) for the creatine treated was analyzed, and a subgroup of 8 (male-n=4, female-n=4) for the placebo treated was analyzed. The study was conducted under the approval of the Human Ethics Committee of McMaster University, and all subjects provided written, informed consent prior to participating.

Experimental Protocol

All subjects completed 3 d food records (including one weekend day) from which each individual's mean daily energy and protein intake was calculated using a commercially available computer program (Nutritionist V, San Bruno, California). Based on this information, an individual's isoenergetic diet was designed for the experimental trials. Lean body mass measurements were made prior to and after the intervention using dual energy x-ray absorptiometry (DEXA; Hologic QDR-1000/W). On each occasion measurements were made at the same time of day.

The subjects did not perform any strenuous exercise for two days before the experiment. At 1600 h the subjects reported to the laboratory. A 22 – gauge plastic catheter was inserted into an antecubital vein, and a baseline blood sample was obtained for alpha – ketoisocaproic acid (alpha KIC) background enrichment. An expired gas sample was collected, simultaneously, and used for background enrichment of $^{13}CO_2$. Gas samples were collected into 60 L bags with duplicate samples injected into 10 ml evacuated tubes for subsequent analysis of $^{13}CO_2/^{12}CO_2$ ratios, to be used for the calculation of whole body protein synthesis (not reported in this study). At t= -120min a priming dose of L-[1-^{13}C]leucine (1mg/kg) and [^{13}C] sodium bicarbonate (0.295 mg/kg) (both 99% atom percent; CDN Isotopes, Pointe Claire, Quebec) was administered over 1 min, followed by a constant infusion of L-[1-^{13}C]leucine (1 mg/kg) for 14 h, delivered by a calibrated syringe pump (Cole Parmer). The L-[1-^{13}C]leucine was diluted into sterile saline on the day of the infusion and the [^{13}C] sodium bicarbonate was diluted immediately prior to the infusion, both under aseptic conditions, and both were sterilized

by micro-filtration immediately prior to the infusion (0.2 um; Acrodisc). All subjects received both the leucine and the sodium bicarbonate from the same batch.

Blood samples were placed into heparinized, chilled tubes and centrifuged immediately, and the plasma was stored at -70°C until the analysis. At t = -15 min (before biopsy), and at t = 0 blood samples were taken to ensure that isotopic plateau had been reached. Blood samples were also taken at t = 4 h, t = 11.75 h, and t = 12 h. Breath samples were also taken simultaneously at these time points. Following the t = 0 collection subjects rested over night while being infused. A protein free snack was provided for each subject during the infusion at approximately t = 3 h (crackers and diet coke). Percutaneous muscle biopsies, using a suction modified Bergstrom biopsy needle (Stille), were extracted from an incision in the *vastus lateralis* approximately 20 cm above the knee. This procedure was conducted under local (2% lidocaine) anesthesia. The first biopsy was taken at t = 0 h, and the second at t = 12 h in the opposite leg (Table 5). Muscle biopsies were frozen in liquid nitrogen, and stored at -70°C before analysis.

Following this trial, subjects were randomly assigned in a double blind manner to either a placebo group or a creatine group. The creatine group consumed 4x5g/d of creatine monohydrate (ISA, Mississauga, Ont) for 6 days, and then were put on a maintenance dose of 5g/day for 2-3 days before returning to the laboratory for their second infusion. Subjects in the placebo group consumed isocaloric quantities of a commercially available glucose polymer (Polycose) in the same manner as the creatine group. All subjects were instructed to consume their supplement dissolved in juice or a

soda beverage. Following the 8 or 9 d supplementation phase, subjects returned to the laboratory for a second overnight infusion as described above.

Prior to both infusions, subjects followed a meat-free checklist diet for two days based on their habitual caloric intake. On the day of the infusion subjects consumed a pre-packaged diet which contained no meat products.

Analytic Techniques

Mixed Muscle Protein Extraction Method

Approximately 25 – 30 mg of wet tissue was removed from storage in a -70°C freezer. With all apparatus and equipment pre-cooled with liquid nitrogen the muscle samples were pulverized using a mortar and pestle. 1.0 ml of 10% Trichloroacetic acid (TCA) was added to each tube, and vortexed for 10 sec. The samples were then incubated on ice for 10 min. Samples were then homogenized using a glass mortar and pestle, and centrifuged at 2500 rpm for 10 – 15 min. The supernatant was poured off and stored for tissue fluid (free pool) amino acid analysis. 2 ml of normal saline was injected into the sample pellet and vortexed to remove all residual TCA. Tubes were again centrifuged at 2500 rpm for 15 min in the same manner described above. The supernatant was again poured off, and 2 ml of saline added in the same manner previously described. This was repeated twice more to ensure that all TCA residue was removed. 1 ml of 6N HCl was added to each tube and vortexed for 10 seconds. Tubes were capped and heated at 110°C in a heating block for 24 h to hydrolyze protein bound

amino acids. Tubes were then removed from the heating block and allowed to cool to room temperature.

Amino Acid Isolation via Cation Exchange Chromatography

Disposable sep columns were primed by adding 2 ml of deionized water, and allowed to drain. 1 ml of a slurry of 100-200 mesh hydrogen cation exchange resin (Biorad AG 50W-X8), and 1M acetic acid was added to the column. The muscle hydrolysate was poured over the column, allowing the amino acids to bind the resin. The columns were then rinsed twice with 2 ml of 0.001N HCl to maintain the amino acids in a protonated state. The amino acids were then eluted off the resin into 4.0 ml pre-labeled Wheaton vials using 2 x 1.0 ml 6N NH_4OH rinses. Samples were then evaporated to dryness (~ 4 h) in a speedvac.

Leucine Derivitization

To make the leucine volatile it was chemically derivitized to its propyl-ester. The acid functional group is derivitized first by adding 200 ul of a combination of 3 parts 1-propanol and 1 part 12N HCl to the Wheaton vials. The samples were vortexed for approximately 10 sec and heated at 110° for 1 h. Samples were vortexed periodically during the hour. Samples were removed from the heat and rotary evaporated for 1 h, or until completely dry. The amino group was then derivitized by adding 200 ul of a mixture of 5 parts acetone, 2 parts triethylamine, 1 part acetic anhydride . Samples were then vortexed for 10 sec and heated at 70°C for 20 min with periodic vortexing during the

incubation. Samples were removed from the heat and dried under nitrogen for approximately 5 min, or until crystals formed. No more than 5 samples were dried at any one time as to avoid over-drying and losing the now volatile propyl-esters of the aa. When samples were ready to inject into the Isotope Ratio Mass Spectrometer (IRMS), the samples were resuspended in ~ 50 ul of ethyl acetate. Depending on the concentration of amino acids in the sample, the amount of ethyl acetate used to resuspend the sample was adjusted appropriately.

Alpha-Ketoisocaproic acid Preparation

1.0 ml cation exchange columns were primed by adding 1.0 ml of a 50%by volume slurry of 100-200 mesh cation exchange resin (Biorad AG 50W-X8 hydrogen form) and 1M acetic acid. 400 ul of sample plasma was added into the columns, collecting the eluate into disposable borosilicate glass culture tubes. The columns were washed with 2 x 1.0 ml aliquots of 0.001N HCl to ensure that all of the KIC was washed off of the column. 300 ul of 6N $HCLO_4$ was used to deproteinize the sample, which was then vortexed at 2800 rpm for 10 min. An additional drop of acid was added to ensure that all the protein had precipitated and the supernatant was then decanted into labeled screw top tubes and the pellet was discarded. 1.0 ml of a derivitizing reagent was added which was comprised of 100 parts o-phenylenediamine (OPDA) dihydrochloride salt (Sigma), 5 parts H_2O, and 15 parts 12N HCl. The samples were vortexed for 10 s and heated at 110°C for 45 min. Samples were removed from the heat and allowed to cool completely. Once the sample had cooled, 2.0 ml of CH_2Cl_2 was added and allowed to

extract for one minute. The aqueous phase (top layer) was discarded by vacuum and the tubes were placed in the -70°C freezer overnight to precipitate the remaining H_2O/HCl. The CH_2Cl_2 was decanted into pre-labeled 4.0 ml flat bottom vials and allowed to evaporate overnight in a ventilated hood. Samples were derivatized using 50 ul of a combination of 5 parts of BSTFA reagent to one part of pyridine by heating at 60°C for 15 min. Samples were then analyzed in a GC/MS.

High Energy Phosphate Analysis

After a muscle biopsy was taken, and divided into a piece for electron microscopy [~5 mg wet weight (ww)], and light microscopy (~50 mg ww), a sample (~10-30 mg ww) was frozen in liquid nitrogen (-170 °C). Samples were freeze-dried over night, and stored at –50 °C until subsequent analysis.

Muscle Powdering

Samples were powdered using two pairs of tweezers, altered such that the heads were bent at 45 degree angles for improved handling of the muscle. All powdering procedures were performed at 4 °C, with 15-30% of relative humidity to prevent rehydration of the muscle sample. Each sample was inspected for surface blood, and subsequently removed by carefully scraping the muscle. The sample was crushed between the tweezers and repeatedly rubbed together pulverizing the muscle into a fine powder. Periodic inspections were performed to ensure that all connective tissue was

removed. 5-10 mg of powder was weighed out into a 1.5ml polyethylene tube, for PCA extraction.

Metabolite Extraction

Muscle metabolites were extracted using 0.5 mol/l perchloric acid containing 1mmol/l EDTA.$2H_2O$ at a ratio of 800ul to every 10mg of powder for about 5 min on ice, while periodically vortexing. Extracts were then centrifuged for 5 min at 7000 rpm, and neutralized using 2 mol/l $KHCO_3$, for 5 min, while periodically vortexing. Extracts were then centrifuged for 15 min at 7000 rpm, and the supernate was stored in a 1.5ml polyethylene tube at –50 °C. 1ml of the extract was equivalent to 10mg of powder. No more than 10 samples were extracted at any given time.

Phosphocreatine and Adenosine Triphosphate

Extracts were assayed successively for ATP and PCr in the presence of 50.0 mmol triethanolamine buffer, pH-7.4; 1.0 mmol/ magnesium chloride, 0.5 mmol dithiothreitol, 100 umol glucose, 50.0 umol NADP, 0.02 U/ml glucose-6-phosphate dehydrogenase, 0.14U/ml hexokinase, ADP, and 25 U/mg creatine kinase. Both the creatine kinase and the hexokinase was stabilized using 10% BSA.

The assay was carried out in 13 x 75 glass screw top tubes using 10ul of sample to 1ml of reagent. The reactant solution was vortexed, and read at a wavelength of 340nm, in a Shimadzu fluorometer. For ATP analysis, 25ul of Hexokinase was then added, the tube was again vortexed, and incubated in the dark at room temperature for 30 min.

Samples were again read in the fluorometer. 20ul of creatine kinase was then added to the tubes, vortexed, and incubated in the dark at room temperature for 60 min. Samples were again read in the fluorometer. Following each incubation, enzyme activity was stable at its endpoint.

Creatine

Extracts were assayed for creatine in the presence of 50.0 mmol imidazole buffer, pH 7.4; 5 mmol magnesium chloride, 30 mmol potassium chloride, 25umol phosphoenolpyruvate, 200 umol ATP, 45 umol NADH, 0.240 U/ml lactate dehydrogenase, 0.75 U/ml pyruvate kinase, 3.6 U/ml creatine kinase. The creatine kinase was stabilized using 10% BSA.

The assay was carried out in 13 x 75 glass screw top tubes using 10ul of sample in 1ml of reagent. Following the addition of the sample to the reagent, the reactant solution was vortexed, and incubated at room temperature in the dark for 15min. Samples were then read in a shimadzu fluorometer (Taiwan) at a wavelength of 340nm. 25ul of creatine kinase was added to the sample, vortexed, and incubated at room temperature in the dark for 30 min. Samples were again read in the fluorometer. At the end of the second incubation enzyme activity was stable at its endpoint.

Intra-assay coefficient of variations for ATP and PCr were 4.0% and 5.4% respectively. Similarly intra-assay c.v. for creatine was 6.4%.

FSR Calculation

Fractional synthetic rate was calculated using the following equation:

$$FSR = \frac{[E2-E1] * 11}{KIC_E * T}$$

Where E = muscle enrichment at time 2(12h) and time 1(0h), KIC_E = alpha-KIC enrichment, and T = infusion time between biopsies (12h). The factor 11 is used to correct the values back to tracer/tracee enrichment from the values obtained by Gas-chromatography-combustion-isotope ratio mass spectrometry.

Statistical Analysis

All values are expressed as mean ± standard deviation. All analysis were performed using the STATISTICA statistical package (STATISTICA for Win v5.0). Alpha level was set at 0.05 for all statistical analysis. Differences in FSR, and muscular creatine concentration were analyzed using a 3-way, repeated measures mixed [Factor 1: Condition (2 levels: placebo; creatine); Factor 2: Gender (2 levels: male; female); Factor 3: Time (2 levels: pre; post)] analysis of variance (ANOVA). Differences in LBM were also analyzed using a 3-way, repeated measures, mixed [Factor 1: Gender (2 levels: male; female); Factor 2: Condition (2 levels: placebo; creatine); Factor 3: Time (2 levels: pre; post)] analysis of variance (ANOVA). Differences in subject characteristics were analyzed using a 1-way [Factor 1: Condition (2 levels: placebo; creatine)] analysis of variance (ANOVA) to ensure that our two groups were comparable. Where no differences were found between genders, data were collapsed over condition.

Results

Subject Characteristics

At baseline the groups were comparable with respect to age, height, body weight, %body fat, and LBM (Table 1). The age range of the subjects was 21-26 y for the females, and 20-31 for the males.

Plasma Alpha Ketoisocaproic Acid Enrichment

All subjects reached isotopic plateau by 120 minutes into the ^{13}C-leucine infusion. Isotopic plateau was maintained throughout the remaining 12 h of infusion (Fig 1).

Intra-muscular Creatine Values

There were no significant gender differences for intra-muscular free creatine ($p=0.27$; Male: pre – 54 ± 9.3; pst – 66 ± 9.2; Female: pre – 62 ± 10.3; pst – 68.1 ± 15.3) phosphocreatine ($p=0.17$; Male: pre – 72 ± 12.8; pst-70 ± 9.9; Female: pre-71 ± 13.2; pst-74 ± 14.5), total creatine ($p=0.8$; Male: pre-126.8 ± 11.6; pst-136.4 ± 11.5; Female: pre-132 ± 12.9; pst-143 ± 21.4), or ATP ($p=0.59$; Male: pre-20 ± 2.7; pst-21 ± 3.0; Female: pre-22 ± 2.2; pst-21 ± 2.0) following supplementation. There was also no gender differences at baseline for free creatine ($p=0.16$), phosphocreatine (0.7), total creatine ($p=0.2$), or ATP ($p=0.25$). Intra-muscular total Cr concentration increased 13.1% in the creatine supplemented group ($p=0.02$; Creatine: pre-129 ± 11.6; pst-146 ± 20; Placebo: pre-129 ± 13.2; pst-132 ± 10.8), and the total creatine concentration was 10.6% higher in the creatine group versus the placebo group following supplementation ($p>0.05$).

Phosphocreatine concentration increased 10% in the creatine treated group, however this was not significant ($p>0.05$). Following supplementation phosphocreatine concentration was higher in the creatine group (14.9%) than in the placebo group ($p=0.05$; Creatine: pre-70 ± 8; pst-77 ± 15; Placebo: pre-72 ± 16.7; pst-67 ± 4.9). Free creatine concentration increased 16.9% following creatine supplementation, however this was not significant as the placebo group also increased 14% ($p>0.05$; Creatine: pre-59 ± 10.1; pst-69 ± 12.8; Placebo: pre-57 ± 11.2; pst-65 ± 12.4) (Fig 2-4). Refer to table 1.

Pearson R correlations were performed to compare the changes in Cr, PCr, and total Cr concentrations to their pre supplementation values following supplementation. The correlation between PCr and its pre-supplementation value was $r= - 0.52$ ($p>0.05$). The correlation between the increase in free Cr, and its pre-supplementation value was significant at $r= - 0.61$ ($p<0.05$), and the correlation between total Cr and its pre-supplementation value was $r= -0.2$ ($p=0.2$) (Fig 5-7).

Table 1. Intramuscular Creatine Concentrations

Group		PCr (mmol/kg/dm)		Cr (mmol/kg/dm)		Total Cr (mmol/kg/dm)		ATP (mmol/kg/dm)	
		Pre	Pst	Pre	Pst	Pre	Pst	Pre	Pst
Creatine									
	Males	72.0 (6.7)	73.0 (12.5)	53.4 (6.7)	68.1 (10.0)	125.5 (7.9)	141.2 (12.4)	21.6 (2.3)	21.0 (3.2)
	Females	69.7 (9.8)	81.2 (18.3)	64.5 (10.2)	70.3 (15.8)	134 (13.5)	151.5 (25.6)	21.5 (2.1)	21.4 (2.4)
Placebo									
	Males	73.9 (18.3)	65.7 (3.5)	54.3 (12.2)	65.0 (8.6)	128 (15.6)	130 (7.7)	19.9 (3.0)	21.9 (2.9)
	Females	71.6 (16.6)	68.7 (5.8)	59.7 (10.5)	65.9 (15.6)	131.3 (13.1)	134.7 (13.2)	22.4 (2.4)	22.2 (1.5)

Body Mass Changes with Creatine Supplementation

There was no significant effect of creatine supplementation on LBM ($p>0.05$; Creatine: pre- 57.2kg ± 12.5; pst- 57.9 ± 13; Placebo: pre-51.7 ± 10.1; pst- 52.3 ± 10.7). Nor was there a significant effect of supplementation on TBM ($p>0.05$; Creatine: pre – 75.3kg ± 11.6; pst – 75.75kg ± 11.5; Placebo: pre – 68.5kg ± 5.9; pst – 69.2 ± 5.5). There was also no effect of supplementation between gender ($p>0.05$). There was, however, a main effect for time in both groups ($p<0.05$), as both the placebo and creatine groups increased significantly following supplementation (Figure 8-9). Refer to table 2.

Table 2. Body Composition

Group		Body Composition			
		TBM (kg)		FFM (kg)	
		Pre	Pst	Pre	Pst
Creatine					
	Males	87.3 (15.9)	88 (15.7)	66.9 (8.0)	67.9 (9.0)
	Females	63.3 (7.3)	63.5 (7.4)	44.5 (2.9)	45 (3.8)
Placebo					
	Males	76.1 (8.3)	76.6 (7.6)	63.0 (3.6)	63.8 (3.4)
	Females	61.0 (3.5)	61.8 (3.4)	46.0 (4.1)	46.5 (4.8)

Mixed Muscle Fractional Synthetic Rate

There was no significant difference in resting muscle protein fractional synthetic rate between males and females ($p>0.05$; Males: pre – 0.05885 %/h ± 0.02; pst – 0.058 %/h ± 0.023; Females: pre – 0.0613 %/h ± 0.02; pst – 0.0728 %/h ± 0.015). Females resting baseline FSR was 3.9% higher than the females baseline FSR. Following creatine supplementation males resting FSR was 8.1% higher than females. There was also no difference between the placebo and creatine groups ($p>0.05$; Placebo: pre – 0.063 %/h ± 0.02; pst – 0.071 %/h ± 0.016; Creatine: pre – 0.056 %/h ± 0.02; pst – 0.058 %/h ± 0.023) (Fig 10-14). Refer to table 3. At baseline, FSR for the placebo group was 11% higher than the creatine group. Following supplementation, FSR for the placebo group was 18% higher than the creatine group.

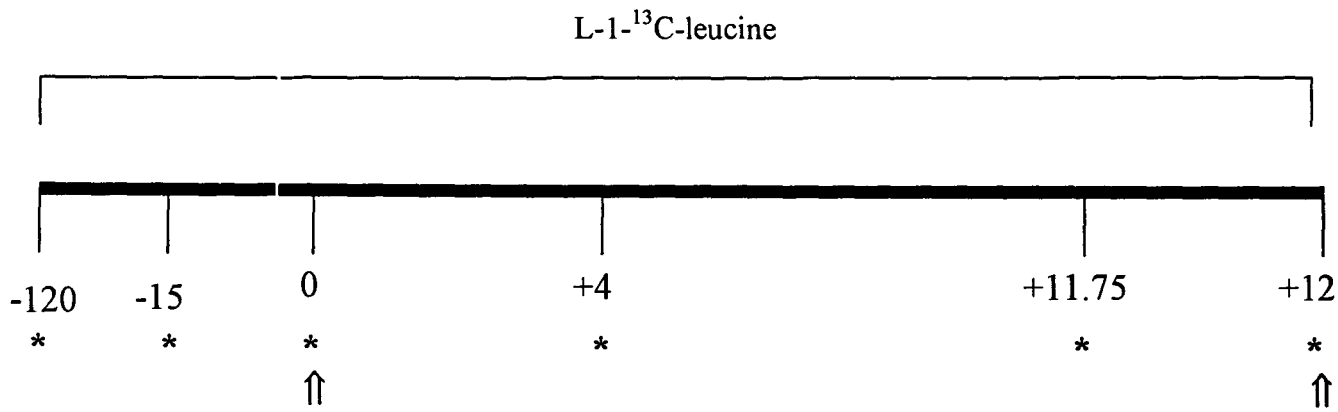
Table 3. Mixed Muscle Fractional Synthetic Rate

Group		FSR (%/h)	
		Pre	Pst
Creatine			
	Males	0.0553 (0.02)	0.0614 (0.02)
	Females	0.0572 (0.02)	0.0564 (0.02)
Placebo			
	Males	0.0624 (0.03)	0.0546 (0.02)
	Females	0.0654 (0.01)	0.0892 (0.02)

TABLE 4. SUBJECT CHARACTERISTICS

	Age (yr)	Height (cm)	Mass (kg)	%BF	LBM (kg)	Kcal/Kg/d
Placebo (n=8)	23.4 (2.1)	176.25 (13.6)	69 (4.3)	16.2 (3.3)	54.5 (2.6)	36.8 (14.2)
Creatine (n=14)	22.4 (4.7)	174 (11.4)	75.3 (10.4)	15 (2.9)	55.6 (3.7)	30.76 (10.9)

TABLE 5. **Testing Protocol**



* - blood and gas samples

⇑ - muscle biopsy

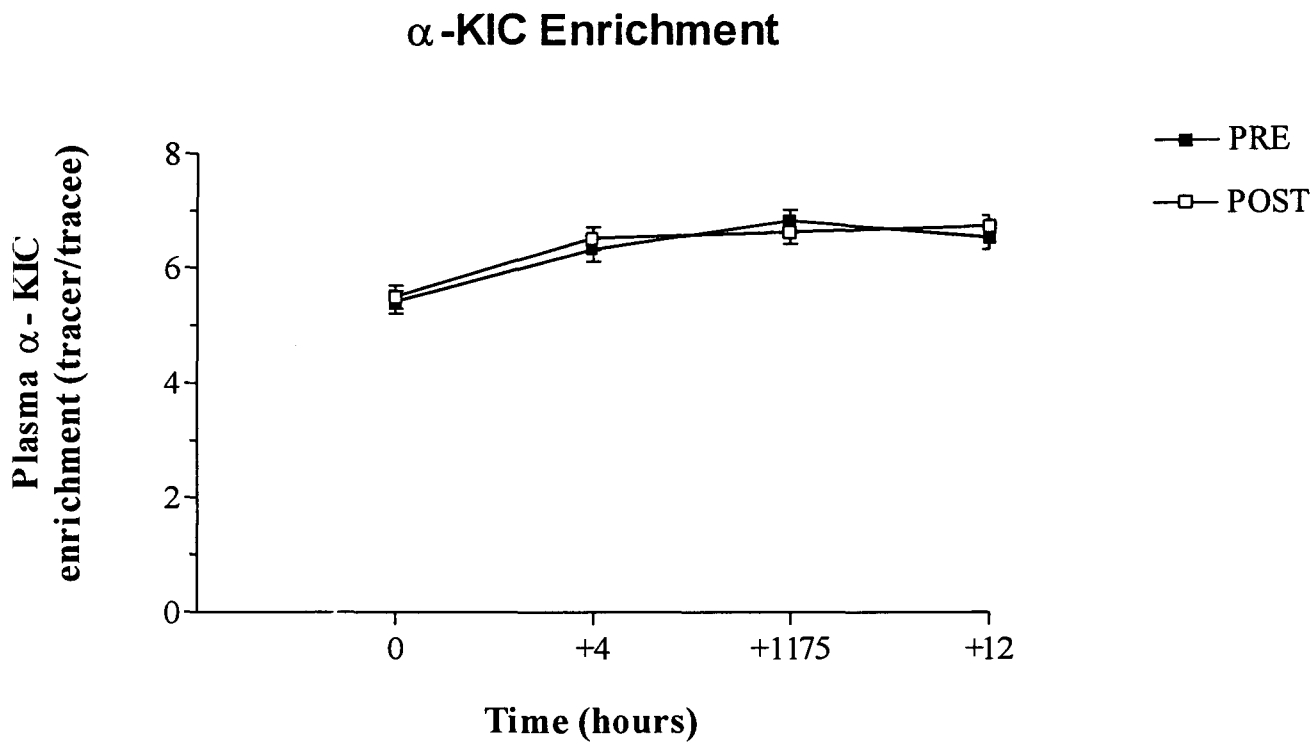


Figure 1. Alpha KIC enrichment during both trials for both the Cr and placebo group.

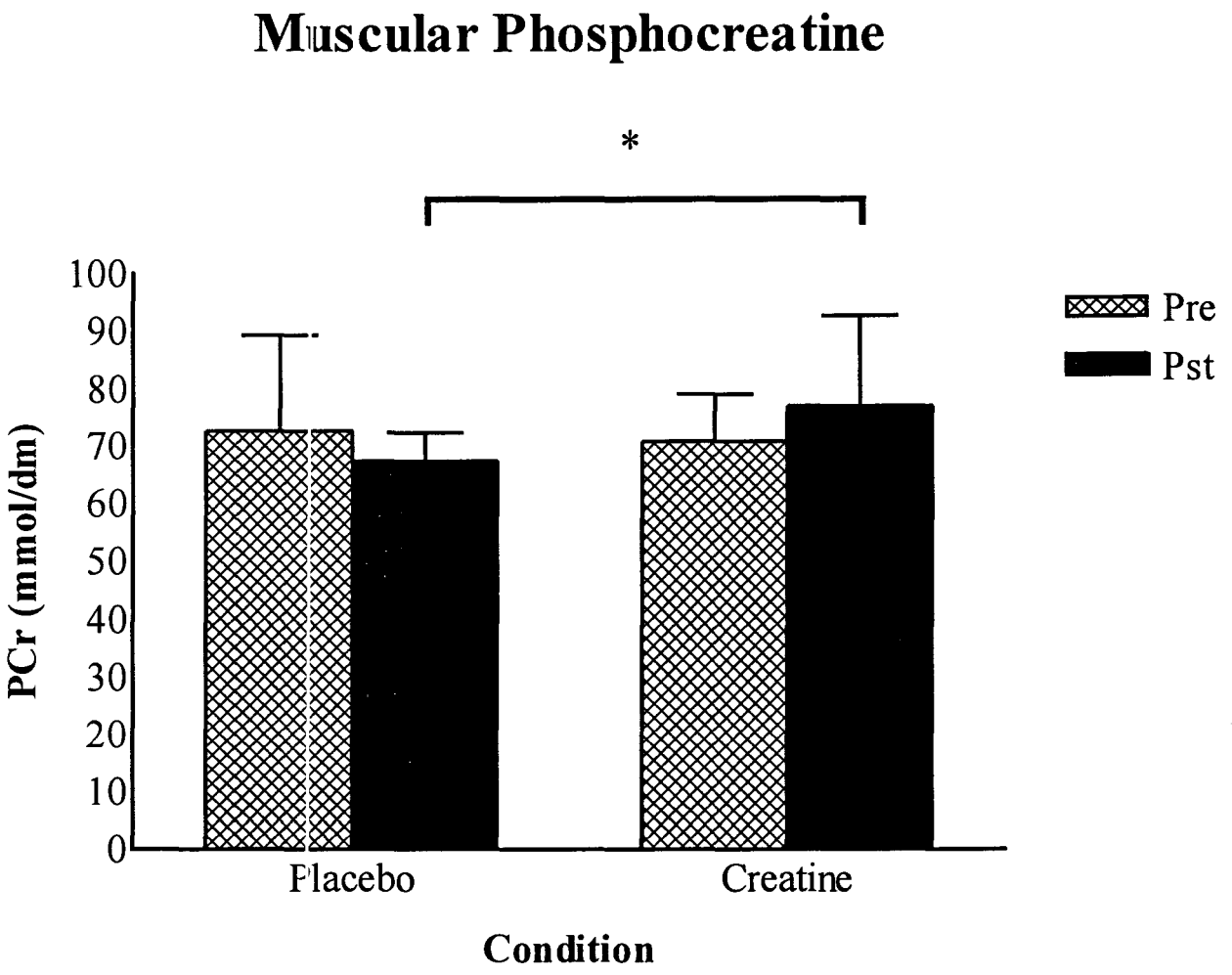


Figure 2. Muscular Phosphocreatine before and after supplementation in both groups expressed in mmol/dm. The symbol *, denotes a significant ($p<0.05$) difference between PCr in the creatine group, and PCr in the placebo group following supplementation.

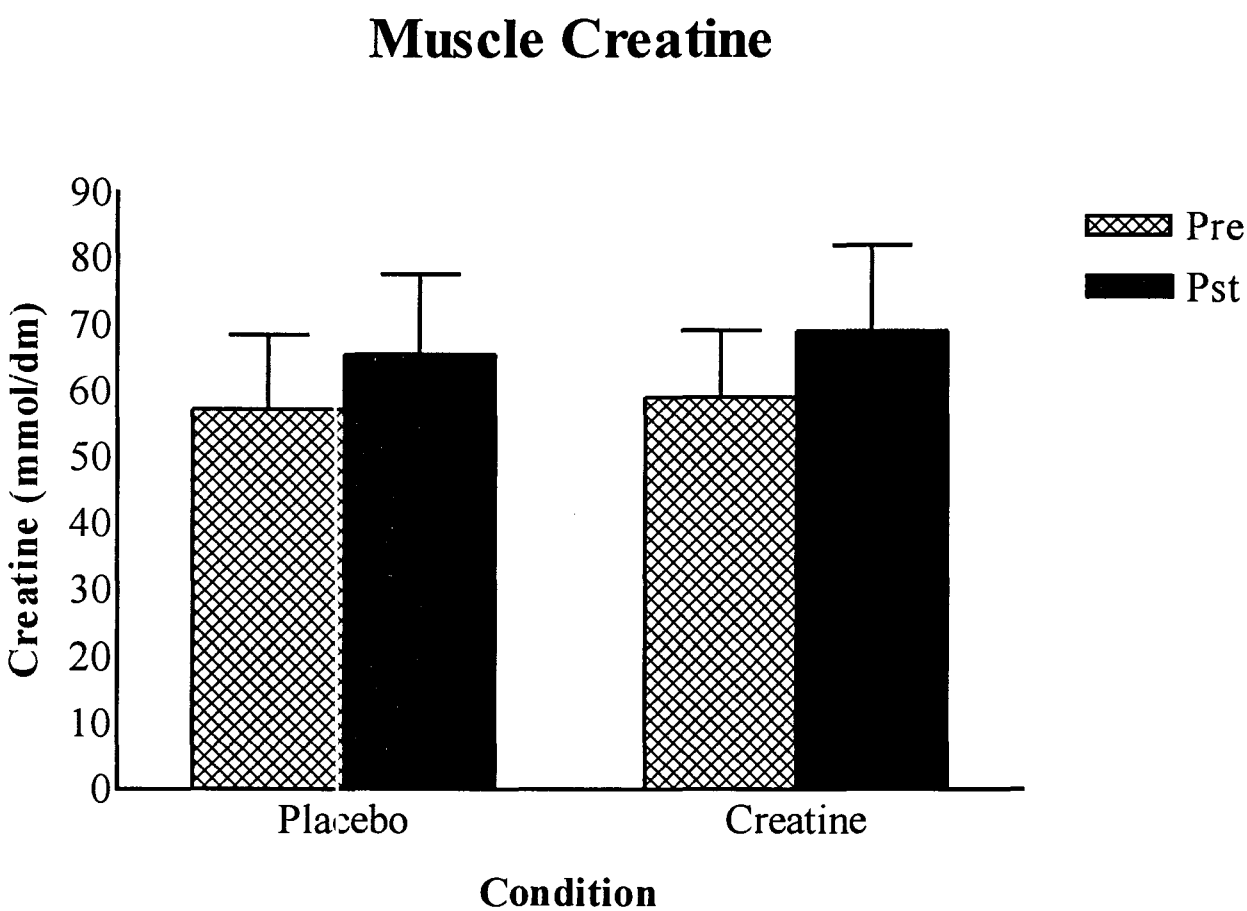


Figure 3. Muscular creatine before and after supplementation in both groups expressed in mmol/dm.

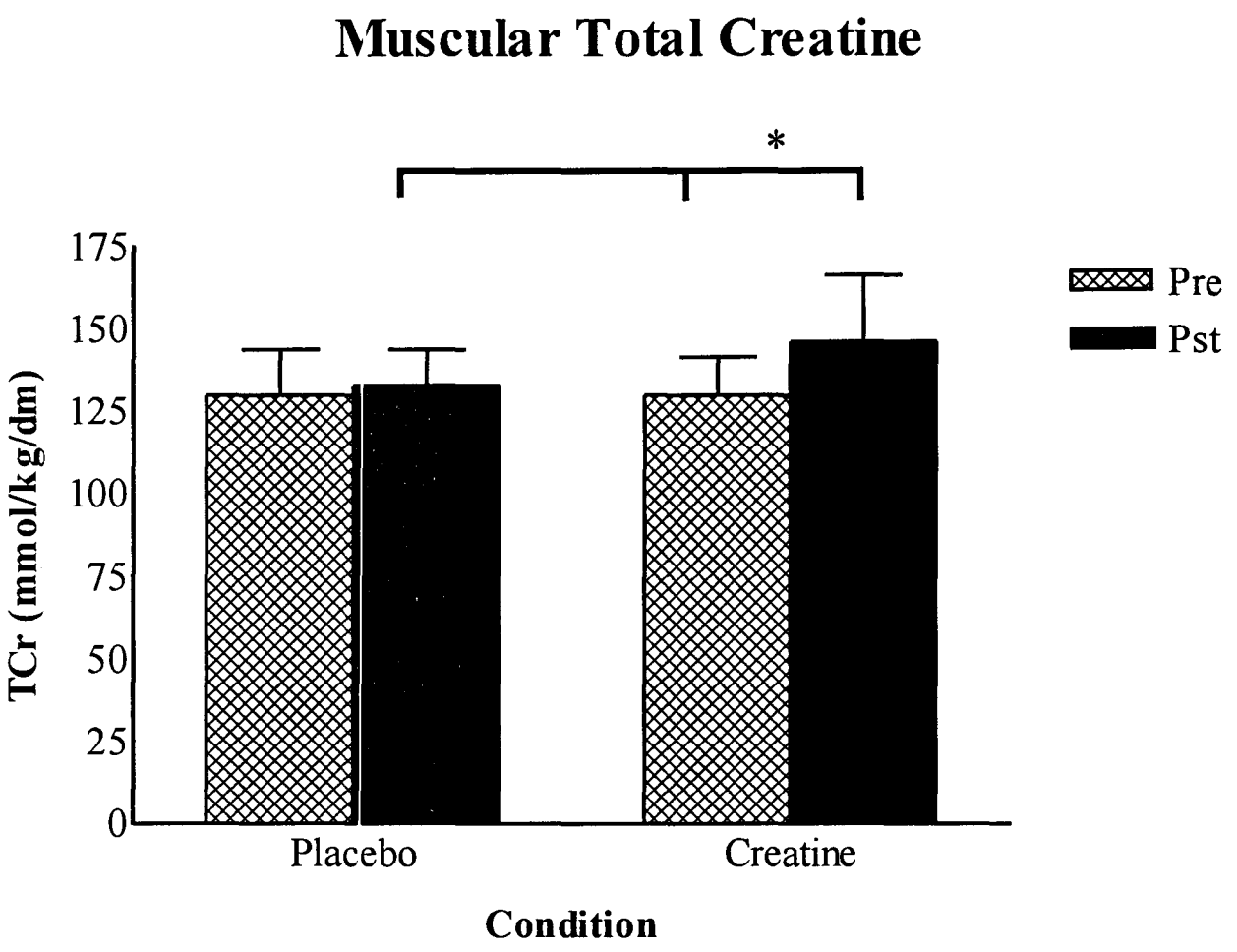


Figure 4. Muscular total creatine before and after supplementation expressed in mmol/dm. The symbol *, denotes a significant ($p<0.05$) difference between the creatine and placebo group following supplementation for total creatine, and a significant change in the creatine supplemented group pre to post.

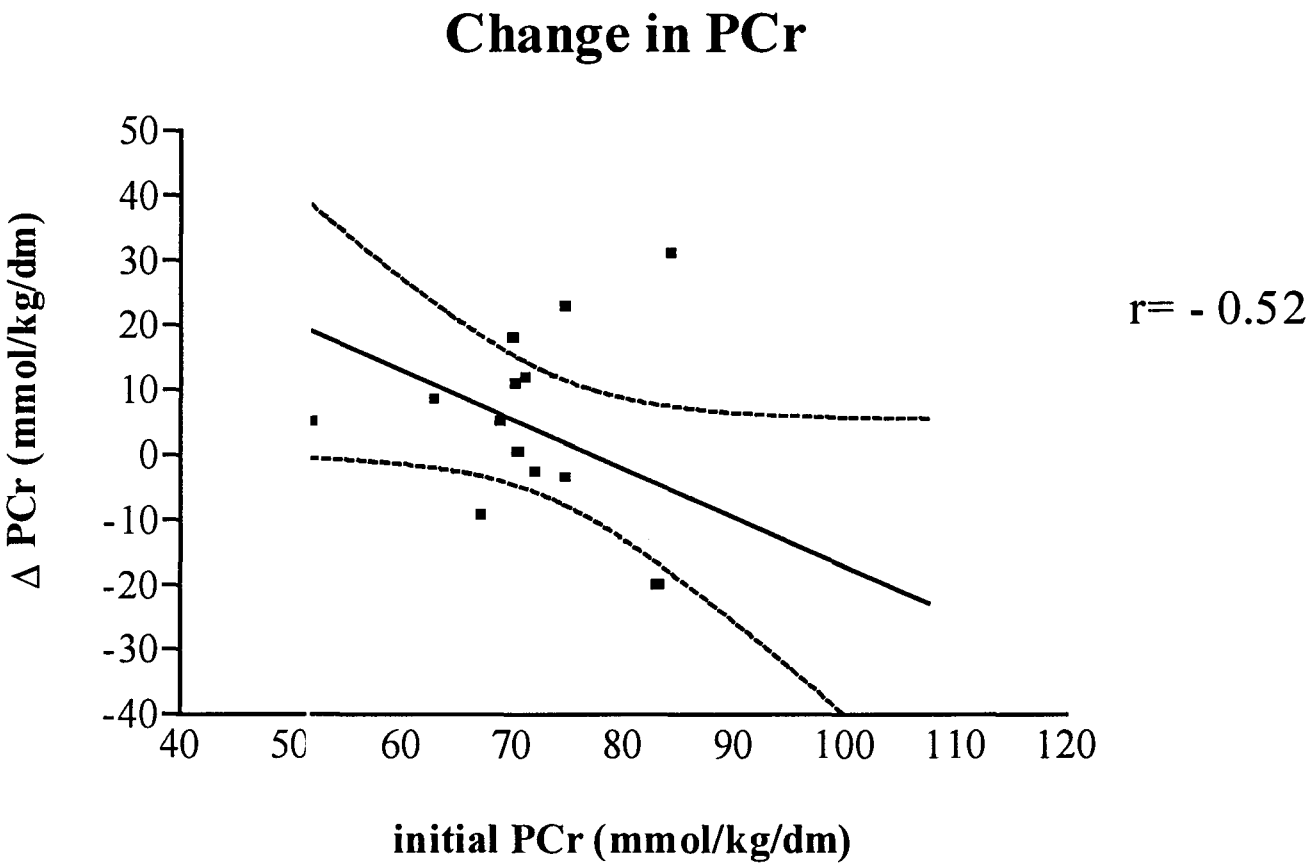


Figure 5. Correlation between initial PCr and post supplementation values expressed in mmol/kg/dm ($p>0.05$).

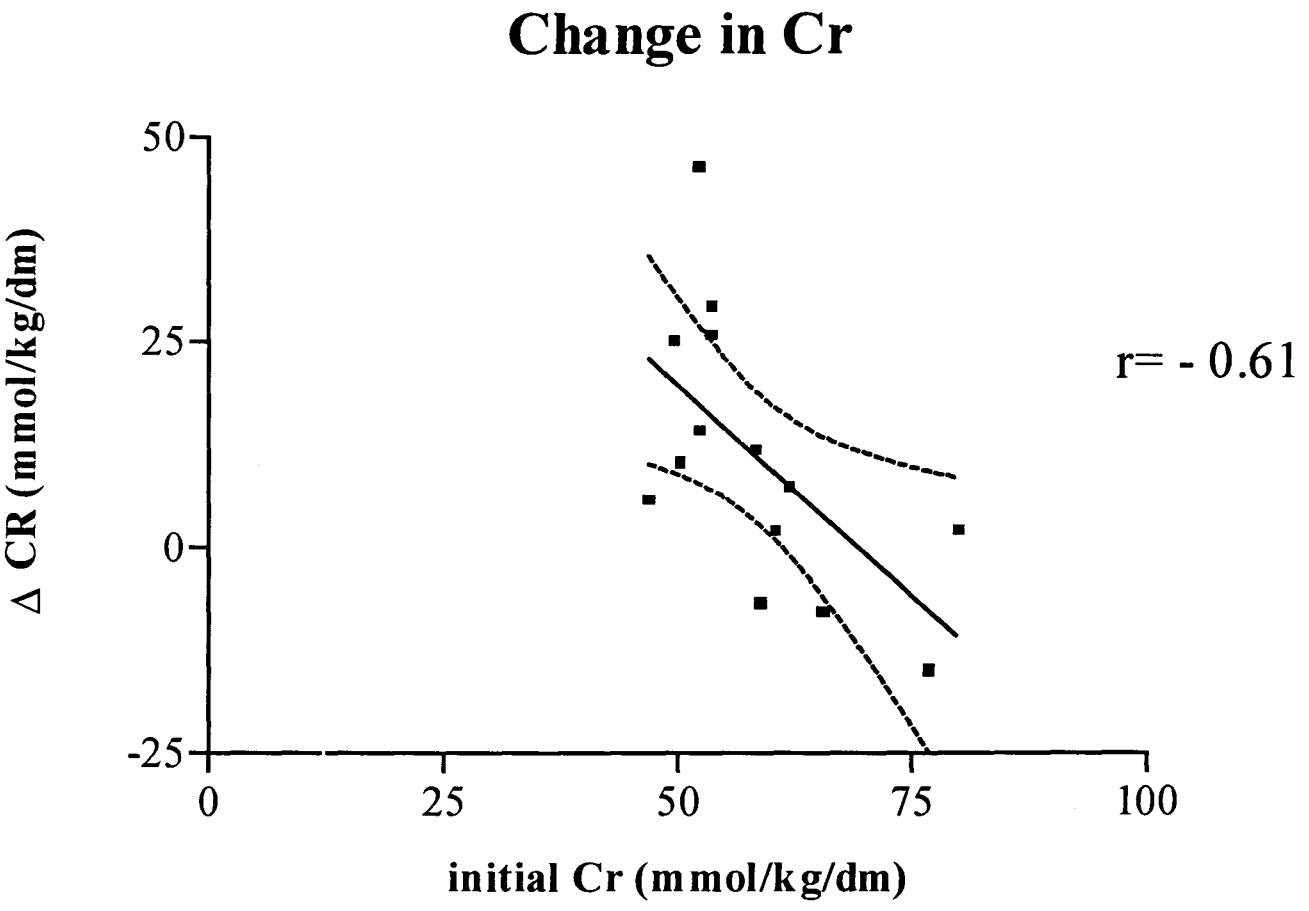


Figure 6. Correlation between initial Cr and post supplementation values expressed in mmol/kg/dm ($p<0.05$).

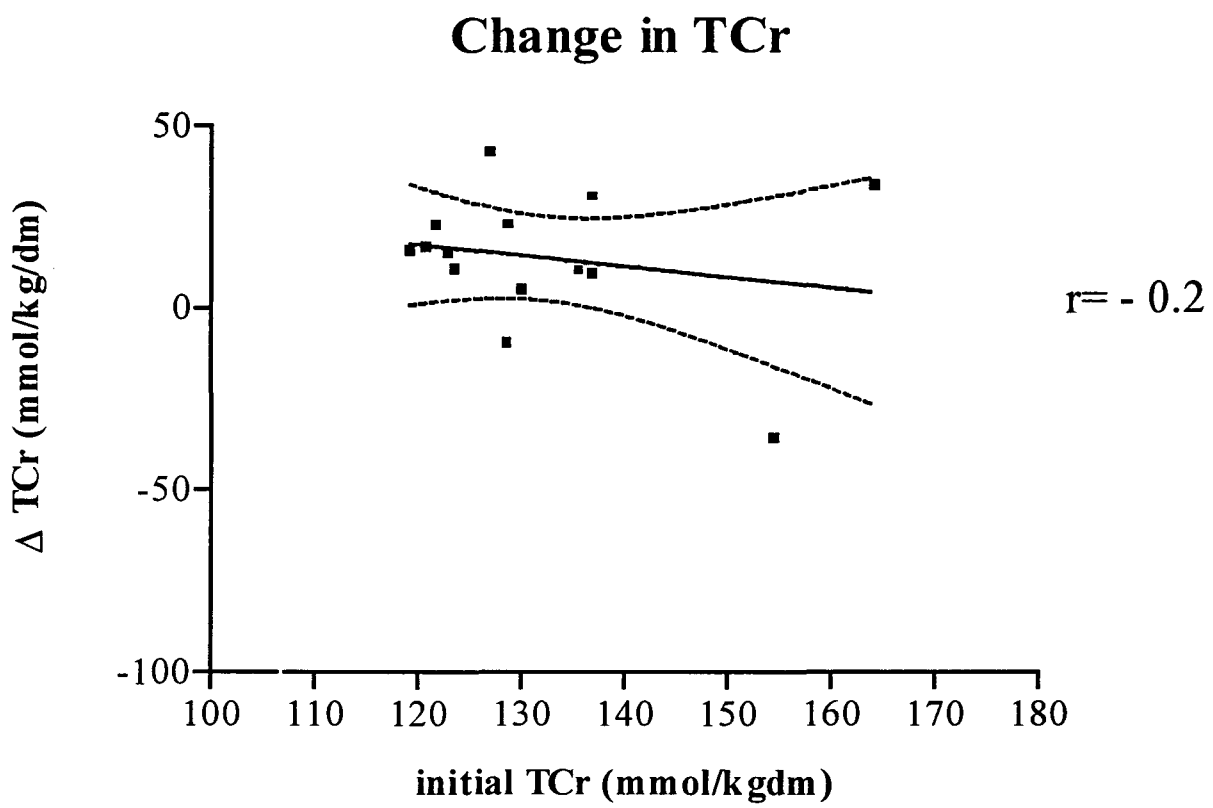


Figure 7. Correlation between initial TCr and post supplementation values expressed in mmol/kg/dm ($p>0.05$).

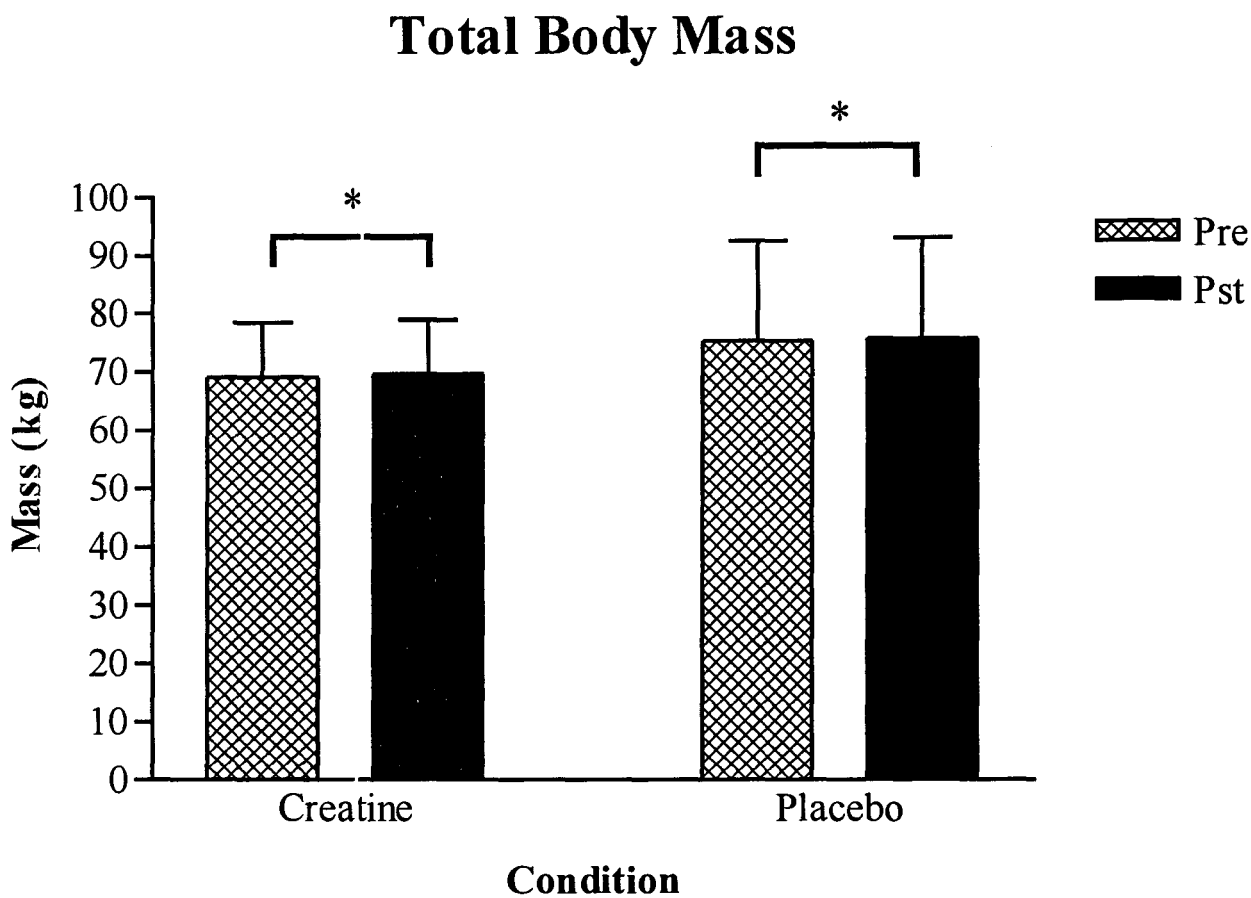


Figure 8. Total body mass changes expressed in kg following supplementation. The symbol *, denotes a significant change ($p<0.05$) pre to post in both groups for total body mass.

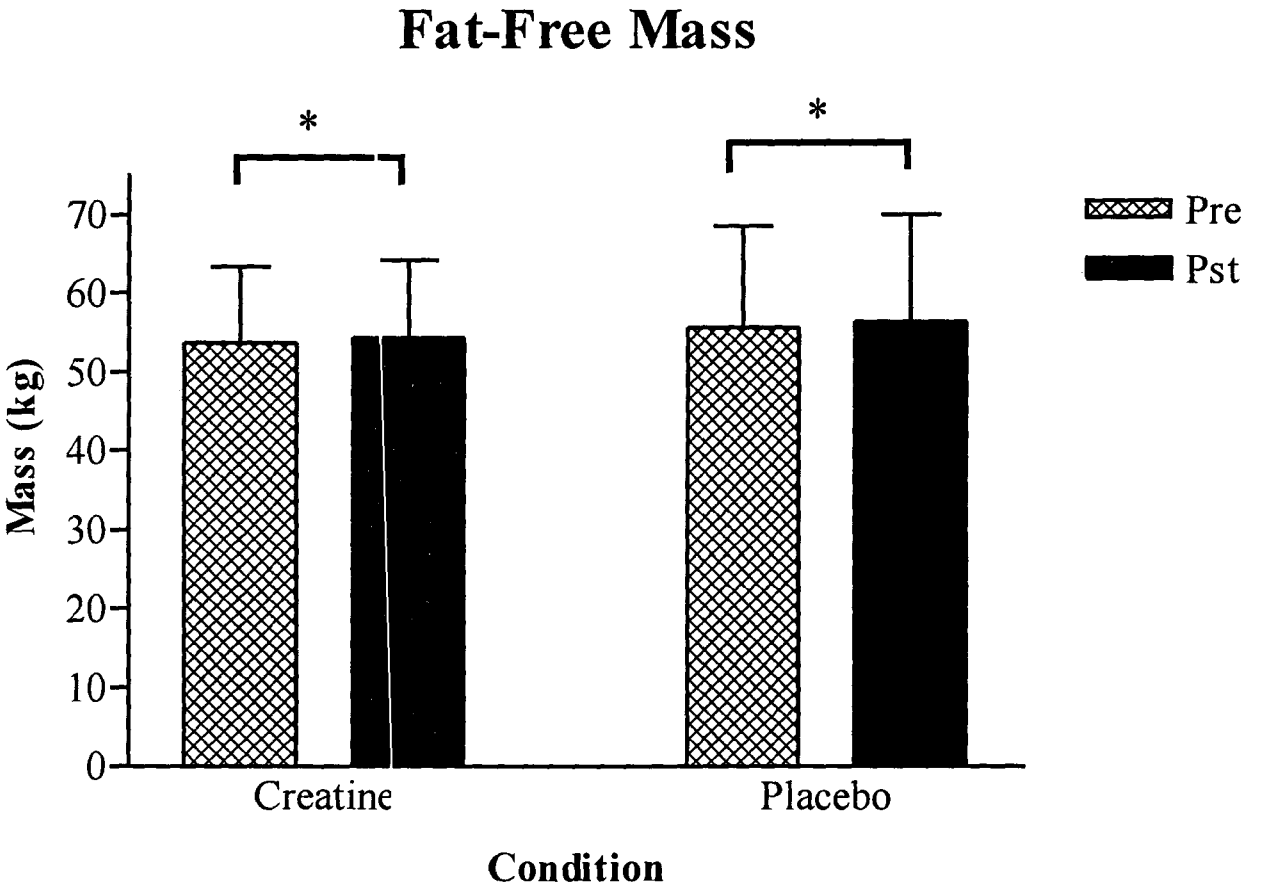


Figure 9. Fat-free mass changes following supplementation, assessed by DEXA, expressed in kg. The symbol *, denotes a significant ($p<0.05$) change pre to post in both groups following supplementation.

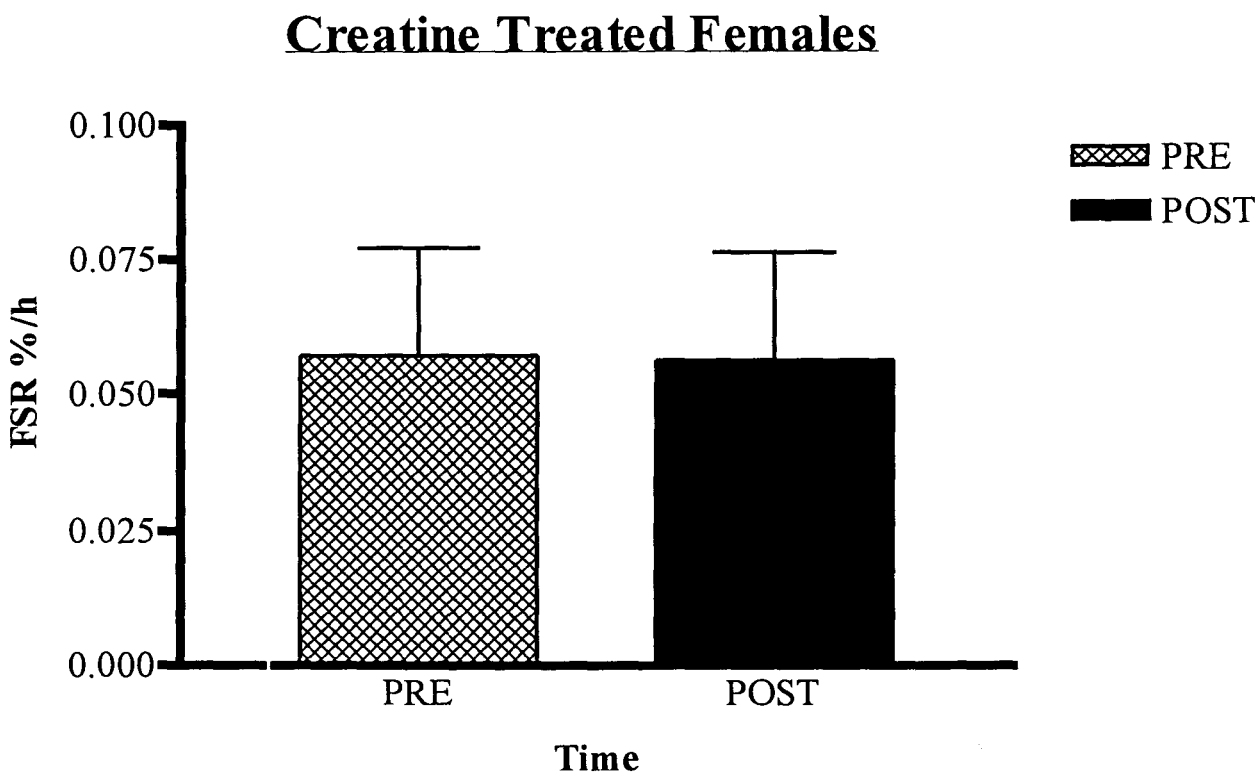


Figure 10. Creatine treated females resting mixed muscle protein FSR expressed in %/h.

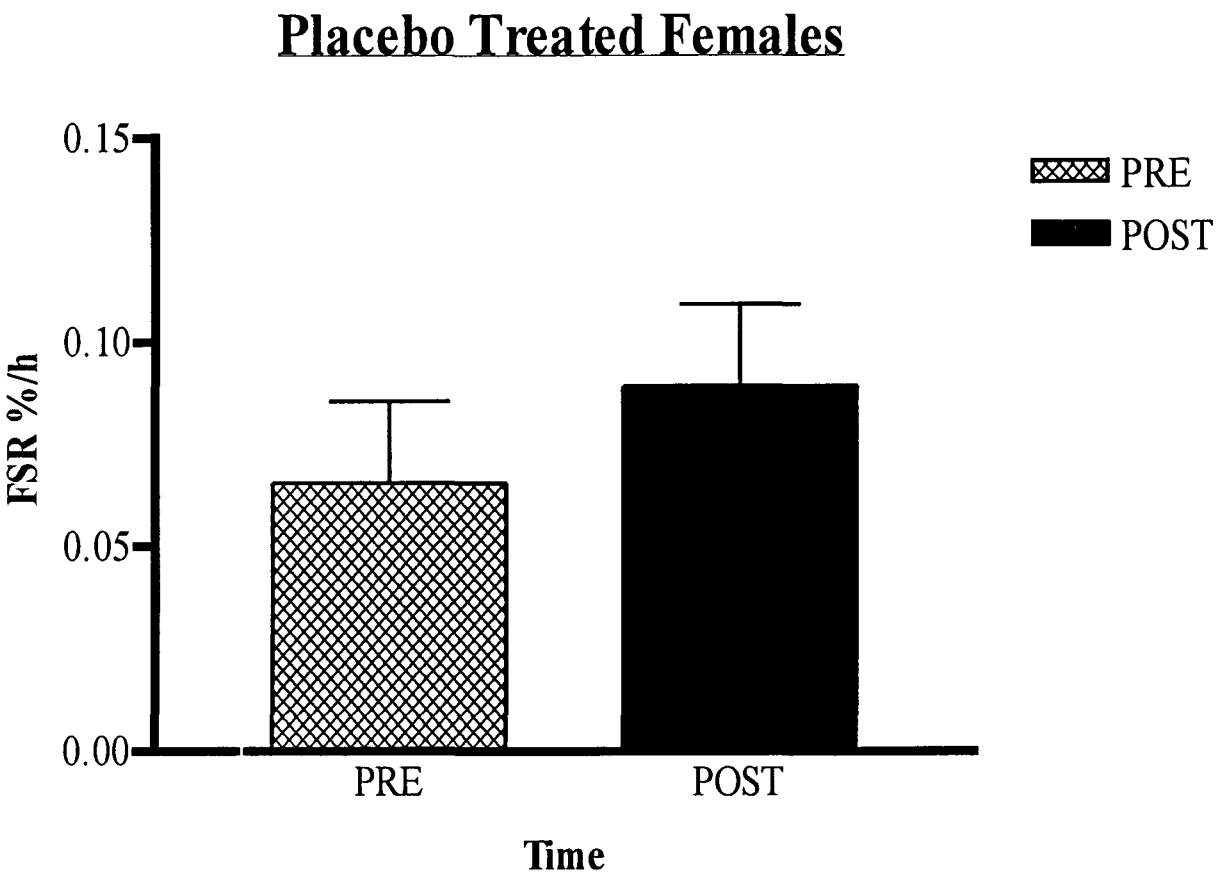


Figure 11. Placebo treated females resting mixed muscle FSR expressed in %/h.

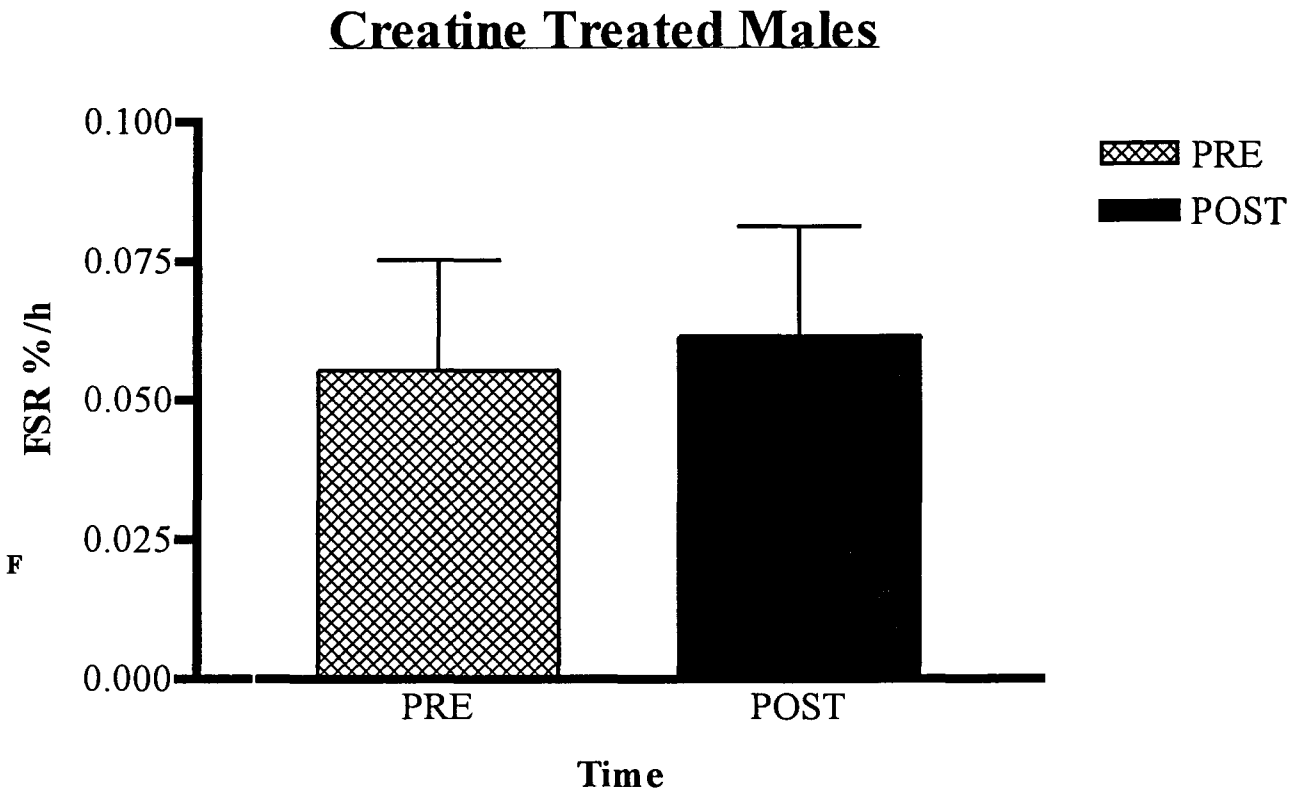


Figure 12. Creatine treated males resting mixed muscle protein FSR expressed in %/h.

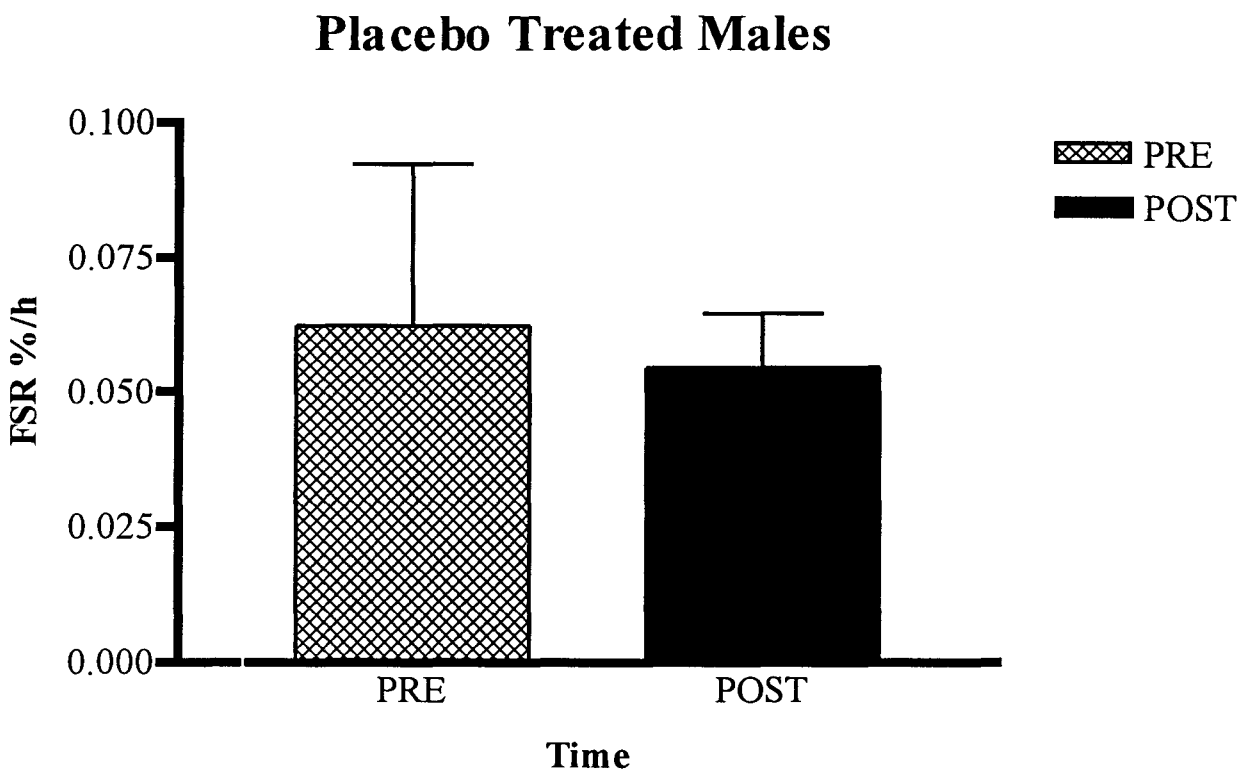


Figure 13. Placebo treated males resting mixed muscle protein FSR expressed in %/h.

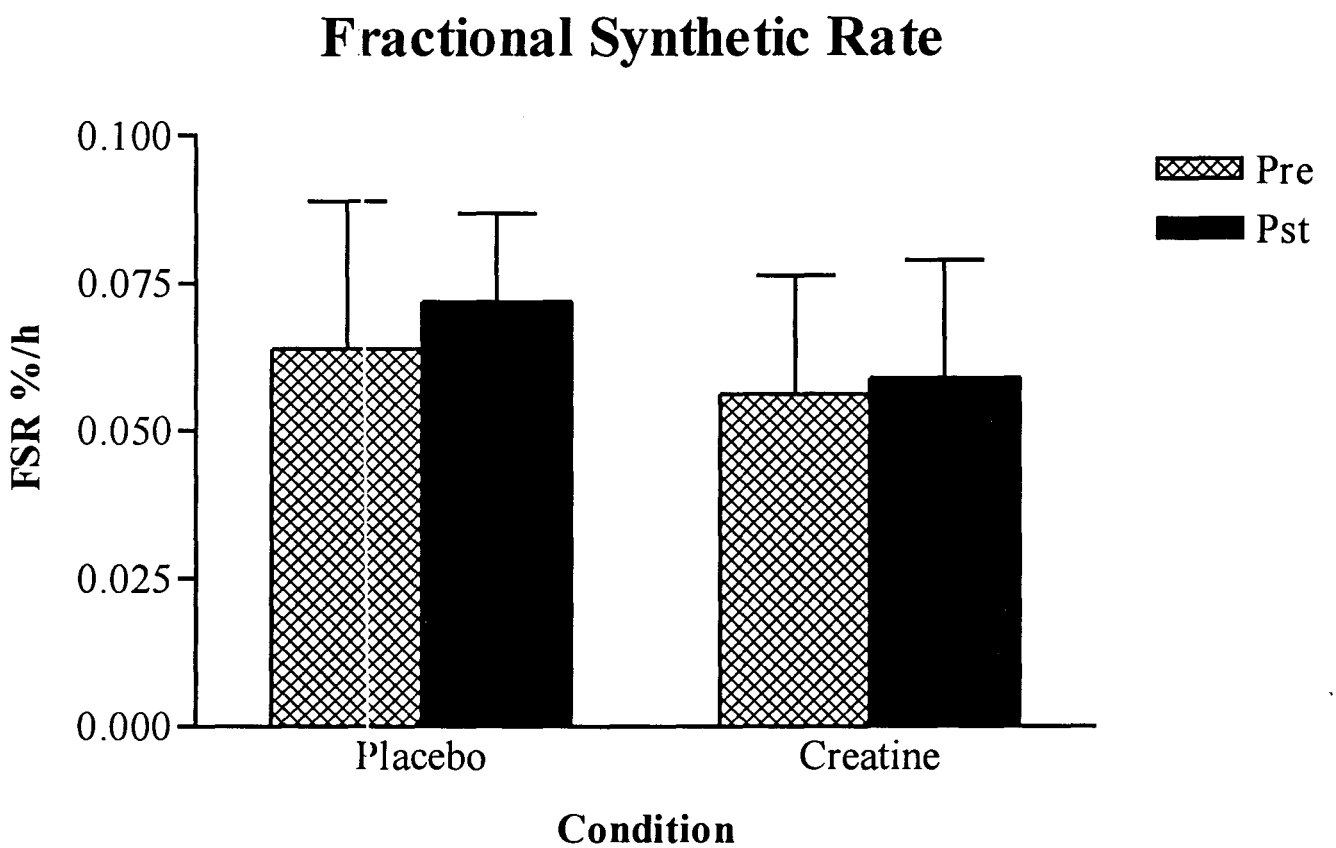


Figure 14. Placebo and Creatine muscle protein FSR expressed in %/h.

Discussion

In this study we determined that creatine supplementation (20g/d) for 7 d had no effect on resting mixed skeletal muscle protein FSR in humans, which does not support the literature which suggests that creatine may stimulate contractile protein FSR (18). Ingwall (18), as well as Young and Denome (19), have reported that when myotubes were incubated in a creatine rich medium, myofibrillar protein synthesis is significantly increased. In Ingwall's (18) experiments, myosin and actin specific synthetic rates were established, whereas in the present study mixed muscle protein synthesis was measured. Interestingly, Ingwall (18) reported an increase in actin and myosin specific protein synthesis in response to exposure to creatine *in vitro*, however, total protein synthesis was not reported to be significantly increased. These results (18) would suggest that creatine acts specifically to increase contractile protein synthesis. In vivo, however, it has been established by Yarasheski and colleagues (26) that mixed muscle protein synthesis, and myosin and actin specific protein synthesis rates respond in a directionally similar manner to the physiological stresses of exercise

Ingwall (18) had suggested, creatine may act as a chemical signal for transcriptional regulation, thus, mRNA analysis *in vivo* might be a more sensitive mediator of the potential for increasing muscle protein FSR. Under these circumstances, creatine may increase the mRNA for myofibrillar protein, however, there may be a time delay between transcription and an up-regulation of FSR. In contrast, it has been shown that the exercise-induced acute increase in FSR is mediated at the translational level (27).

Thus, if creatine increased the mRNA for myofibrillar proteins, the potential increase in FSR may not be realized over the duration of the current study.

Many *in vivo* studies examining creatine supplementation, have reported significant weight gains following acute creatine supplementation (4, 5, 9, 11), however, the source of the weight gain has not been elucidated. Our results are in accordance with the suggestion of Hultman and colleagues (6), who reported a significant decrease in urine excretion while subjects supplemented creatine, implying that the creatine induced water retention. In the present investigation, muscle protein FSR at rest was not elevated following acute creatine supplementation, therefore the increase in weight is likely attributable to increased intramuscular water retention.

In the present study, total Cr concentrations were significantly elevated following creatine supplementation of 20g/d for 7 d. Increases were in a similar magnitude to those shown by Harris and colleagues (5), and it was apparent that those with initially lower free Cr concentrations increased to a greater extent as suggested by Harris (5), and Odland (28). Despite the higher concentrations in muscular PCr, and total Cr concentrations following supplementation, no increases in muscle protein FSR resulted, suggesting that there is no relation between Cr concentrations and FSR. This of course, may be precluded by the nature of the measurement and the inherent variability using the FSR technique to determine muscle protein synthetic rate.

The question of whether or not a combination of creatine and resistance training would increase muscle protein synthetic rates above that of resistance training alone has not yet been answered. It has been shown by Kreider (14), and Vandenbergh (13) that

the administration of creatine during a strength training program resulted in a greater increase in LBM. Since creatine supplementation has been implicated as playing an integral role during high intensity activity in potentiating performance (3), and this type of exercise can stimulate protein synthesis (17), creatine supplementation may act indirectly to stimulate protein synthesis by allowing a greater volume of training. The literature provides several lines of evidence supporting the idea that acute creatine supplementation has a performance enhancing effect (1). Thus, it likely that this acute effect may persist during long term supplementation, which would allow one to work at a higher intensity on a daily basis. Since the present study shows that PCr concentration was significantly higher in the Cr group compared to the placebo group following supplementation, then an increase in total lifting volume may be possible. This claim is supported by Gordon *et al.* (29) who showed that an increase in PCr concentration was directly related to performance during one legged cycle ergometry in patients with congestive heart failure. Both 1-legged cycling endurance and peak torque were reported to increase significantly following supplementation. Thus, a greater total lifting volume secondary to Cr supplementation might result in greater total protein accretion over a given period of time.

Although cellular hydration was not measured in this experiment, and thus the hypothesis of cellular swelling on protein metabolism was not tested, it is unlikely that creatine would stimulate protein synthesis via an increase in cell water. In the present study LBM increased 0.7kg following Cr supplementation, this however, was non-significant, and thus it is difficult to decipher whether or not we induced any

intramuscular water retention in the present study. In a study examining the effect of hydration on protein synthesis Haussinger and colleagues (15), reported that patients in varying diseased states showed a positive correlation between cellular hydration and nitrogen balance. The results by Haussinger and colleagues (15), included only those in a hypovolemic and euvolemic state, and thus did not really address the question as to whether this correlation extends beyond euvolemia to those in a hyper-hydrated state. There have been several studies that have investigated cellular swelling as either an anabolic or anti-catabolic stimulus for protein. Few have shown an effect of cell swelling as a stimulator for synthesis (16), yet several have reported an anti-catabolic effect of increasing cell volume (26, 27, 28). However, there have been no reports supporting that increased cellular water acts as a stimulus for increasing muscle protein metabolism.

One issue could be the difficulties in extrapolating from data gathered on hepatocytes and mammarcytes to muscle, however it seems clear that a uniform directional change in this area of research does not exist. The manner in which muscle cells respond to cell volume changes may not be the same as hepatocytes and mammarcytes. In fact, proteins in the same type of tissue may act directionally different in response to swelling. Quillard and colleagues (25) demonstrated that the response of to swelling in hepatocytes is highly dependent on the protein showing a decrease in phosphoenolpyruvate carboxykinase mRNA following exposure to a hypo-osmotic solution.

Since the effect of creatine on protein metabolism is still undeveloped, there are several lines of research which need to be clarified. The role of creatine in muscle

protein anabolism has been established by Sipila in 1981 (4), who reported increases in type II fiber area following one year of creatine supplementation in patients diagnosed with gyrate atrophy of the choroid and retina. It has been suggested (5) that those with initially low creatine levels were most likely to benefit from creatine supplementation. Since creatine may play a regulatory role in protein metabolism, it is possible that people with low resting [PCr] may demonstrate increases in muscle protein FSR with creatine supplementation.

The effect of creatine on resting muscle protein fractional synthetic rate has been measured in the present study, however, the effect of creatine on protein degradation remains unknown. It has been emphasized that it is important to consider breakdown rate when determining muscle protein kinetics(21). During an acute bout of exercise it appears that breakdown increases less than synthesis and returns to resting levels far sooner than synthesis, inducing a more positive protein balance following exercise (21). Creatine may attenuate muscle protein breakdown, and thus induce a larger increase in protein accretion over a given period of time. Vandenbergh (13), and Kreider (14) have both demonstrated that significant gains in LBM can be achieved when supplementing creatine while resistance training. If no interaction is observed between resistance training and creatine on muscle protein FSR, then FBR may be affected by creatine supplementation.

Although we report no change in mixed muscle FSR following short-term creatine supplementation, there still may be some benefit for athletes and patients to be supplementing their diet with creatine. There appears to be little doubt that creatine

induces a metabolic advantage (1), and there is some evidence supporting an interaction between creatine and exercise, which results in a greater lean body mass (13, 14). Further investigation is needed involving direct measurement of protein synthesis following long-term creatine supplementation and resistance training, to determine if creatine does induce an increased muscle protein FSR.

REFERENCES

1. **Ellington WR.** Phosphocreatine represents a thermodynamic and functional improvement over other muscle phosphagens. *J. Exp. Biol.* 143:177-94, 1989.

2. **Linder MC.** Nutritional Biochemistry and Metabolism with Clinical Applications. East Norwalk. Connecticut: Appleton & Lange. 1991, pp. 96.

3. **Juhn MS, and MA Tarnopolsky.** Oral creatine supplementation and athletic performance: a critical review. *Cli. J. Sport. Med.* 8(4):286-97, 1998.

4. **Sipila I, Rapola J, Simell O, Vannas A.** Supplementary creatine as a treatment for gyrate atrophy of the choroid and retina. *N. Engl. J. Med.* 304(15):867-70, 1981.

5. **Harris RC, Soderlund K, Hultman E.** Elevation of creatine in resting and exercised muscle of normal subjects by creatine supplementation. *Clin. Science.* 83:367-374, 1992.

6. **Hultman E, Soderlund K, Timmons A, Cederblad JG, and PL Greenhaff.** Muscle creatine loading in men. *J. Appl. Physiol.* 81(1):232-237, 1996.

7. **Earnest CP, Snell PG, Rodriguez R, Almada AL, and TL Mitchell.** The effect of creatine monohydrate ingestion on anaerobic power indices, muscular strength and body composition. *Acta. Physiol. Scand.* 153:207-209, 1995.

8. **Birch R, Noble D, and PL Greenhaff.** The influence of dietary creatine supplementation on performance during repeated bouts of maximal isokinetic cycling in man. *Eur. J. Appl. Physiol.* 69:268-270, 1994.

9. **Redondo D, Williams M, Dowling E, Graham B, Jones S, and A Almada.** The effect of oral creatine supplementation on running velocity. *Med. Sci. Sports Exerc.* 26:S23, 1994.

10. **Burke LM, Pyne DB, Telford RD.** Effect of oral creatine supplementation on single-effort sprint performance in elite swimmers. *Int. J. Sport Nutr.* 6(3):222-33, 1996.

11. **Balsom PD, Harridge SDR, Soderlund K, Sjodin B, and B Eckblom.** Creatine supplementation pre se does not enhance endurance exercise performance. *Acta. Physiol. Scand.* 149:531-533, 1993.

13. **Vandenbergh K, Goris M, Van Hecke P, Van Leemputte M, Vangerven L, and P Hespel.** Long-term creatine intake is beneficial to muscle performance during resistance exercise. *J. Appl. Physiol.* 83(6):2055-63, 1997.

14. **Kreider RB, Ferriera M, Wilson M, Grindstaff P, Plisk S, Reinardy J, Cantler E, and AL Almada.** Effects of creatine supplementation on body composition, strength and sprint performance. *Med. Sci. Sports. Exerc.* 30(1):73-82, 1998.

15. **Haussinger D, Roth E, Lang F, and W Gerok.** Cellular hydration state: an important determinant of protein catabolism and disease. *Lancet.* 341(8856):1330-2, 1993.

16. **Millar ID, Barber MC, Lomax MA, Travers MT, Shennan DB.** Mammary protein synthesis is acutely regulated by the cellular hydration state. *Biochem. Biophys. Res. Commun.* 230(2):351-5, 1997.

17. **Phillips SM, Tipton KD, Aarsland A, Wolf SE, and RR Wolfe.** Mixed muscle protein synthesis and breakdown after resistance exercise in humans. *Am. J. Physiol.* 273:E99-107, 1997.

18. **Ingwall J.** Creatine and the control of muscle-specific protein synthesis in cardiac and skeletal muscle. *Circ. Research.* 38(5):I115-I122, 1976.

19. **Young RB, and RM Denome.** Effect of creatine on contents of myosin heavy chain and myosin heavy chain mRNA in steady-state chicken muscle-cell cultures. *Biochem. J.* 218(3):871-6, 1984.

20. **Chesley A, MacDougall JD. Tarnopolsky MA, Atkinson SA, and K Smith.** Changes in human muscle protein synthesis after resistance exercise. *J. Appl. Physiol.* 73(4):1383-8, 1992.

21. **Phillips SM, Tipton KD, Ferrando AA, and RR Wolfe.** Resistance training reduces the acute exercise-induced increase in muscle protein turn-over. *Am. J. Physiol.* 276:E118-24, 1997.

22. **Biolo G, Tipton KD, Klein S, and RR Wolfe.** An abundant supply of amino acids enhances the metabolic effect of exercise on muscle protein.Am. *J. Physiol.*273 E122-E129, 1997.

23. **Biolo G, Maggi SP, Williams BD, Tipton KD, and RR Wolfe.** Increased rates of muscle protein turnover and amino acid transport after resistance exercise in humans. *Am. J. Physiol.* 268:E514-E520, 1995.

24. **Tipton KD, Ferrando AA, Phillips SM, Doyle DJr, and RR Wolfe.** Postexercise net protein synthesis in human muscle from orally administered amino acids. *Am. J. Physiol.* 276:E628-34, 1999.

25. **Quillard M, Husson A, Chedeville A, Fairand A, Lavoinne A.** Protein synthesis is involved in the modulation of the level of the phosphoenolpyruvate carboxykinase mRNA by changes in cell volume in isolated rat hepatocytes. *FEBS Lett.* 423(2): 125-8, 1998.

26. **Yarasheski KE, *et al.*** (1999).Personal Communication.

27. **Welle S, Bhatt K, Thornton CA.** Stimulation of myofibrillar synthesis by exercise is mediated by more efficient translation of mRNA. *J. Appl. Physiol.* 86(4):1220-5, 1999.

28. **Odland LM, MacDougall JD, Tarnopolsky MA, Elorriaga A, A Borgmann.** The effect of oral creatine supplementation on muscle [PCr] and short-term maximum power output. *Med. Sci. Sports. Exerc.* 29:216-9, 1997.

29. **Gordon A, Hultman E, Kaijser L, Kristjansson S, Rolf CJ, Nyquist O, C Sylv'en.** Creatine supplementation in chronic heart failure increases skeletal muscle creatine phosphate and muscle performance. *Cardiovasc. Res.* 30:413-18, 1995.

4.0 APPENDICES

APPENDIX A

Information and Consent Form

CREATINE SUPPLEMENTATION: EFFECTS ON MUSCLE PROTEIN SYNTHESIS AND MUSCULAR PERFORMANCE

INFORMATION AND CONSENT FORM

INVESTIGATORS:	DEPARTMENT:	CONTACT:
Dr. Mark Tarnopolsky	Medicine and Kinesiology	x24465
Mr. Sasa Mihic	Kinesiology	x27037
Mr. Dan MacLennan	Kinesiology	x27037
Mr. Gianni Parise	Kinesiology	x27037

PURPOSE:

Oral creatine supplementation has been a popular practice among athletes involved in anaerobic, short-duration, high-intensity activities (e.g. jumping, sprinting, weight-lifting etc.). It has been suggested that oral ingestion of creatine monohydrate of 20g per day for 5-6 days may benefit physical performance by enhancing volitional strength and anaerobic power. It has also been shown that this protocol is usually accompanied by an increase in body mass, and that males tend to gain significantly more weight than females. These changes in mass are likely due to greater water retention by the muscles, which suggests that lean tissue (fat-free mass) is affected. Indeed, a recent

work in our lab (Mihic and colleagues, unpublished data, 1997) has demonstrated that the increase in whole body weight was primarily due to increased lean body mass. Body fat was not affected by the supplementation. Therefore, this study will try to address the following questions:

1. Does creatine supplementation have an effect on muscle protein synthesis?
2. Will creatine supplementation benefit muscle performance in short-term, high-intensity activities?

OUTLINE:

You will be one of the 28 male and female volunteers participating in the study. After having recorded your 4-day dietary intake, you will first be familiarized with the performance testing. You will also be asked to abstain from exercise on specific days during the trial.

Following an overnight fast, you will report to the Human Performance Laboratory in IWC for the performance protocol (PPRE). You will undergo the following tests: a) 60- sec. handgrip strength test b) a 2-min strength test by the m.tibialis anterior (front of your shin - lifts your toes off the ground) c) knee extension fatigue protocol (Cybex apparatus), and d) two consecutive 30 sec. all-out bike sprints (Wingate), interspersed with a 4 min. recovery. Prior to and following exercise, small amounts of blood will be sampled (~10ml) through a plastic catheter. The catheter will have been

inserted into the antecubital vein of your dominant arm by a trained, certified lab member.

After a three-day rest, you will undergo a stable isotope infusion protocol (ISOPRE). Prior to and on the day of infusion, it will be required that you eat pre-packaged food designed to mach your habitual intake. A prime (1mg/kg body mass) dose of sterile amino acid (leucine) tracer will then be infused via a plastic catheter (see above), followed by a constant infusion (1mg/kg/hr) for the next several hours. Another catheter will be inserted into the other arm for blood collection at different time-points (total amount ~120ml). ~90 min. after the onset of the infusion, Dr. Tarnopolsky will perform a muscle biopsy on the outer portion of your leg (m. vastus lateralis). As well, expired gas will be collected a number of times. The infusion protocol will be completed following another muscle biopsy of the contralateral m.vastus lateralis. A 24hr urine collection will again take place on the day of testing.

On the following day, you will have your body composition (fat-free mass, body fat %, bone mineral density) assessed by DEXA (x-ray scan). At this point you will be supplemented with either creatine (CR) or placebo (PL; sugar-like substance), as assigned in a randomized, double-blind fashion. The substance will be ingested 4 x 5g/d x 5d, and 5g/d for the subsequent 5 days (total of 10 days). To control for dietary creatine intake, the consumption of food will be pre-determined (check-list diet) for the duration of the supplementation.

On Day 6 of the protocol, you will again perform a performance test, identical to that previously described. You will undergo another DEXA scan on Day 9. On Day 10,

supplementation will cease, and a leucine infusion will take place in the same way as ISOPRE (see above). Like for the ISOPRE, pre-packaged diet will be consumed, and 24hr urinary sample collected.

DETAILS OF THE PROCEDURES AND POSSIBLE RISKS:

1. Blood sampling. There may be slight bruising at the site of insertion of the plastic venous catheter. The catheter itself is designed to allow for blood to be drawn safely with minimal discomfort to you. It will be inserted by a trained physician or by a trained and certified member of the lab. The total amount of blood taken will be up to 120ml per testing (1/3 cup).

2. Needle biopsy procedure. Involves the local injection of an anesthetic ("freezing") into the skin of the outer thigh area, after which a small (4mm) incision will be made and a small (50-100mg) piece of muscle will be removed using a sterile hollow needle. After the procedure, a suture will be used to close the skin, and ice and pressure will be applied to minimize bruising. The procedure will be performed by Dr. Tarnopolsky who has done it more than 7,000 times. Complications with the procedure are rare. However, in our experience with athletes, 4/7,000 experience a local skin infection, 6/7,000 have a small lump at the site of biopsy (all disappear with massage after ~1 week), 1/400 have temporary (up to 4 months) localized loss of sensation in the skin at the site of incision, and a few subjects have mild bruising around the incision for 4-5 days. In theory, one could damage a small motor branch of the m. vastus lateralis, which should not affect

function (knee extension). Nevertheless, this has not been seen in any of the patients/subjects biopsied by Dr. Tarnopolsky.

3. Stable isotope infusion. ^{13}C leucine has been widely used to examine whole body protein metabolism, as well as muscle protein synthetic rates. The isotope is stable (i.e. non-radioactive), with the natural abundance of ~1.11% of the total body carbon pool. The slight increase in your isotopic enrichment will return to baseline after 24hr. The infusion solution is guaranteed sterile by the manufacturer.

4. Creatine monohydrate supplementation. Administration of creatine similar to that of this study's has been used in a number of experiments (Harris et al., 1992; Hultman et al., 1996). No side-effects have been observed, except for a transient increase in total body mass (~1kg). This weight gain is mostly due to water retention by the lean tissue, and will return to baseline shortly after the cessation of the supplementation. Anecdotal reports of increased blood pressure and altered kidney function have not been supported by a recent well-controlled, randomized, double-blind trial done by our lab (Mihic et al., unpublished data, 1997). However, it is not known what impact creatine ingestion may have in conditions of dramatic changes in body mass, typical for some combat sports (wrestling, judo, boxing etc.). The press recently reported deaths of two elite wrestlers who had been losing weight in an extremely short period of time. The autopsies revealed severe cases of dehydration and it was stated that both athletes were taking creatine as they were losing weight. This report suggests that caution should be exercised when major weight reduction programs are combined with creatine supplementation.

BENEFITS:

You should be aware that the results of this study will be made available to the scientific community, although neither your name nor any reference to you will be used in compiling or publishing these results. You may withdraw from the study at any time without any adverse repercussions, even after signing this form.

You will receive an honorarium of $195 upon the completion of the study to compensate for your time commitment. Additionally, you will have access to your own data (body composition, average dietary intake etc.), as well as the group data when it becomes available.

You will be able to contact student investigators at 525-9140 (x27037) and/or Dr. Mark Tarnopolsky at any time regarding your questions or concerns about the study. Dr. Tarnopolsky can be contacted at: W- 521-2100 (x6593, or x6443 pager: 2888); H- 527-1295.

_____

_____

I,_____, HAVE READ AND UNDERSTAND THE ABOVE EXPLANATION OF THE PURPOSE AND PROCEDURES OF THE PROJECT, AND AGREE TO PARTICIPATE AS A SUBJECT.

_____	_____
SIGNATURE	DATE
_____	_____
WITNESS	DATE

APPENDIX B:

Statistical Summary Tables

ANOVA table 1: Age

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Group	1	5.27	20	6.55	0.805	0.38

ANOVA table 2: Mass

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Group	1	206.2	20	230.7	0.895	0.3553

ANOVA table 3: Height

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Group	1	25.77	20	127.8	0.202	0.66

ANOVA table 6: Kcal/kg/d

Effect	df Effect	MS Effect	Df Error	MS Error	F	p-level
Group	1	185.46	20	61.6	3.01	0.099

ANOVA table 4: %BF

Effect	df Effect	MS Effect	Df Error	MS Error	F	p-level
Group	1	7	20	50.59	0.1385	0.714

ANOVA table 5: LBM

Effect	df Effect	MS Effect	Df Error	MS Error	F	p-level
Group	1	5.18	20	142.8	0.036	0.8514

ANOVA table 6: FSR

Effect	df Effect	MS Effect	Df Error	MS Error	F	p-level
Condition	1	0.0011	18	0.00046	2.4	0.14
Gender	1	0.00075	18	0.00046	1.6	0.22
Time	1	0.00029	18	0.00028	1.06	0.32
Conditon x Gender	1	0.0011	18	0.00046	2.3	0.15
Conditon x Time	1	0.00075	18	0.00028	0.27	0.61
Gender x Time	1	0.00039	18	0.00028	1.4	0.25
Condition x Gender x Time	1	0.00094	18	0.00028	3.4	0.08

ANOVA table 8: PCr

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Gender	1	35.12	23	214.17	0.16	0.689
Condition	1	212.23	23	214.17	0.99	0.329
Time	1	1.56	23	109.13	0.01	0.905
Gender x Condition	1	22.88	23	214.17	0.11	0.747
Gender x Time	1	209.82	23	109.13	1.92	0.179
Conditon x Time	1	469.17	23	109.13	4.29	0.049
Gender x Conditon x Time	1	22.17	23	109.13	0.2	0.656

ANOVA table 9: Cr

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Gender	1	322.85	23	152.39	2.12	0.16
Condition	1	109.26	23	152.39	0.72	0.41
Time	1	1176.16	23	120.63	9.75	0.005
Gender x Condition	1	39.77	23	152.39	0.26	0.614
Gender x Time	1	151.26	23	120.63	1.25	0.274
Conditon x Time	1	10.09	23	120.63	0.08	0.775
Gender x Conditon x Time	1	16.55	23	120.63	0.14	0.715

ANOVA table 10: TCr

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Gender	1	571.69	23	330.77	1.73	0.202
Condition	1	628.12	23	330.77	1.89	0.181
Time	1	1262.55	23	102.72	12.3	0.002
Gender x Condition	1	122.64	23	330.77	0.37	0.55
Gender x Time	1	4.96	23	102.72	0.05	0.83
Conditon x Time	1	615.1	23	102.72	5.98	0.022
Gender x Conditon x Time	1	0.36	23	102.72	0	0.95

ANOVA table 11: ATP

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Gender	1	8.3	23	5.93	1.39	0.249
Condition	1	0.48	23	5.93	0.08	0.778
Time	1	0.74	23	7.11	0.1	0.75
Gender x Condition	1	4.98	23	5.93	0.84	0.368
Gender x Time	1	2.08	23	7.11	0.29	0.593
Conditon x Time	1	5.1	23	7.11	0.72	0.405
Gender x Conditon x Time	1	6.4	23	7.11	0.9	0.353

ANOVA table 12: Total Body Mass

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Gender	1	4807.5	23	196.59	24.5	0
Condition	1	417.4	23	196.59	2.12	0.158
Time	1	3.5	23	0.609	5.76	0.024
Gender x Condition	1	379.8	23	196.59	1.93	0.177
Gender x Time	1	0.522	23	0.609	0.86	0.364
Conditon x Time	1	0.021	23	0.609	0.04	0.853
Gender x Conditon x Time	1	0.013	23	0.609	0.02	0.883

ANOVA table 13: Fat-Free Mass

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Gender	1	5327.15	23	59.27	89.9	0
Condition	1	21.422	23	59.27	0.36	0.554
Time	1	5.857	23	0.9958	5.88	0.024
Gender x Condition	1	99.931	23	59.27	1.69	0.207
Gender x Time	1	0.515	23	0.9958	0.52	0.479
Conditon x Time	1	0.077	23	0.9958	0.08	0.783
Gender x Conditon x Time	1	0.127	23	0.9958	0.13	0.724

APPENDIX C

Raw Data

MIXED MUSCLE ANALYSIS

Female Code	Subject	Atom%	Atom% Excess	Male Code	Subject	Atom%	Atom% Excess
Pl	1-pre-0	1.0765	0.00425	Pl	17-pre-0	1.0775	0.0026
	1-pre-12	1.0808			17-pre-12	1.0801	
	1-pst-0	1.0823	0.0043		17-pst-0	1.0823	0.0035
	1-pst-12	1.0866			17-pst-12	1.0858	
Pl	2-pre-0	1.0772	0.00305	Pl	10-pre-0	1.0768	0.0052
	2-pre-12	1.0803			10-pre-12	1.082	
	2-pst-0	1.082	0.0049		10-pst-0	1.0827	0.00325
	2-pst-12	1.0869			10-pst-12	1.086	
Pl	23-pre-0	1.0759	0.00585	Cr	11-pre-0	1.0759	0.0049
	23-pre-12	1.0817			11-pre-12	1.0808	
	23-pst-0	1.0836	0.00765		11-pst-0	1.0824	0.00385
	23-pst-12	1.0912			11-pst-12	1.0862	
Pl	22-pre-0	1.0774	0.00325	Cr	12-pre-0	1.0778	0.0029
	22-pre-12	1.0806			12-pre-12	1.0807	
	22-pst-0	1.0825	0.0053		12-pst-0	1.0837	0.00645
	22-pst-12	1.0878			12-pst-12	1.0901	
Cr	6-pre-0	1.0765	0.00245	Cr	13-pre-0	1.0767	0.00345
	6-pre-12	1.079			13-pre-12	1.0801	
	6-pst-0	1.0823	0.0027		13-pst-0	1.0832	0.0031
	6-pst-12	1.085			13pst-12	1.0863	
Cr	7-pre-0	1.0773	0.0021	Cr	15pre-0	1.0772	0.0031
	7-pre-12	1.0794			15-pre-12	1.0803	
	7-pst-0	1.0817	0.00275		15-pst-0	1.0841	0.0038
	7-pst-12	1.0845			15-pst-12	1.0881	
Cr	8-pre-0	1.0761	0.0054	Pl	9-pre-0	1.0772	0.0032
	8-pre-12	1.0815			9-pre-12	1.0804	
	8-pst-0	1.0842	0.00235		9-pst-0	1.0844	0.0017
	8-pst-12	1.0865			9-pst-12	1.0861	

MIXED MUSCLE ANALYSIS

Female				Male			
Code	Subject	Atom%	Atom% Excess	Code	Subject	Atom%	Atom% Excess
Cr	18-pre-0	1.0764	0.0055	Cr	24-pre-0	1.0769	0.00305
	18-pre-12	1.0819			24-pre-12	1.08	
	18-pst-0	1.0843	0.0055		24-pst-0	1.0827	0.0027
	18-pst-12	1.0898			24-pst-12	1.0854	
Cr	4-pre-0	1.0768	0.004	Cr	25-pre-0	1.0784	0.0017
	4-pre-12	1.0808			25-pre-12	1.0801	
	4-pst-0	1.0831	0.0041		25-pst-0	1.0812	0.0045
	4-pst-12	1.0872			25-pst-12	1.0857	
Cr	19-pre-0	1.0775	0.00315	Cr	26-pre-0	1.0757	0.00485
	19-pre-12	1.0807			26-pre-12	1.0805	
	19-pst-0	1.085	0.00375		26-pst-0	1.0816	0.0042
	19-pst-12	1.0887			26-pst-12	1.0858	
Cr	21-pre-0	1.0756	0.00485	Pl	16-pre-0	1.0766	0.002
	21-pre-12	1.0804			16-pre-12	1.0786	
	21-pst-0	1.0821	0.0059		16-pst-0	1.0816	0.00275
	21-pst-12	1.088			16-pst-12	1.0844	

MUSCLE CREATINE

Subject #	Gender	Condition	PCr-pre	Cr-pre	TCr-pre	ATP-pre	PCr-pst	Cr-pst	TCr-pst	ATP-pst
1	f	PI	56.6	65.3	121.9	21.2	67.2	54.2	121.4	22.9
2	f	PI	97.1	60.9	157.9	17.6	64.6	88	152.7	23
3	f	PI	48.6	75.9	124.5	24.3	65.4	53.8	119.2	20.2
4	f	PI	72.1	49.6	121.7	24.7	69.6	74.6	144.2	24.2
20	f	PI	84.7	43.5	128.2	23.2	81.4	48.7	130.1	23.1
22	f	PI	64.4	61.9	126.3	22.9	68.2	59.8	128	22.1
23	f	PI	77.9	61.1	139	23.2	64.7	82.8	147.5	19.97
5	f	Cr	65.8	59.9	125.8	22.6	72.1	69.3	141.4	18.4
7	f	Cr	71.1	58.9	130	18.2	83.1	52.2	135.3	22.3
13	f	Cr	70.2	58.4	128.7	20.4	81.2	70.3	151.5	21.5
8	f	Cr	84.4	80	164.3	24.1	115.7	82.1	197.8	23.5
18	f	Cr	74.7	52.3	127	23.2	71.2	98.7	169.9	25.4
19	f	Cr	51.8	76.8	128.6	20	57	61.8	118.8	19.5
21	f	Cr	70	65.6	135.6	22.5	88.1	57.8	145.9	19.8
9	m	PI	55.1	78	133.1	21.7	59.5	77.4	136.9	24
11	m	PI	107.6	46.9	154.5	22.9	65.8	52.8	118.6	18.5
14	m	PI	68.4	45.6	114.1	16.6	65.1	59.4	124.4	24.3
16	m	PI	64.1	47.4	111.5	16.9	70	64.3	134.3	20.8
17	m	PI	80.5	51.5	132	23.4	66.8	71.5	138.3	25.2
27	m	PI	68.2	56.5	124.6	18.3	67.3	64.9	132.2	18.6
10	m	Cr	76.7	41.8	118.6	25.5	75.8	55.1	130.9	18.6
12	m	Cr	67.1	53.6	120.7	18.55	57.9	79.4	137.3	18.7
7	m	Cr	70.5	52.4	122.8	22.5	71.1	66.8	137.9	18.3
15	m	Cr	69	50.2	119.2	20.4	74.1	60.6	134.8	25.5
24	m	Cr	74.7	62.1	136.8	22.7	97.7	69.5	167.2	19.5
25	m	Cr	63	60.5	123.6	19.6	71.4	62.6	134	25.7
26	m	Cr	83.2	53.7	136.9	22.6	63.3	83.1	146.3	20.8

Mean-PI	72.7154	57.2385	129.946	21.3	67.3538	65.554	132.908	22.0669
SD-PI	16.7754	11.2325	13.7814	2.91004	4.98341	12.401	10.836	2.23519
Mean-Cr	70.8714	59.0143	129.9	21.6321	77.1214	69.236	146.357	21.25
SD-Cr	8.17514	10.1277	11.628	2.13286	15.6653	12.803	20.0896	2.77482

APPENDIX D

Expanded Methodology

Gas Chromatography – Combustion – Isotope Ratio Mass Spectrometry

Theory and Principles

The mass spectrometer equation can be defined as:

$$M/Z = B^2R^2/2V$$

Where an ion of mass (M) and charge (Z) is accelerated in a potential (V) and injected into a uniform magnetic field (B) then the ion experiences a force and moves in a circular orbit of radius (R). For singly charged ions the radius is determined by the choice of magnetic and electric field. The combination of fields selects ions of particular mass and forms a mass filter. This principal is the basis of all magnetic-sector mass spectrometers. There are several steps in analyzing a sample in a mass spectrometer and they are defined by ionizing a sample gas, forming a beam, accelerating that beam by an electric field, deflecting it by a magnetic field, and finally detecting it. All of these events take place in three different sections of the IRMS. The source is responsible for ionization, beam formation and acceleration. The flight tube is responsible for magnetic deflection, and the collector detects the sample. When the sample is first ionized a beam of electrons is passed through the gas and through a series of collisions or close encounters one or more electrons can adhere to the molecule and form a negative ion, or detach from the molecule and leave a positive ion. In most cases it is the molecules which have lost an electron and thus carry a positive ion which are analyzed. The positive ions are now accelerated and formed into a beam by increasing the ionization chamber to a positive potential. The ions are accelerated through a slit in the source

towards a second slit (alpha) at ground potential. The flight tubes forms an arc, which passes between the poles of a magnet. As the ion beam travels down the tube the beam is separated into different beams dependent on the mass of the ions. A particular radius, and therefore mass is selected by a slit at either end of the flight tube. In the collector the ions pass through a resolving slit and detected by a faraday cup. The ion current in the cup is proportional to the number of incident ions, and therefore to the partial pressure of the isotopic molecular species in the sample gas. Normally, during isotope ratio measurements more than one faraday cup is used for different masses. Therefore, the measurement of $^{12}CO_2$ of mass 44 can be compared to a labeled $^{13}CO_2$, resulting in a mass 45. The sensitivity in such an instrument is that which allows us to measure the difference of even 1 mass unit (SIRA Series II handbook).

APPENDIX E

Expanded Discussion

FSR Variability

The FSR method is typically known for having a large degree of variability, and thus only stimuli that induce dramatic changes in protein synthetic rate can be detected. For example, exercise (Chesley *et al.* 1991; Phillips *et al.* 1997, 1999), amino acids (Biolo *et al.* 1994, 1995; Tipton *et al.* 1999), and insulin (Biolo *et al.* 1999), have all been shown to have measurable effects on muscle protein metabolism, however all of these act with either a mechanical stress on the muscle tissue, or are directly involved in muscle anabolism as either a substrate or a signal. Furthermore, current literature demonstrates the variability of measuring muscle protein synthesis using stable isotope methods. Roy and colleagues (1997) reported a non-significant 36% increase in FSR following a sub-maximal bout of resistance exercise. Similarly, Tipton and colleagues (1996) reported FSR values in which a combination of endurance and resistance exercise resulted in a 73% increase above resistance exercise alone, and a 30% increase above swimming alone. Despite the pronounced divergence in stimuli, the FSR values were non-ignificant. Thus, even if creatine did induce a protein synthetic effect on muscle, unless it increased synthesis at least 30% above normal, it is unlikely that we would detect it using these methods. There are two possisble reasons for the large degree of variability associated with the measure of FSR. One major assumption we make concerning muscle biopsies, is that the muscle is completely homogenous. That is, that the center of the muscle will have the same concentrations of intramuscular metabolites as the peripheral sites in the muscle. This, however, may not be the case. A second issue concerning tracer methodology, is the concentration of tracer and tracee that are being measured. At

the muscle level we are detecting differences between hundreds of molecules, which requires a great amount of sensitivity. Although the current technology is very sensitive and accurate, it is almost impossible to measure differences between hundreds of molecules with complete certainty.

Furthermore, if creatine exerts a similar effect on protein metabolism as it does on human performance (4% increase), and body weight (3% increase), then changes would not have been detected using the FSR method of measurement due its inherent variability (25%).

Alpha-Ketoisocaproic Acid as a Pre-cursor Pool for Protein Synthesis

One key element in determining muscle protein FSR, is the precursor pool that is used to determine the protein synthetic potential. The obligatory precursors of proteins are amino acids that are acylated to tRNA. Since at least 1 g of muscle tissue is needed to isolate tRNA in muscle several surrogate measures of precursor pool enrichment have been used in the calculation of fractional muscle protein synthesis in humans. Plasma alpha-KIC (alpha ketoisocaproic acid) is used as a surrogate measure because once leucine enters the cell it is transaminated quickly, forming KIC, which is then delivered back into the plasma. Some controversy has surfaced regarding the use of either KIC or tissue leucine, concerning the accuracy in calculating muscle protein FSR. In our study KIC was the precursor used to calculate FSR. The use of this precursor as a surrogate measure of leucyl-tRNA is supported by several other researchers. Using a swine model skeletal muscle FSR was calculated using different precursor pools (Baumann et

al. 1994). Following a labeled tracer infusion FSR was calculated using leucyl-tRNA, and several other pools including plasma KIC, tissue fluid leucine, arterial leucine, and arterial KIC. The calculations showed that plasma KIC was the closest value to leucyl-tRNA accounting for ~ 90% of the FSR calculated with leucyl-tRNA. Tissue fluid leucine on the other hand only accounted for ~ 80% of the FSR calculated using leucyl-tRNA as the gold standard. It should be noted however, that the results were almost completely reversed in the liver. To summarize, the study demonstrated that the relationships between the various precursor pools in skeletal muscle, heart and liver differ significantly. More importantly, although tissue fluid leucine and plasma KIC were similar to leucyl-tRNA, KIC appeared to be the more accurate surrogate pool. Similarly, Watt and colleagues (1991) demonstrated that plasma KIC is very similar to leucyl-tRNA in human erector spinae muscle, and human placental tissue. In patients undergoing orthopedic spinal surgery, and scheduled cesarean sections, between 1-5 g of tissue were removed for protein synthetic rate determination following a primed constant infusion of [^{13}C]leucine. Interestingly, both the tissue free leucine, and the plasma KIC resulted in difference of about 10% from leucyl-tRNA labeling. This particular study acknowledges that both pools will result in about a 10% deviation from "true" muscle protein synthetic rate, however, the procedure used in determining plasma KIC is easier and more convenient than determining tissue free leucine. Thus, it would appear as though KIC as a surrogate measure of leucyl-tRNA labeling can be considered a valid method for the determination of muscle protein synthesis.

REFERENCES

Balsom PD, Harridge SDR, Soderlund K, Sjodin B, and B Eckblom. Creatine supplementation pre se does not enhance endurance exercise performance. *Acta. Physiol. Scand.* 149:531-533, 1993.

Bangsbo, J. regulation of glycogenolysis and glycolysis during intense exercise: in vivo studies using repeated intense exercise. Biochemistry of exercise Nine. Human Kinetics, Champaign, IL., 1995.

Baquet A, Maisin L, L Hue. Swelling of rat hepatocytes activates acetyl-CoA carboxylase in parallel to glycogen synthase. *Biochem. J.* 278:887-90, 1991.

Baquet A, Hue L, Meijer AJ, van Woerkom GM, PJ Plomp. Swelling of rat hepatocytes stimulates glycogen synthesis. *J. Biol. Chem.* 265(2):955-9, 1990.

Bennet WM, Connacher AA, Scrimegeour CM, MJ Rennie. The effect of amino acid infusion on leg protein turnover assessed by [L-1-^{15}N]phenylalanine and [L-1-^{13}C]leucine exchange. *Eur. J. Clin. Invest.* 20:41-50, 1990.

Bennet WM, Connacher AA, Scrimegour CM, Smith K, MJ Rennie. Increase in anterior- tibialis muscle protein synthesis in healthy men during mixed amino acid infusion: studies of incorporation of [1-13C]leucine. *Clin. Sci. Lon.* 76: 447-454, 1989.

Bergstrom, J., and Hultman, E. Muscle glycogen synthesis after exercise. An enhancing factor localized to the muscle cells in man. *Nature* (Lond.) 210, 309, 1966.

Bessman, S.P., and C.L. Carpenter. The Creatine-Creatine Phosphate Energy Shuttle. *Annu Rev Biochem.* 54:831-62, 1985.

Bessman, S.P., and F. Savabi. The role of the phosphocreatine energy shuttle in exercise and muscle hypertrophy. In Biochemistry of exercise VII, A.W Taylor, P.D. Gollnick, H.J. Green, C.D. Ianuzzo, E.G. Noble, G. Metivier, and J.R. Sutton, (Eds.). Champaign, IL: Human Kinetics, 1990, pp. 167-178.

Biolo G, Williams BD, Fleming RY, RR Wolfe. Insulin action on muscle protein kinetics and amino acid transport during recovery after resistance exercise. *Diabetes.* 48(5): 949-57, 1999.

Biolo G, Tipton KD, Klein S, and RR Wolfe. An abundant supply of amino acids enhances the metabolic effect of exercise on muscle protein. *Am. J. Physiol.* 273:E122-E129, 1997.

Biolo G, Maggi SP, Williams BD, Tipton KD, and RR Wolfe. Increased rates of muscle protein turnover and amino acid transport after resistance exercise in humans. *Am. J. Physiol.* 268:E514-E520, 1995.

Bhasin S, Storer TW, Berman N, Yarasheski KE, Clevenger B, Phllips J, Lee WP, Bunnell TJ, R Casaburi. Testosterone replacement increases fat-free mass and muscle size in hypogonadal men. *J. Clin. Endocrinol. Metab.* 82(2):407-13, 1997.

Bhasin S, Storer TW, Berman N, Callegari C, Clevenger C, Phillips J, Bunnell TJ, Tricker R, Shirazi A, R Cassaburi. The effects of supraphysiologic doses of testostrone on muscle size and strength in normal men. *N. Engl. J. Med.* 335(1): 1-7, 1996.

Birch R, Noble D, and PL Greenhaff. The influence of dietary creatine supplementation on performance during repeated bouts of maximal isokinetic cycling in man. *Eur. J. Appl. Physiol.* 69:268-270, 1994.

Bosco C, Tihanyi J, Pucspk J, Kovacs I, Gabossy A, Colli R, Pulvirenti G, Tranquilli C, Foti C, Viru M, Viru A. Effect of oral creatine supplementation on jumping and running performance. *Int. J. Sports. Med.* 18(5):369-72, 1997.

Brodsky IG, Balagopal P, KS Nair. Effects of testosterone replacement on muscle mass and muscle protein synthesis in hypogonadal men-a clinical research center study. *J. Clin. Endocrinol. Metab.* 81(10):3469-75, 1996.

Burke LM, Pyne DB, Telford RD. Effect of oral creatine supplementation on single-effort sprint performance in elite swimmers. *Int. J. Sport Nutr.* 6(3):222-33, 1996.

Campbell WW, Crim MC, Young VR, Joseph LJ, WJ Evans. Effects of resistance training and dietary protein intake on protein metabolism in older adults. *Am. J. Physiol.* 268: E1143-E1153, 1995.

Chanutin, A. The fate of creatine when administered to man. *J. Biol. Chem.* 67:29-37, 1926.

Chesley A, MacDougall JD. Tarnopolsky MA, Atkinson SA, and K Smith. Changes in human muscle protein synthesis after resistance exercise. *J. Appl. Physiol.* 73(4) 1383-8, 1992.

Cooke WH, Grandjean PW, WS Barnes. Effect of oral creatine supplementation on power output and fatigue during bicycle ergometry. *J. Appl. Physiol.* 78(2): 670-3, 1995.

Cortiella J, Matthews DE, Hoerr RA, Bier DM, VR Young. Leucine kinetics at graded intakes in young men: quantitative fate of dietary leucine. *Am. J. Clin. Nutr.* 48(4):998-1009, 1988.

Crim, M. C., D. H. Calloway, and S. Margen. Creatine metabolism in men: creatine pool size and turnover in relation to creatine intake. *J. Nutr.* 106:371-381, 1976.

Dawson B, Cutler M, Moody A, Lawrence S, Goodman C, N Randall. Effects of oral creatine loading on single and repeated maximal short sprints. Aust. J. Sci. Med. Sport. 27(3):56-71, 1995.

Delanghe J, De Slypere JP, DE Buyzere M, Robbrecht J, Wieme R, Vermeulen A. Normal reference values for creatine, creatinine, and carnitine are lower in vegetarians. *Clin. Chem.* 35(8): 1802-1803, 1989.

Earnest CP, Snell PG, Rodriguez R, Almada AL, and TL Mitchell. The effect of creatine monohydrate ingestion on anaerobic power indices, muscular strength and body composition. *Acta. Physiol. Scand.* 153:207-209, 1995.

Edwards, R.H.T., Harris, R.C., Hultman, E., Kaijser, L., Koh, D., and Nordesjo, L-O. Effect of temperature on muscle energy metabolism and endurance during successive isometric contractions sustained to fatigue of the quadriceps muscle in man. *J. Physiol.* (Lond) 220, 335, 1972.

Ellington WR. Phosphocreatine represents a thermodynamic and functional improvement over other muscle phosphagens. *J. Exp. Biol.* 143:177-94, 1989.

Febbraio MA, Flanagan TR, Snow RJ, Zhao S, MF Carey. Effect of creatine supplmementation on intramuscular TCr, metabolism and performance during intermittent, supramaximal exercise in humans. Acta. Physiol. Scand. 155(4): 387-95, 1995.

Fern EB, Bielinski RN, Schutz Y. Effects of exaggerated amino acid and protein supply in man. *Experentia.* 47(2):168-172, 1991.

Ferrando AA, Tipton KD, Doyle D, Phillips SM, Cortiella J, RR Wolfe. Testosterone injection stimulates net protein synthesis but not tissue amino acid transport. *Am. J. Physiol.* 275:E864-E871, 1998.

Ferrando AA, Tipton KD, Bamman MM, and RR Wolfe. Resistance exercise maintains skeletal muscle protein synthesis during bed rest. *J. Appl. Physiol.* 82(3):807-810, 1997.

Ferrando AA, Lane HW, Stuart CA, Davis-Street J, and RR Wolfe. Prolonged bed rest decreases skeletal muscle and whole body protein synthesis. *Am. J. Physiol.* 270:E627-33, 1996.

Fry, D.M., and M.F. Morales. A reexamination of the effects of creatine on muscle protein synthesis in tissue culture. *J. Cell. Biol.* 84(2):294-7.

Fryburg DA, Gelfand RA, EJ Barrett. Growth hormone acutely stimulates forearm muscle protein synthesis in normal humans. *Am. J. Physiol.* 260: E499-E504, 1991.

Fryburg DA, Barrett EJ, Louard RJ, RA Gelfand. Effect of starvation on human muscle protein metabolism and it's response to insulin. *Am. J. Physiol.* 259: E477-82, 1990.

Fukagawa NK, Minaker KL, Rowe JW, Goodman MN, Matthews DE, Bier DM, VR Young. Insulin-mediated reduction of whole body protein breakdown. Dose-response effects on leucine metabolism in postabsorptive men. *J. Clin. Invest.* 76(6): 2306-2311, 1985.

Fulks RM, Li JB, and AL Goldberg. Effects of insulin, glucose, and amino acids on protein turnover in rat diaphragm. *J. Biol. Chem.* 250(1):290-8, 1975.

Gelfand RA, and EJ Barrett. Effect of physiologic hyperinsulinemia on skeletal muscle protein synthesis and breakdown in men. *J. Clin. Invest.* 80: 1-6, 1987.

Green AL, Simpson EJ, Littlewood JJ, Macdonald IA, Greenhaff PL. Carbohydrate ingestion augments creatine retention during creatine feeding in humans. *Acta Physiol Scand* 158:195-202, 1996.

Greenhaff, P.L., A. Casey, A. H. Short, R. Harris, K. Soderlund, and E. Hultman. Influence of oral creatine supplementation on muscle torque during repeated bouts of maximal voluntary exercise in man. *Clin. Sci.* 84:565-571, 1993.

Greenhaff PL, Bodin K, Soderlaund K, Hultman E. Effect of oral creatine supplementation on skeletal muscle phosphocreatine resynthesis. *Am. J. Physiol.* 266(Endocrinol. Metab. 29): E725-E730,1994.

Griggs RC, Kingston W, Jozefowicz RF, Herr BE, Forbes G, Halliday D. Effect of testosterone on muscle mass and muscle protein synthesis. *J. Appl. Physiol.* 66(1): 498-503, 1989.

Grindstaff PD, Kreider R, Bishop R, Wilson M, Wood L, Alexander C, Almada A. Effects of creatine supplementation on repetitive sprint performance and body composition in competitive swimmers. *Int. J. Sports. Nutr.* 7(4):330-46, 1997.

Guimbal C, Kilimian MW. A Na^+-dependent creatine transporter in rabbit brain, muscle heart and kidney. *J. Biol. Chem.* 268:8418-8421, 1993.

Guthmiller P, Van Pilsum JF, Boen JR, DM McGuire. Cloning and sequencing of rat kidney L-arginine: glycine amidinotransferase. Studies on the mechanism of regulation by growth hormone and creatine. *J. Biol. Chem.* 269:17556-60, 1994.

Harris RC, Soderlund K, Hultman E. Elevation of creatine in resting and exercised muscle of normal subjects by creatine supplementation. *Clin. Science.* 83:367-374, 1992.

Haugland, R. B., and D. T. Chang. Insulin effect on creatine transport in skeletal muscle. *Proc. Soc. Exp. Biol. Med.* 148:1-4, 1975.

Haussinger D. The role of cellular hydration in the regulation of cell function. *Biochem. J.* 313: 697-710, 1996.

Haussinger D, Roth E, Lang F, and W Gerok. Cellular hydration state: an important determinant of protein catabolism and disease. *Lancet.* 341(8856):1330-2, 1993.

Horber FF, and MW Haymond. Human growth hormone prevents the protein catabolic side effects of prenisone in humans. *J. Clin. Invest.* 86(1):265-72, 1990

Horber FF, Scheideggar JR, Grunig BE, FJ Frey. Thigh muscle mass and function in patients treated with glucocorticoids. *Eur. J. Clin. Invest.* 15(6):302-7, 1985.

Hortobagyi T, Barrier J, Beard D, Braspennincx J, Koens P, Devita P, Dempsey L, J Lambert. Greater initial adaptations to submaximal muscle lengthening than maximal shortening. *J. Appl. Physiol.* 81(4):1677-1682, 1996.

Hultman E, Soderlund K, Timmons A, Cederblad JG, and PL Greenhaff. Muscle creatine loading in men. *J. Appl. Physiol.* 81(1):232-237, 1996.

Hultman, E., P.L. Greenhaff, J-M. Ren, and K. Soderlund. Energy metabolism and fatigue during intense muscle contraction. *Biochem. Soc. Trans.* 19:347-353, 1991.

Hultman E, Del Canale S, Sjoholm H. Effect of induced metabolic acidosis on intracellular pH, buffer capacity and contraction force of human skeletal muscle. *Clin. Sci.* 69:505-510, 1985.

Ingwall J. Creatine and the control of muscle-specific protein synthesis in cardiac and skeletal muscle. *Circ. Research.* 38(5):I115-I122, 1976.

Ingwall, J. C.D. Weiner, M.F. Morales, E. Davis, and F.E. Stockdale. Specificity of creatine in the control of muscle protein synthesis. *J. Cell. Physiol.* 63:145-151, 1974.

Ingwall JS, Morlaes MF, Stockdale FE, K Wildenthal. Creatine: a possible stimulus skeletal cardiac muscle hypertrophy. *Recent. Adv. Stud. Cardiac. Struct. Metab.* 8:467-481, 1975.

Ingwall, J.S., M.F. Morales, and F.E. Stockdale. Creatine and the control of myosin synthesis in differentiating skeletal muscle. *Proc. Nat. Acad. Sci.* USA. 69:2250-2253, 1972.

Jefferson LS, Li JB, SR Rannels. Regulation by insulin of amino acid release and protein turnover in the perfused rat hemicorpus. *J. Biol. Chem.* 252(4): 1476-1483, 1977.

Juhn MS, and MA Tarnopolsky. Oral creatine supplementation and athletic performance: a critical review. *Cli. J. Sport. Med.* 8(4):286-97, 1998.

Katz, A., K. Sahlin, and J. Henrickson. Muscle ATP turnover rate during isometric contraction in humans. *J. Appl. Physiol.* 60(6): 1839-1842, 1986.

Krause U, Rider MH, L Hue. Protein kinase signaling pathway triggered by cell swelling and involved in the activation of glycogen synthase and acetyl-CoA carboxylase in solated rat hepatocytes. *J. Biol. Chem.* 271:16668-73, 1996.

Kreider RB, Ferriera M, Wilson M, Grindstaff P, Plisk S, Reinardy J, Cantler E, and AL Almada. Effects of creatine supplementation on body composition, strength and sprint performance. *Med. Sci. Sports. Exerc.* 30(1):73-82, 1998.

Lemon PW, Tarnopolsky MA, MacDougall JD, SA Atkinson. Protein requirement and muscle mass/strength changes during intensive training in novice body builders. *J. Appl. Physiol.* 73(2):767-75, 1992.

Li JB, and LS Jefferson. Influence of amino acid availability on protein turnover in perfused skeletal muscle. *Biochim. Biophys. Acta.* 544(2):351-9, 1978.

Linder MC. Nutritional Biochemistry and Metabolism with Clinical Applications. East Norwalk, Connecticut: Appleton & Lange. 1991., pp. 96.

Loike JD, Zalutsky DL, Kaback E, Miranda AF, Silverstein SC. Extracellular creatine regulates creatine transport in rat and human cells. *Proc. Natl. Acad. Sci.* 85:807-811. 1988.

Lourdes Guerrero-Ontiveros M, and T Walliman. Creatine supplementation in health and disease. Effects of chronic ingestion in vivo: Down-regulation of the expression of creatine transporter isoforms in skeletal muscle. *Mol. Cell. Biochem.* 184: 427-437, 1998.

Mujika I, Chatard JC, Lacoste L, Barale F, Geyssant A. Creatine supplementation does not improve sprint performance in competitive swimmers. *Med. Sci. Sports. Exerc.* 28: 1435-41, 1996.

Low SY, Rennie MJ, PM Taylor. Signalling elements involved in amino acid transport responses to altered muscle cell volume. *FASEB. J.* 11(13): 1111, 1997.

Low SY, Taylor PM, MJ Rennie. Responses of glutamine transport in cultured rat skeletal muscle to osmotically induced changes in cell volume. *J. Physiol.* 492:877-85, 1996.

Lundholm K, and T Scherston. Incorporation of leucine into human skeletal muscle proteins. *Acta. Physiol. Scand.* 93(4):433-41, 1975.

MacDougall JD, Gibala MJ, Tarnopolsky MA, MacDonald JR, Interisano SA, Yarasheski KE. The time course for elevated muscle protein synthesis following heavy resistance exercise. *Can. J. Appl. Physiol.* 20(4):480-6, 1995.

Mainwood, G.W., and J.T. De Zepetnek. Post-tetanic responses in creatine depleted rat EDL muscle. *Muscle and Nerve.* 8:774-782, 1985.

Matthews DE, Marano MA, Campbell RG. Spanchnic bed utilization of glutamine and glutamic acid in humans. *Am. J. Physiol.* 264:E848-E854, 1993.

McCall GE, Byrnes WC, Dickinson A, Pattany PM, SJ Fleck. Muscle fiber hypertrophy, hyprplasia, and capillary density in college men after resistance training. *J. Appl. Physiol.* 81(5):2004-12.

McNurlan A, Essen P, Thorell A, Calder AG, Andersen SE, Ljungqvist O, Sandgren A, Grant I, Tjader I, PE Ballmer. Response of protein synthesis in human skeletal muscle to insulin: an investigation with [L-2H5]phenylalanine. *Am. J. Physiol.* 267:E102-E108. 1994.

Meijer AJ, Gustafson LA, Luiken JJ, Blommaart PJ, Caro LH, Van Woerkom GM, Spronk C, L Boon. Cell Swelling and the sensitivity of autophagic proteolysis to inhibition by amino acids in isolated rat hepatocytes. *Eur. J. Biochem.* 215: 449-454, 1993.

Meyer, R. A., H.L. Sweeney, and M.J. Kushmerick. Phosphagen and intracellular pH changes during muscular contraction of creatine-depleted rat muscle. *Am. J. Physiol.* 250:C264-C274, 1986.

Millar ID, Barber MC, Lomax MA, Travers MT, Shennan DB. Mammary protein synthesis is acutely regulated by the cellular hydration state. *Biochem. Biophys. Res. Commun.* 230(2):351-5, 1997.

Mosoni L, Houlier ML, Mirnad PP, Bayle G, J Grizard. Effect of amino acids alone or with insulin on muscle and liver protein synthesis in adult and old rats. *Am. J. Physiol.* 264:E614-E620, 1993.

Mujika, I., J-C., Chatard, L. Lacoste, F. Barale, and A. Geysssant. Creatine supplementation does not improve sprint performance in competitive swimmers. *Med. Sci. Sports Exerc.* 28(11):1435-1441, 1996.

Needham DM. *Machina Carnis. The biochemistry of Muscular Contraction in Its Historical Development.* Cambridge, U.K: Cambridge University Press, 1971.

Odland LM, MacDougall JD, Tarnopolsky MA. The effect of oral creatine supplementation on muscle phosphocreatine concentration and short-term power output. *Med. Sci. Sp. Exer.* 2(2):216-219, 1997.

Oopik V, Paasuke M, Timpmann S, Medijainen L, Ereline J, Smirnova T. Effect of creatine supplementation during rapid body mass reduction on metabolism and isokinetic muscle performance capacity. *Eur. J Appl. Physiol.* 78(1): 83-92, 1998.

Pacy PJ, Nair KS, Ford C, D Halliday. Failure of insulin infusion to stimulate fractional muscle protein synthesis in type I diabetic patients. Anabolic effect of insulin and decreased proteolysis. *Diabetes.* 38(5):618-24, 1989.

Pannemans DL, Halliday D, Westerterp KR, AD Kester. Effect of variable protein intake on whole-body protein turnover in young men and women. *Am. J. Clin. Nutr.* 61(1):59-74, 1995.

Pannemans DL, Halliday D, KR Westerterp. Whole-body protein turnover in eldely men and women: responses to two protein intakes. *Am. J. Clin. Nutr.* 61(1): 33-38, 1995.

Peyrebrune MC, Nevill ME, Donaldson FJ, Cosford DJ. The effects of oral creatine supplementation on performance in single and repeated sprint swimming. *J. Sports. Sci.* 16(3):271-9, 1998.

Phillips SM, Tipton KD, Aarsland A, Wolf SE, and RR Wolfe. Mixed muscle protein synthesis and breakdown after resistance exercise in humans. *Am. J. Physiol.* 273:E99-107, 1997.

Phillips SM, Tipton KD, Ferrando AA, and RR Wolfe. Resistance training reduces the acute exercise-induced increase in muscle protein turn-over. *Am. J. Physiol.* 276:E118-24, 1997.

Prevost MC, Nelson, AG, Morris GS. Creatine supplementation enhances intermittent work performance. *Res. Q. Exerc. Sport.* 68(3):233-40, 1997.

Quillard M, Husson A, Chedeville A, Fairand A, Lavoinne A. Protein synthesis is involved in the modulation of the level of the phosphoenolpyruvate carboxykinase mRNA by changes in cell volume in isolated rat hepatocytes. *FEBS Lett.* 423(2): 125-8, 1998.

Redondo D, Williams M, Dowling E, Graham B, Jones S, and A Almada. The effect of oral creatine supplementation on running velocity. *Med. Sci. Sports Exerc.* 26:S23, 1994.

Roman WJ, Fleckenstein J, Stray-Gunderson J, Always SE, Peshock R, WJ Gonyea. Adaptations in the elbow flexors of elderly males after heavy-resistance training. *J. Appl. Physiol.* 74(2):750-4, 1993.

Roy BD, Tarnopolsky MA, MacDougall JD, Fowles J, KE Yarasheski. Effect of glucose supplement timing on protein metabolism after resistance training. *J. Appl. Physiol.* 82(6): 1882-1888, 1997.

Schneider DA, McDonough PJ, Fadel PJ, Berwick JP. Creatine supplementation and the total work performed during 15-s and 1-min bouts of maximal cycling. *Aust. J. Sci Med. Sport.* 29(3):65-8, 1997.

Sipila I, Rapola J, Simell O, Vannas A. Supplementary creatine as a treatment for gyrate atrophy of the choroid and retina. *N. Engl. J. Med.* 304(15):867-70, 1981.

Snow RJ, McKenna MJ, Selig SE, Kemp J, Stathis CG, S Zhao. Effect of creatine supplementation on sprint exercise performance and muscle metabolism. *J. Appl. Physiol.* 84 1667-1673, 1998.

Staron RS, Karapondo DL, Kraemer WJ, Fry AC, Gordon SE, Falkel JE, Hagerman FC, RS Hikida. Skeletal muscle adaptations during early phase of heavy-resistance training in men and women. *J. Appl. Physiol.* 76(3):1247-55, 1994.

Steenge GR, Lambourne J, Casey A, MacDonald IA, PL Greenhaff. Stimulatory effect of insulin on creatine accumulation in human skeletal muscle. *Am. J. Physiol.* 275:E974-E979, 1998.

Stoll B, Gerok W, Lang F, D Haussinger. Liver cell volume and protein synthesis. *Biochem. J.* 287: 217-222, 1992.

Storey, K.B., and P.W., Hochachka. Activation of muscle glycolysis. A role for creatine phosphate in phosphofructokinase regulation. *FEBS. Letters.* 46:337-339, 1974.

Stroud, M. A., D. Holliman, D. Bell, A.L. Green, I. A. MacDonald, and P.L. Greenhaff. Effect of oral creatine supplementation on gas exchange and blood lactate accumulation during steady -state incremental treadmill exercise and recovery in man. *Clin. Sci.* 87:707-710, 1994.

Svanberg E, Moller-Loswick AC, Matthews DE, Korner U, Andersson M, K Lundholm. Effects of amino acids on synthesis and degradation of skeletal muscle proteins in humnans. *Am. J. Physiol.* 271:E718-E724, 1996.

Tarnopolsky MA, Atkinson SA, MacDougall JD, Chesley A, Phillips S, HP Schwarcz. Evaluation of protein requirments for trained strength athletes. *J. Appl. Physiol.* 73(5): 1986-95, 1992.

Thompson CH, Kemp GS, Sanderson AL, Dixon RM, Styles P, Taylor DJ, Radda GK. Effect of creatine on aerobic and anaerobic metabolism in skeletal muscle in swimmers. *Br. J. Sports. Med.* 30(3): 222-5, 1996.

Tipton KD, Ferrando AA, Phillips SM, Doyle DJr, and RR Wolfe. Postexercise net protein synthesis in human muscle from orally administered amino acids. *Am. J. Physiol.* 276:E628-34, 1999.

Tipton KD, Ferrando AA, Williams BD, and RR Wolfe. Muscle protien metabolism in female swimmers after a combination of resistance and endurance exercise. *J. Appl. Physiol.* 81(5):2034-2038, 1996.

Tohyama Y, Kameji T, S Hayashi. Mechanisms of dramatic fluctuations of ornithine decarboxylase activity upon tonicity changes in priamary cultured rat hepatocytes. *Eur. J. Biochem.* 202(3):1327-31, 1991.

Urban RJ, Bodenburg YH, Gilkison C, Foxworth J, Coggan AR, Wolfe RR, AA Ferrando. Testosterone administration to elderly men increases skeletal muscle strength and protein synthesis. *Am. J. Physiol.* 269:E820-E826, 1995.

Vandenbergh K, Goris M, Van Hecke P, Van Leemputte M, Vangerven L, and P Hespel. Long-term creatine intake is beneficial to muscle performance during resistance exercise. *J. Appl. Physiol.* 83(6):2055-63, 1997.

Volek, J. S. and Kraemar, W. J. Creatine supplementation: Its effect on human muscular performance and body composition. *Journal of Strength and Human Conditioning Research* 10(3):200-210, 1996.

Volpi E, Ferrando AA, Yeckel CW, Tipton KD, and RR Wolfe. Exogenous amino acids stimulate net muscle protein synthesis in the elderly. *J. Clin. Invest.* 101(9):2000-2007. 1998.

Walliman, T., M. Wyss, D. Brdiczka, K. Nicolay, and H.M. Eppenberger. Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: The phosphocreatine circuit for cellular energy homeostasis. *Biochem. J.* 281:21-40, 1992.

Walker, J. B. Creatine: Biosynthesis, regulation and function. Adv. *Enzymol. Relat. Areas Mol. Med.* 50:177-242, 1979.

Watt PW, Corbett ME, MJ Renie. Stimulation of protein synthesis in pig skeletal muscle by infusion of amino acids during constant insulin availability. *Am. J. Physiol.* 263(26): E453-E460, 1992.

Welle S, Thornton CA. High-protein meals do not enhance myofibrillar synthesis after resistance exercise in 62- to 75-yr-old men and women. *Am. J. Physiol.* 274:E677-E683, 1998.

Welle S, Bhatt K, Thornton CA. Stimulation of myofibrillar synthesis by exercise is mediated by more efficient translation of mRNA. *J. Appl. Physiol.* 86(4):1220-5, 1999

Williams BD, Wolfe RR, Bracy DP, DH Wasserman. Gut proteolysis contributes essential amino acids during exercise. *Am. J. Physiol.* 270: E85-E90, 1996.

Yarasheski KE, *et al.* (1999). Personal Communication.

Yarasheski KE Zachweija JJ, Angelopoulos TJ, DM Bier. Short-term growth hormone treatment does not increase muscle protein synthesis in experienced weight lifters. *J. Appl. Physiol.* 74(6):3073-6, 1993.

Yarasheski KE, Zachweija JJ, and DM Bier. Acute effects of resistance exercise on muscle protein synthesis rates in young and elderly men and women. *Am. J. Physiol.* 265:E210-E214, 1993.

Yarasheski KE, Campbell JA, Smith K, Rennie MJ, Holloszy JO, DM Bier. Effect of growth hormone and resistance exercise on muscle growth in young men. *Am. J. Physiol.* 262 E261-E267, 1992.

Young, R.B., and R.M. Denome. Effects of creatine on conetents of myosin heavy chain and myosin-heavy-chain mRNA in steady-state chicken muscle-cell cultures. *Biochem. J.* 218(3):871-6.