

Effects of Creatine Availability on Skeletal Muscle Metabolism

Submitted by

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Master of Science (Nutrition)
Bachelor of Science (Nutrition)

A thesis submitted in total fulfilment of the requirements of the degree of

Doctor of Philosophy

(With Publication)

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Submitted December 2016

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Statement of Authorship and Sources

This thesis contains no material published elsewhere or extracted in whole or in part from a thesis by which I have qualified for or been awarded another degree or diploma. No parts of this thesis have been submitted towards the award of any other degree or diploma in any other tertiary institution.

No other person's work has been used without due acknowledgment in the main text of the thesis. All research procedures reported in the thesis received the approval of the relevant Ethics/Safety Committees.

A handwritten signature in black ink, appearing to read 'Kristylen Andre Tomcik'.

9 December 2016

Kristylen Andre Tomcik

Date

Acknowledgements

To John,

Thank you for giving me the opportunity to undertake the adventure of a lifetime and to learn and grow, as a person and as a scientist, under your tutelage. Whether it was because I was lucky enough to have been part of a few studies that landed me on PubMed or because Jack enjoyed the hot chocolate and cookie I bought him in our first NYC meeting, thank you for “going with your gut” and taking a chance on me. Maybe we’ll publish another fenugreek paper together one day (let’s not and say we did)!

To Orly,

More than a supervisor, you are a confidant, a role model and a guardian angel. My gratitude for your teaching, patience and care falls short of words and this final year could not/would not have been completed without you. Your selfless nature and protective spirit in the midst of adversity is an example of the person I can only hope to one day become. Thank you for pushing me to always be better. You will always be like family to me. Thank you for everything.

To Louise,

The months spent at the AIS were tough, but also some of my fondest PhD memories. Thank you for the opportunity to learn from the best in the business. The organisation, quality and precision of the work you lead is unmatched. I’m truly grateful to have had the opportunity to work alongside you, the athletes and the A-Team you lead in Canberra (also the cakes!).

To my CEN Family,

Girls Club – **Brooke, Evelyn and Jill** – and Boys Club – **Bill, Bobby, Marcus, Nolan and Sam**: thank you for your friendship, for sharing your knowledge, lending a hand when two weren’t enough and constantly encouraging me through good days and especially the bad days. To **Donny and Will**: There are MANY words I could use to describe my time with you knuckleheads but I think one word in particular sums it all up: blessed. Thank you for being like brothers to me, for the numerous (dumb) jokes, guidance in the lab and for answering every “quick question” I ever had about real mechanistic research. I will miss all of you.

To ACU Exercise Science & the Mary MacKillop Institute for Health Research

It would be remiss of me if I didn’t extend my gratitude to the staff and post-graduate students at both ACU and the ‘tute for not only forming collaborative work partnerships over the last few years but also building bonds of friendship which extend far beyond research.

To the Cr/Gly Team,

Thank you for the support you all gave me from the moment I arrived (4 beds in 1 week) to the last trial day. To **Meg**: thank you for your methodical approach to teaching (especially the differences in bike pedals); to **Julia**: thank you for being my partner in crime on this beast; to **the Dietitians**: thank you for the hours of dedication to meal planning and prep-work; to **Luc** and **Joan**: thank you for your assistance in transporting and analysing precious cargo; and lastly, to **the Participants**: thank you for your muscle, sweat and incredible efforts during a challenging protocol. This venture could not have been done without you all.

To my CWRU Nutrition Family,

Four years ago I could not have imagined life in Australia pursuing my doctorate and I owe it to the vision of a brilliant group of mentors and advisors pushing me. To **Dr. Brunengraber**: thank you for allowing me to work in your lab and giving me my first taste of research; to **Guofang** and **John**: thank you for trusting me to run my first experiments and nurturing a passion for science; to **Stephanie** (aka grandma): thank you for being a role model for the hard work, passion and dedication required to pursue a PhD; and to **Dr. Barkoukis**: thank you for never losing faith in me, for pushing me to go beyond my comfort zone and for constantly reminding me of the value of hard work and tenacity.

To my COAH Family,

Thank you for providing me with a home away from home, surrounding me with love (and good food) and for being a constant support system (even when I went M.I.A. for a while). I thank God for putting each of you into my life (even the ones from Michigan).

To Friends Back Home,

Despite being oceans apart, our friendships never wavered. Thank you for years of letters, messages, care packages and visits (ok that last one was only **Drew** - thanks man!). To **John, Omar, Jessi, Steve, Kevin** and **Cory** in the Land and to the Case “Bros” **Matt, Dennis, Brandon, Nicole** and **Becca**: thank you for your love, for never letting me forget why I started down this path and for forgiving me for all the missed weddings; to my brother from a Croatian mother **Dennis**: thank you for being someone I could always count on to be there – not just for me but also for my family while I’ve been away; to **Keri**: “The younger, more attractive, improved version of myself. Thank you for (your unwavering faith in me), (your) crazy texts and (listening to my PhD)-induced (rants). You are truly (great).”

To my Everythings,

To **Mom and Dad:** My rocks. Words simply cannot do justice to express my love and gratitude for all you've done. Simply put: Thank you for the courage you had that allowed me to follow my own dreams, make my own path and live my own life wherever that ended up taking me and supporting me unconditionally throughout. Thank you for all the sacrifices you made that provided the opportunities for me to learn and grow as a son, a brother, a colleague and friend. Thank you for instilling in me the value of a good work ethic and the importance of showing kindness to others. This thesis stands as a testament to the love you poured into me.

To **Daniel:** My hero. Thank you for your constant encouragement and late night phone calls despite not having any clue as to what I'm doing. The way you handle adversity and uncertainty in your career is a standard I measure my own attitude by. Thank you for teaching me patience, generosity, humbleness and optimism in the face of deadlines, selfishness, pride and doubt. Thank you for being an example of passion that flows from a place of love and joy. Now, go for a run!

Lastly, and most importantly

Only by the grace of God from whom all blessings flow have I been humbly brought to the completion to this course of study. All the gifts I have been given I have used to the best of my abilities in order that this work may bring glory, honour and praise to Him and Him alone.

“In all this you greatly rejoice, though now for a little while you may have had to suffer grief in all kinds of trials. These have come so that the proven genuineness of your faith - of greater worth than gold, which perishes even though refined by fire - may result in praise, glory and honour when Jesus Christ is revealed.”

1 Peter 1:6-7

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Publications Related to Thesis

1. **Tomcik KA**, Smiles WJ, Camera DM, Hügel HM, Hawley JA, Watts R (2016). Fenugreek increases insulin-stimulated creatine content in L6C11 muscle myotubes. *European Journal of Nutrition*. pp 1-7 DOI:10.1007/s00394-015-1145-1. Published January 2016.

Manuscripts Related to the Thesis Currently Under Review

1. **Tomcik KA**, Bone JL, Ross ML, Jeacocke NA, Camera DM, Tachtsis B, Senden J, van Loon LJC, Hawley JA, Burke LM (2016). Effects of Combined Creatine and Carbohydrate Loading on Cycling Time Trial Performance. *Medicine & Science in Sports and Exercise*. Under second review (manuscript # MSSE-D-16-01178).
2. **Tomcik KA**, Hawley JA, Lacham-Kaplan O (2016). The *in vitro* Effects of Creatine and Insulin on Skeletal Myotubes Cultured in Different Glucose Concentrations. *Journal of Nutritional Biochemistry*. Under Review (manuscript # JNB_2016_593)
3. **Tomcik KA**, Hawley JA, Burke LM (2016). The Interactive Roles of Muscle Creatine and Glycogen: New Insights. *Sports Medicine*. Under review.

Other Relevant Manuscript

1. Bone JL, Ross ML, **Tomcik KA**, Jeacocke NA, Hopkins WG, Burke LM (2016). Manipulation of Muscle Creatine & Glycogen Changes DXA Estimates of Body Composition. *Medicine & Science in Sports & Exercise*. Accepted November 2016. DOI: 10.1249/MSS.0000000000001174 (Epub ahead of print)
2. Bone JL, Ross ML, Hopkins WG, **Tomcik KA**, Jeacocke NA, Burke LM (2016). Ultrasound determination of muscle glycogen content fails to correlate with biopsy-derived estimates. In Preparation.

Conference Presentations

1. **Tomcik KA**, Smiles WJ, Camera DM, Hügel HM, Hawley JA, Watts R. Additive Effect of Fenugreek Increases Creatine Uptake in L6C11 Muscle Cells. *Cell Symposium: Exercise Metabolism* - Amsterdam, THE NETHERLANDS. July 2015.
2. **Tomcik KA**, Bone JL, Ross ML, Jeacocke NA, Camera DM, Tachtsis B, Hawley JA, Burke LM . Effects of Creatine and Carbohydrate Loading on Prolonged Cycling to Exhaustion. *Exercise and Sports Science Australia: Research to Practice* - Melbourne, Victoria, AUSTRALIA. April 2016.
3. **Tomcik KA**, Hawley JA, Lacham-Kaplan O. Creatine and Insulin Augment the Thickness of C2C12-Derived Myotubes Following Long Term Cultures in Low Glucose Medium. *Federation of American Societies for Experimental Biology (FASEB) Science Research Conference: Skeletal Muscle Satellite Cells and Regeneration* – Keystone, Colorado, UNITED STATES of AMERICA. July 2016

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List of Abbreviations and Nomenclature

4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
4-HIL	(2S,3R,4S)-4-hydroxyisoleucine
ADP	Adenosine diphosphate
AGAT	L-Arginine:glycine amidinotransferase
AKT (PKB)	Protein kinase B
AMP	Adenosine monophosphate
AMPK	5' adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
AS160	AKT substrate of 160 kDa
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BM	Body mass
Ca ²⁺	Calcium
CaMK	Ca ²⁺ /calmodulin-dependent protein kinase
CCS	Creatine and carbohydrate supplemented
CCSE	Creatine and carbohydrate supplemented and exercise
cDNA	Complementary deoxyribonucleic acid
Cl	Chloride
CO ₂	Carbon dioxide
CoA	Coenzyme A
CK	Creatine kinase
Cr/CR	Creatine
CrT/CT	Creatine transporter

DHEA	Dehydroepiandrosterone
DNA	Deoxyribonucleic acid
DM	Dry mass
DMEM	Dulbecco's modified eagle's medium
DTT	Dithiothreitol
DXA	Dual-energy X-ray absorptiometry
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EIF4E	Eukaryotic translation initiation factor 4E
ERK	Extracellular-signal-regulated kinases
EtOH	Ethanol
FAD/FADH	Flavin adenine dinucleotide (oxidized/half reduced)
FBS	Foetal bovine serum
FEN	Fenugreek
GAA	Guanidinoacetate (also known as Glycocyamine)
GAMT	Guanidinoacetate N-methyltransferase
GAP	GTPase-activating proteins
GLUT4	Glucose transporter type 4
GOI	Gene of interest
GSK3	Glycogen synthase kinase 3
GTP	Guanosine-5'-triphosphate
GTPase	Guanosine triphosphate hydrolase enzyme
HG	High glucose
IGF-1	Insulin-like growth factor 1
INS	Insulin

IRS1	Insulin receptor substrate 1
IR- β	Insulin receptor beta
K	Potassium
Kcal	Kilocalorie
KOH	Potassium hydroxide
LG	Low glucose
MAPK	Mitogen-activated protein kinases
MEF2	Myocyte enhancer factor-2
MGF	Mechano growth factor
MHC	Myosin heavy chain
MRF	Myogenic regulatory factor
mRNA	Messenger RNA
mTOR	Mechanistic (or mammalian) target of rapamycin
mTORC 1/2	Mechanistic target of rapamycin complex 1/2
Myf5	Myogenic factor 5
Myf6	Myogenic factor 6
MyoD	Myogenic differentiation factor
Na	Sodium
NAD ⁺ /NADH	Nicotinamide adenine dinucleotide (oxidized/reduced)
NaOH	Sodium hydroxide
p38 MAPK	P38 mitogen-activated protein kinases
p70S6K	P70 S6 ribosomal protein kinase
Pax7	Paired box protein 7
PBS	Phosphate buffered saline
PCr	Phosphocreatine

PDK1	3-phosphoinositide dependent protein kinase-1
PGC-1 α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3-K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC	Protein kinase C
PMSF	Phenylmethanesulfonylfluoride
PT	Performance trial
RNA	Ribonucleic acid
S6K	Ribosomal protein kinase
SAM	S-Adenosyl methionine
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SLC6	Solute carrier family 6
SLC6A8	Solute carrier family 6 member 8
SLC6A10	Solute carrier family 6 member 10
SPSS	Statistical package for the social sciences
TCA	Tricarboxylic acid
TSC1/2	Tuberous sclerosis 1/2
TT	Time trial
TTE	Time to exhaustion
VO _{2max}	Maximum volume of oxygen used
VO _{2peak}	Peak volume of oxygen used
W	Watt
WW	Wet weight

Abstract

Nutritional strategies which increase energy substrate availability enhance exercise capacity and affect cell signalling and muscle morphology. The co-ingestion of carbohydrate with creatine has been shown to enhance both intramuscular creatine as well as glycogen storage, with the former being thoroughly investigated with respect to resistance-based exercise. Despite evidence of synergy, the direct performance benefits of this co-supplementation strategy on endurance-based exercise and its metabolic effect(s) on cellular signalling remained to be investigated. The work comprising this thesis represents a series of independent yet related studies that determined the effects of manipulating creatine along with carbohydrate (glucose) and/or insulin availability using both *in vivo* (human) and *in vitro* (rodent cell lines) models to better understand creatine's role in metabolism and performance.

Study I determined the *in vivo* effects of a concurrent creatine and carbohydrate loading regimen on endurance cycling performance. A clinical trial was undertaken in which 18 well-trained male cyclists completed three performance trials (PT) consisting of a 120-km time-trial with intermittent high-intensity sprints followed by an inclined ride to volitional fatigue. Participants were pair-matched following a baseline PT into creatine ($20 \text{ g}\cdot\text{d}^{-1}$ for 5 d + $3 \text{ g}\cdot\text{d}^{-1}$ for 9 d) or placebo loaded groups and consumed, in a cross-over allocation, either a moderate ($6 \text{ g}\cdot\text{kg}^{-1} \text{ BM}\cdot\text{d}^{-1}$) or carbohydrate loaded ($12 \text{ g}\cdot\text{kg}^{-1} \text{ BM}\cdot\text{d}^{-1}$) diet prior to the final two PTs. A significant treatment effect was observed with creatine on power output for both 1-km ($P<0.001$) and 4-km ($P<0.01$), with a significant increase above baseline observed with creatine in the last 4-km sprint ($P<0.05$). No change in overall PT performance time was observed. Carbohydrate loading elevated intramuscular glycogen stores ($P<0.01$) with no difference between supplement groups. These results demonstrated that creatine loading may facilitate higher power outputs during sprints performed within a cycling time-trial.

The effects of creatine and carbohydrate availability on skeletal muscle physiology have previously been independently investigated. However, the combined effects of this novel co-supplementation strategy on cellular signalling events had not been characterised. **Study II** developed an *in vitro* cell culture model to identify the metabolic influence of creatine, insulin and glucose, either independently or combined, on muscle growth in a controlled environment. C2C12 mouse myotubes were differentiated in low ($1 \text{ g}\cdot\text{L}^{-1}$; LG) or high ($4.5 \text{ g}\cdot\text{L}^{-1}$; HG) glucose DMEM supplemented with creatine, insulin, or creatine + insulin for 2 wk. HG with creatine potentiated the greatest myotube size ($P<0.001$) while creatine + insulin with both LG and HG concentrations increased myosin heavy chain (MHC) expression ($P<0.001$). Differences in MHC correlated with p38 MAPK expression but not mTOR, p70S6K or 4E-BP1. These outcomes determined that creatine's anabolic role *in vitro* is more dependent on the prevailing insulin than glucose concentration.

Creatine uptake into muscle is mediated, in part, by insulin. To identify insulin's role in augmenting intramuscular creatine concentrations within a more acute timeframe, **Study III** compared the effect of insulin to fenugreek, a medicinal herb with a demonstrated insulin-mimicking profile, on creatine uptake. Differentiated L6C11 rat myotubes were treated creatine, fenugreek seed extract, or insulin for 24 h. The combination of fenugreek, creatine and insulin increased total cellular creatine concentrations by 4 h ($P<0.05$) with no difference in creatine transporter expression observed between groups. This study determined that fenugreek alone cannot modulate creatine uptake but when added to creatine and insulin treatments, augments total intramuscular creatine levels independent of changes in creatine transport.

Collectively, these studies present novel insights into the interactive effects of creatine combined with other anabolic agents *in vivo* and *in vitro*. The conclusions arising from this research are: 1) *in vivo*, creatine facilitates the ability to generate greater cycling

power output during time-trial cycling; 2) *in vitro*, creatine treatment results in greater myotube size while the addition of insulin results in greater contractile protein expression; and 3) *in vitro*, fenugreek has an additive effect on creatine uptake when co-treated with creatine and insulin. These findings may provide a catalyst for further research which delves into more integrative clinical applications for this beneficial nutritional supplement.

CHAPTER 1: Introduction and Overview

Skeletal muscle plays a vital role in human energy metabolism and as such requires a readily available pool of energy-generating substrates to support skeletal muscle bioenergetics and maintain cellular homeostasis. A significant change in adenosine triphosphate (ATP) concentrations, the body's energy currency, affects skeletal muscle's ability to continue functioning. One compound necessary for the maintenance of skeletal muscle ATP levels is the non-essential amino acid creatine. Creatine is transported from the bloodstream into muscle tissue via an insulin-sensitive creatine transporter where it is then stored as phosphocreatine (PCr). During times of high energy demand and turnover, PCr replenishes intramuscular energy pools by donating a phosphate group to resynthesize ATP. In order to maintain a sufficient turnover of ATP to meet energy demands, a pool of substrates involved in energy regeneration are required to support the various cellular processes of skeletal muscle metabolism.

In athletic populations, the application of nutritional strategies that increase intramuscular pools of energy-generating substrates can help restore ATP concentrations and enhance exercise capacity and subsequent performance. The type of substrate utilized for the generation of energy is dependent on the duration and intensity of activity. Athletes participating in prolonged endurance activity (> 90 min) benefit from carbohydrate-based fuels which increase intramuscular glycogen stores (Hawley et al. 1997), while at the opposite end of the training-spectrum, athletes undertaking brief or intermittent bouts (~10 sec) of high-intensity activity (~95% of maximum heart rate) benefit from creatine supplementation which increases the concentration of muscle PCr available for the rapid resynthesis of ATP (Juhn and Tarnopolsky 1998a).

Creatine uptake into muscle is augmented, in part, by a carbohydrate-induced release of insulin from the pancreas. Moreover, insulin, whose metabolic actions also influence the uptake of glucose into muscle, has been implicated in decreasing proteolysis

and increases DNA replication and protein synthesis by promoting the uptake of circulating amino acids (Robergs and Roberts 2000). The ingestion of carbohydrate concomitant with creatine supplementation has been further reported to enhance both intramuscular creatine and glycogen storage through an increase in cell volume. Changes in cell volume have been associated with changes in signalling pathways involved in protein and glycogen synthesis (Safdar et al. 2008).

A plethora of information on creatine's ergogenic ability has been accumulated over the last two decades, primarily *in vivo* and in tandem with resistance exercise. However, the metabolic consequences of creatine-mediated increases in cell size when co-supplemented with carbohydrates have not been fully characterised. Few studies have looked to investigate the interaction of creatine and carbohydrate co-supplementation with regard to 1) benefits of creatine-mediated glycogen enhancement on endurance exercise performance, 2) alterations in skeletal muscle cell morphology and signalling as a result of hyperosmotic creatine-induced cell swelling, and 3) the role of insulin and insulin-like compounds mediating cell growth in the presence of creatine. The subsequent review of literature will provide background on these matters. The topics addressed in the study chapters aimed to fill gaps in the present understanding of molecular and performance-based changes associated with the manipulation of creatine availability on muscle metabolism.

CHAPTER 2: Literature Review

This chapter is comprised, in part, of the following commentary which under review in

Sports Medicine:

The Interactive Roles of Muscle Creatine and Glycogen: New Insights

Tomcik KA, Hawley JA, Burke LM

2.1 Introduction

Ergogenic aids are techniques or substances used for the purpose of enhancing athletic performance. They can be classified as nutritional, pharmacological, physiological or psychological in nature and range from validated and legal techniques, such as whey protein supplementation, to harmful and illegal practices, such as anabolic steroid use (Thein, Thein, and Landry 1995). Athletes commonly utilise a variety of ergogenic aids in an attempt to gain a competitive edge over their opponents (Thein, Thein, and Landry 1995). Over the past two decades, one of the most commonly utilized, readily available and highly studied sports supplements is creatine (Figure 2.1).

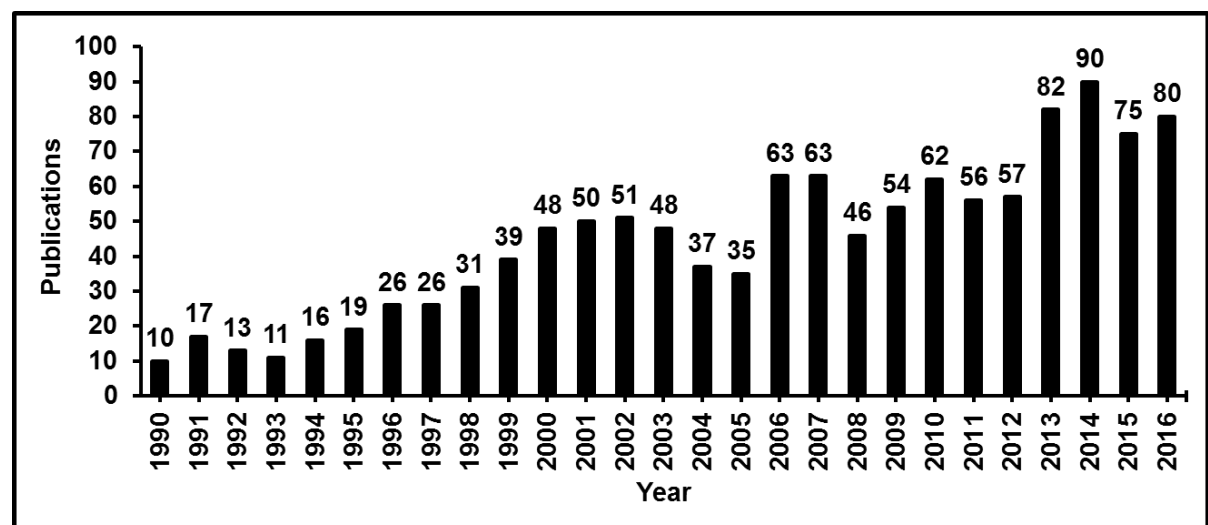


Figure 2.1: Schematic of the increase in creatine supplementation and sports performance-related publications over the last 25 years. (Reproduced from PubMed Comma-Separated Values (CSV) using keywords “Creatine Sports Performance”)

Creatine (*N*-(aminoiminomethyl)-*N*-methyl glycine) is a non-essential nitrogenous organic acid whose name is derived from the Greek word *kreas* meaning “meat/flesh” (Maughan 2013). First discovered in 1835 by the French chemist Michel Eugene Chevreul, creatine is reportedly taken by up to 74% of individuals participating in athletic activities (Sobolewski et al. 2011) while amassing over US\$400 million in global sales annually (Stephens and Greenhaff 2014). Creatine’s appeal stems from its ability to act as a reservoir of energy-generating substrates for the rapid resynthesis of the molecular energy molecule ATP (Volek et al. 1997). Skeletal muscle metabolism, especially during times of increased demand and turnover, requires a tightly coupled generation of energy from a number of substrates in order to maintain ATP stores within a narrow range (Berg, Tymoczko, and Stryer 2002). Creatine acts as an intermediary molecule by which ATP can be re-phosphorylated via the donation of a phosphate group bound to creatine as phosphocreatine (PCr) (Greenhaff 1997). To this end, numerous studies have examined creatine’s capacity for metabolic energy regeneration and subsequent role in increasing strength, decreasing fat mass, and altering muscle morphology, particularly when combined with resistance training (Cooper et al. 2012, Greenhaff 1995, Mujika and Padilla 1997, Feldman 1999, Kreider 2003).

Carbohydrates play an interactive role in enhancing creatine’s ergogenic profile. The ingestion of carbohydrates increases blood glucose concentrations stimulating the release of insulin, which is able to modulate both glucose and creatine transport into muscle (Odoom, Kemp, and Radda 1996). Furthermore, the ingestion of carbohydrates with creatine has been shown to enhance intramuscular creatine storage (Steenge et al. 1998, Green, Hultman, et al. 1996, Odoom, Kemp, and Radda 1996) while also facilitating greater glycogen storage as a result of increased water retention and augmented cell volume (Nelson et al. 2001, Robinson et al. 1999). Cellular swelling caused by the storage of additional water within the muscle

cells has been associated with the upregulation of protein and glycogen synthesis, muscle satellite cell proliferation and differentiation and DNA replication and repair (Kreider et al. 1998, Powers et al. 2003, Ziegenfuss, Lowery, and Lemon 1998, Francaux and Poortmans 1999, Olsson and Saltin 1970b, Baker and Jeukendrup 2014, Sherman et al. 1982, Juhn and Tarnopolsky 1998b, Safdar et al. 2008).

This review provides an overview of present knowledge on the interactive and metabolic effects of creatine. The practical application of creatine and the suggested mechanisms by which it governs activity at a molecular level is the foundation for the work presented in the subsequent chapters.

2.2 Skeletal Muscle Energy Systems

2.2.1 Generation of ATP

The sole substrate that skeletal muscle utilizes for contraction is ATP (Stephens and Greenhaff 2014). Cleavage of a phosphate group from ATP results in approximately 43.3 kilojoules (kJ) of free energy release per mole of ATP broken down (Robergs and Roberts 2000). The concentration of ATP maintains a number of physiological processes, including cell signalling, macromolecule synthesis, cell structure and muscle contraction while also protecting tissue from hypoxia-induced damage (Persky and Brazeau 2001). During times of energy surplus, either from high carbohydrate and/or fat intake, energy-yielding substrates (i.e. glycogen or triglycerides) are stored in various body tissues and mobilised for use during times when metabolic energy demands are high (Robergs and Roberts 2000). During exercise, ATP is hydrolysed to adenosine diphosphate (ADP) and adenosine monophosphate (AMP) (i.e. catabolism). As stores of ATP are limited in skeletal muscle, it must be continually resynthesized from its breakdown substrates ADP, AMP and inorganic phosphate (Stephens and Greenhaff 2014).

The production of ATP in skeletal muscle can occur via one or a combination of three different pathways: 1) the aerobic and anaerobic breakdown of glycogen (i.e. glycolysis), 2) the use of oxygen within the mitochondria (i.e. oxidative phosphorylation/mitochondrial respiration) and/or 3) the transfer of a phosphate group from PCr (i.e. phosphagen energy system). The end products of the anaerobic and aerobic substrate breakdown include molecules of pyruvate and acetyl coenzyme A (CoA), which enter the tricarboxylic acid (TCA) cycle, and/or reduced electron and proton donor molecules nicotinamide adenine dinucleotide (NADH) and flavin-adenine-dinucleotide (FADH) which facilitate the activity of the electron transport chain (Robergs and Roberts 2000). This continual turnover allows for ATP production over the course of prolonged exercise efforts to continue even when more anaerobic means, such as PCr, become depleted. Therefore, the generation of ATP favours the utilization of PCr during times of increased energy turnover, such as during high-intensity exercise.

2.2.2 Phosphagen Energy System

The most rapid energy-generating pathway for ATP resynthesis is through the phosphagen energy system (Persky and Brazeau 2001). Creatine, endogenously synthesized or consumed in the diet, enters the bloodstream and is transported into muscle cells via a specific solute carrier family creatine transporter protein (SLC6A8). Creatine and PCr act as a spatial energy buffer, shuttling ATP from the inner mitochondria into the cytosol (Meyer, Sweeney, and Kushmerick 1984). Once in the muscle cell, 65% of creatine is bound to a phosphate group donated by ATP in a reversible reaction catalysed by the enzyme creatine kinase (CK) and stored as PCr (Persky and Brazeau 2001). This reaction serves as a temporal energy buffer as the formation of PCr keeps creatine in muscle due to the charge that prevents partitioning through the creatine transporter (Greenhaff 1997) (Figure 2.2).

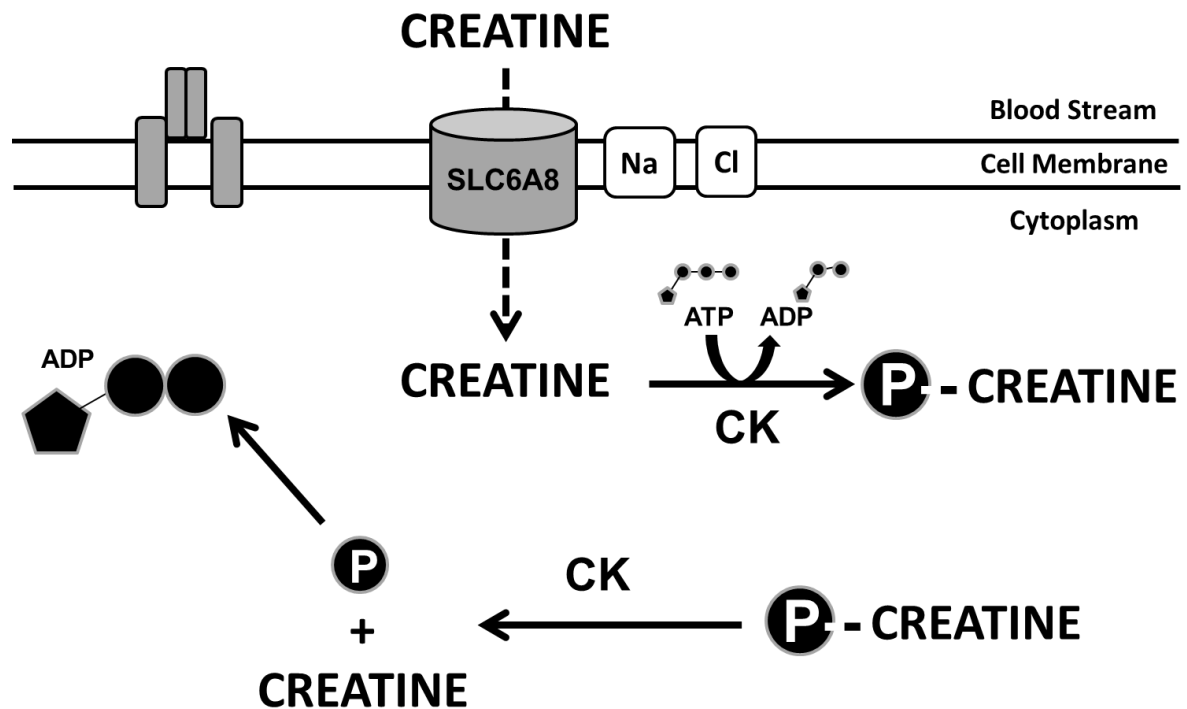


Figure 2.2: The phosphagen energy system. Dietary and endogenously synthesized creatine enters skeletal muscle via transport through the sodium/chloride regulated SLC6A8 creatine transporter. Over 90% of total body creatine is stored in the muscle where ~60% is bound to a phosphate group donated from free adenosine triphosphate (ATP) and stored as potential energy in the form of phosphocreatine (PCr); a reaction catalysed by creatine kinase (CK). During times of High energy demand, PCr replenishes the pool of ATP by donating a phosphate group to adenosine diphosphate (ADP) (or adenosine monophosphate; AMP) via a reversible reaction catalysed by CK, resulting in the generation of the energy molecule ATP.

The availability of PCr is a determining factor in maintaining the cellular ATP:ADP:AMP ratio (Takahashi et al. 1995). This ratio is physiologically relevant because it allows the cell to “sense” the amount of available energy present and control the metabolic pathways that consume or produce ATP (Brooks, Fahey, and White 1996). In muscle (mainly the type II “fast twitch” fibres), a large pool of PCr is available for the immediate regeneration of energy via a reaction catalysed by CK which cleaves the phosphate group allowing for the resynthesis of ATP hydrolysed during short periods of intense work. The presence of inorganic phosphate is important in glycogenolysis (i.e. facilitate the cleavage of glucose residues from glycogen) under conditions in which skeletal muscle relies more on PCr as a means of resynthesizing ATP (Robergs and Roberts 2000). When stores of PCr become significantly depleted (from ~9 to 3 mM), skeletal muscles adapt by increasing the activity of oxidative enzymes (i.e. citrate synthase, hexokinase, phosphofructokinase) which promote aerobic metabolism as a means of generating ATP when anaerobic metabolism is lacking (Ren, Semenkovich, and Holloszy 1993, Berg, Tymoczko, and Stryer 2002). The advantage of the phosphagen energy system is that it provides a large amount of ATP rapidly. As storage capacity is small, PCr is depleted usually within ~10 sec of supra-maximal exercise during short burst (~10 min), high intensity (85-90% maximum heart rate) activity (Kraemer and Volek 1999). Approximately 95% of PCr is resynthesized within 3-4 min following exhaustive exercise (Harris et al. 1976). The pools of total body creatine stores can also be increased through oral supplementation and further augmented via a number of supplementation strategies (Kraemer and Volek 1999).

2.3 Creatine Physiology

Nutritional strategies that enhance energy availability in muscle, including increasing the size of the substrate pools or the efficiency with which they can restore ATP, may enhance subsequent exercise capacity and performance. While all muscle substrates and their

energy generating pathways contribute to the energy demands of most exercise tasks, their relative importance will vary according to the duration and intensity of the activity. Individuals undertaking brief or intermittent high-intensity exercise should therefore target strategies to increase their capacity to utilize the phosphagen pathway (Juhn and Tarnopolsky 1998a).

2.3.1 *Creatine Biosynthesis*

Approximately 50% of whole body creatine stores are obtained through the diet by the consumption of meat and fish (Balsom et al. 1993) or through dietary supplementation (Jager et al. 2011) with the remaining 50% being endogenously synthesised from the amino acids glycine, arginine and methionine (Kingsley et al. 2009, Schoch, Willoughby, and Greenwood 2006). Hence, creatine is sometimes designated as a non-essential amino acid. Endogenous synthesis of creatine occurs via two successive reactions *in vivo* (Walker 1979). The first step of creatine biosynthesis involves the reversible transfer of an amide group from arginine to glycine to form guanidinoacetate (GAA) via the enzyme arginine:glycine amidino-transferase (AGAT). GAA is transferred via the blood to the liver where the irreversible donation of a methyl group from s-adenosyl methionine (SAM) to GAA forms creatine via the enzyme guanidinoacetate N-methyl transferase (GAMT) (Fitch 1977, Walker 1979, Greenhaff 1997). GAA formation via AGAT is the rate-limiting step in the formation of creatine (Walker 1979). The localisation of AGAT and GAMT in tissue is a complex process, with kidneys expressing high levels of AGAT activity but low or undetectable levels of GAMT, giving credence to the idea that endogenous creatine synthesis is a multi-organ process (Brosnan and Brosnan 2007). The pancreas, brain and testes also express both AGAT and GAMT suggesting that these organs may be able to synthesise or play a permissive role in creatine synthesis. However, the majority of the body's creatine is

synthesised by the renal-hepatic axis (Braissant et al. 2001, Moore 2000, Brosnan and Brosnan 2007) (Figure 2.3).

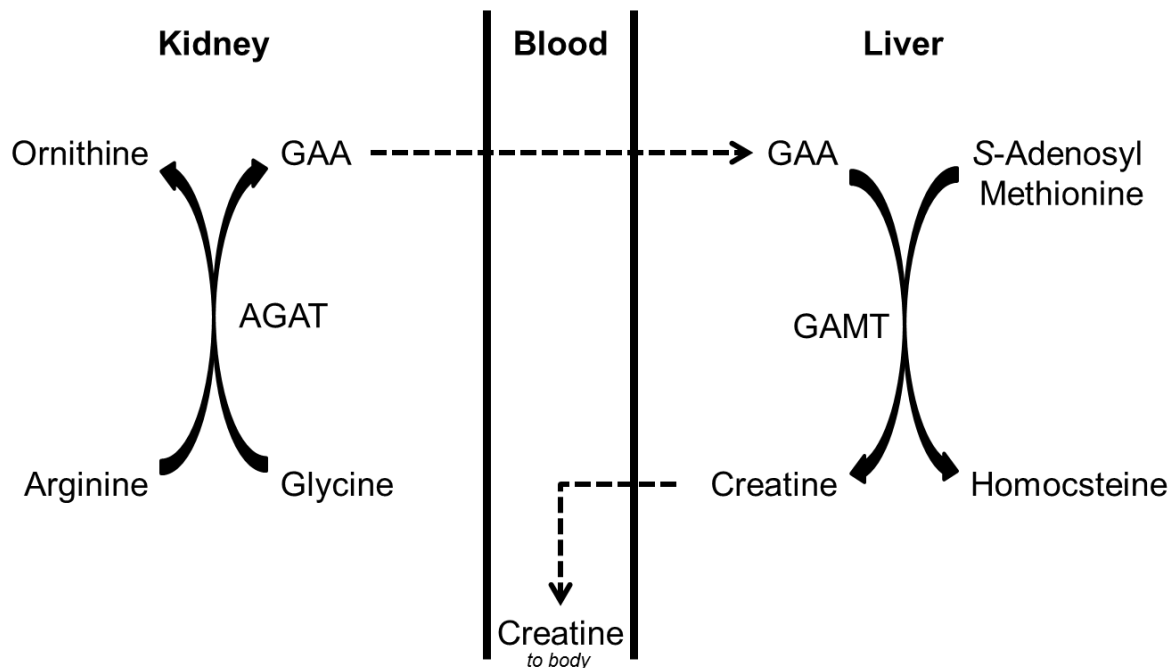


Figure 2.3: Creatine biosynthesis. Endogenous creatine synthesis occurs via a transamination reaction between the amino acids arginine and glycine in the kidney facilitated by the enzyme arginine:glycine amidino-transferase (AGAT) resulting in the formation of guanidinoacetate (GAA). GAA is transported through the blood to the liver where a methylation reaction occurs with s-adenosyl methionine (SAM) catalysed by the enzyme guanidinoacetate N-methyl transferase GAMT, resulting in the formation of creatine which enters the bloodstream and is transported to other tissues including skeletal muscle.

In humans, whole body creatine stores (~120 g) degrade at a rate of approximately 2 g·d⁻¹ and, therefore, pools need to be replenished daily either via ingested or resynthesized creatine (Hoberman, Sims, and Peters 1948). Typically, organs that have high levels of the enzymes AGAT or GMAT have the lowest level of CK enzyme and thus contain very little creatine (Persky and Brazeau 2001, Greenhaff 1997). Coincidentally, tissues that contain the largest quantities of stored creatine, such as skeletal and cardiac muscle, have little capacity to synthesize their own creatine (Brosnan and Brosnan 2007). Therefore, transport from site of synthesis and/or dietary absorption to point of uptake and utilisation must occur (discussed in section 2.3.2).

Recent research has shown that skeletal muscle may be capable of some creatine biosynthesis even in the absence of creatine transport proteins which allow for the passage of endogenous creatine from the blood into skeletal muscle. Russell *et al.* (2014) identified detectable (albeit 80% lower) levels of intramuscular creatine in whole body creatine transporter knockout (CrT^{-y}) mice. Surprisingly, this was concomitant with significantly elevated AGAT protein and mRNA expression and a 1.5 fold increase in biosynthesis of creatine in knockout compared to wild type mice (Russell et al. 2014). These findings suggest that a proportion of the creatine detected in the knockout mouse model is likely to have been synthesised within the muscle. While a small amount of creatine may be produced from skeletal muscle biosynthesis via elevated endogenous intramuscular creatine synthesis (i.e. some activity of AGAT as well as GAMT) (Van Pilsum et al. 1963, Cullen et al. 2006, Russell et al. 2014), its effects may be of little physiological significance. The substantial decrease in creatine content observed in this knockout mouse model suggests that creatine uptake by diffusion (i.e. not transporter-mediated) is insufficient to overcome deficits in the absence of creatine transport machinery (Russell et al. 2014). This indicates that the creatine

transporter protein is important and necessary in maintaining skeletal muscle creatine homeostasis.

2.3.2 *Creatine Transport*

Approximately 95% of total body creatine stores are found within skeletal muscle (Greenhaff 1997). Endogenously-produced as well as exogenously-consumed creatine is shuttled through the bloodstream and transported into skeletal muscle tissue via a sodium-chloride dependent creatine transporter (Persky, Brazeau, and Hochhaus 2003, Wallimann et al. 1992) (mechanism summarised in Figure 2.4). To date, two isoforms of the creatine transporter have been identified, both of which are members of solute-carrier family 6 (SLC6), a group of membrane transporters that facilitate the transport of a number of solutes across plasma membranes via the co-transport of sodium and chloride across a chemical gradient (Chen, Reith, and Quick 2004, Salomons et al. 2001, ZORZANO, FANDOS, and PALACÍN 2000). The SLC6 member 8 (SLC6A8) creatine transporter gene is expressed in most tissues, with the highest levels found in skeletal muscle and kidney and lower levels expressed in the heart, brain, and testes (Salomons et al. 2001, Nash et al. 1993). A second creatine transporter gene, SLC6 member 10 (SLC6A10), is solely expressed in the testes. Despite a 97% similarity in coding between SLC6A8 and SLC6A10, (Eichler et al. 1996, Iyer et al. 1996), creatine ingested through the diet or endogenous biosynthesis is exclusively absorbed into skeletal muscle through the SLC6A8 transporter. As such, all subsequent references to creatine transporters will be to SLC6A8, unless otherwise stated.

The SLC6A8 protein consists of 635 amino acids with 12 membrane-spanning domains whose structure is similar to members of the GABA family of neurotransmitters (Guimbal and Kilimann 1993, Sora et al. 1994, Nash et al. 1993, Saltarelli et al. 1996) with a ~97% similarity at the amino acid level in creatine transporter amongst species (Guimbal and Kilimann 1993, Nash et al. 1993, Sora et al. 1994). The expression of different transporter

isoforms matches closely with the expression of the enzyme creatine kinase, which has been found in the kidney, heart, skeletal muscle, brain, testes and colon but not in the liver, pancreas or intestine (Persky and Brazeau 2001, Iyer et al. 1996).

A number of subsequently described factors regulate creatine transport (Loike, Somes, and Silverstein 1986, Willott et al. 1999, Murphy et al. 2001, Loike et al. 1988, Harris, Soderlund, and Hultman 1992) but the precise mechanism(s) that regulate flux via the creatine transporter are yet to be elucidated. Creatine is co-transported with two sodium and one chloride ions, an example of secondary active transport driven by a gradient established by $\text{Na}^+ + \text{K}^+$ -ATPase (Dai et al. 1999). Creatine transport and uptake is further enhanced by hormones, such as insulin, which promote $\text{Na}^+ + \text{K}^+$ -ATPase activity (Snow and Murphy 2001). A seminal study by Loike *et al.* (1988) in L6 myoblasts showed that the nature of creatine transporter activity is sodium-dependent and further affected by extracellular creatine concentrations (Loike et al. 1988). Later work by Peral *et al.* (2002) identified increased creatine transporter activity and uptake in the presence of higher chloride concentrations in the small intestines of humans, rats, and chickens (Peral et al. 2002). In skeletal muscle, the content of the creatine transporter has also been shown to be dependent on muscle fibre type (Persky and Brazeau 2001) with greater total creatine and PCr concentrations observed in “fast twitch” type II muscle in humans (Meyer, Brown, and Kushmerick 1985, Casey et al. 1996).

Creatine transport is highly substrate specific with the key feature of the carboxyl and guanidino groups of creatine separated by no more than 2-3 carbon atoms (Guimbal and Kilimann 1993, Christie 2007). As such, neither the breakdown product creatinine nor the storage molecule PCr is considered a substrate for creatine transport. However, transport of creatine through the creatine transporter can be saturated, suggesting that the amount of transporter localised to the cell membrane may limit creatine accumulation. The

concentration of intracellular creatine, rather than levels of PCr or extracellular creatine, regulates the rate of creatine uptake and creatine transporter expression (Murphy et al. 2001). Guerrero-Ontiveros and Wallimann (1998) investigated the effects of prolonged creatine supplementation (3-6 months) on creatine transporter expression in rodents. Although the dose used could be considered supra-physiological compared to that normally consumed by humans, their findings suggest that high doses of creatine ingested over an extended period (as practiced by athletes) down-regulates the expression of the creatine transporter due to an over-saturation of intracellular creatine (Schoch, Willoughby, and Greenwood 2006, Guerrero-Ontiveros and Wallimann 1998). Conversely, human investigations have demonstrated that individuals undertaking a 2 month resistance training program with concomitant creatine supplementation ($0.125 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) had significantly greater total creatine concentrations compared to placebo (142.9 ± 8.0 vs $124.1 \pm 7.0 \text{ mmol}\cdot\text{kg}^{-1} \text{ dm}$; $P < 0.05$) without reducing creatine transporter protein or mRNA expression (Tarnopolsky et al. 2003). Whether differences in observations of these two studies was due to differences between human vs animal models as well as a comparative assessment of their intervention time period however was not expounded upon and warrants future investigation,.

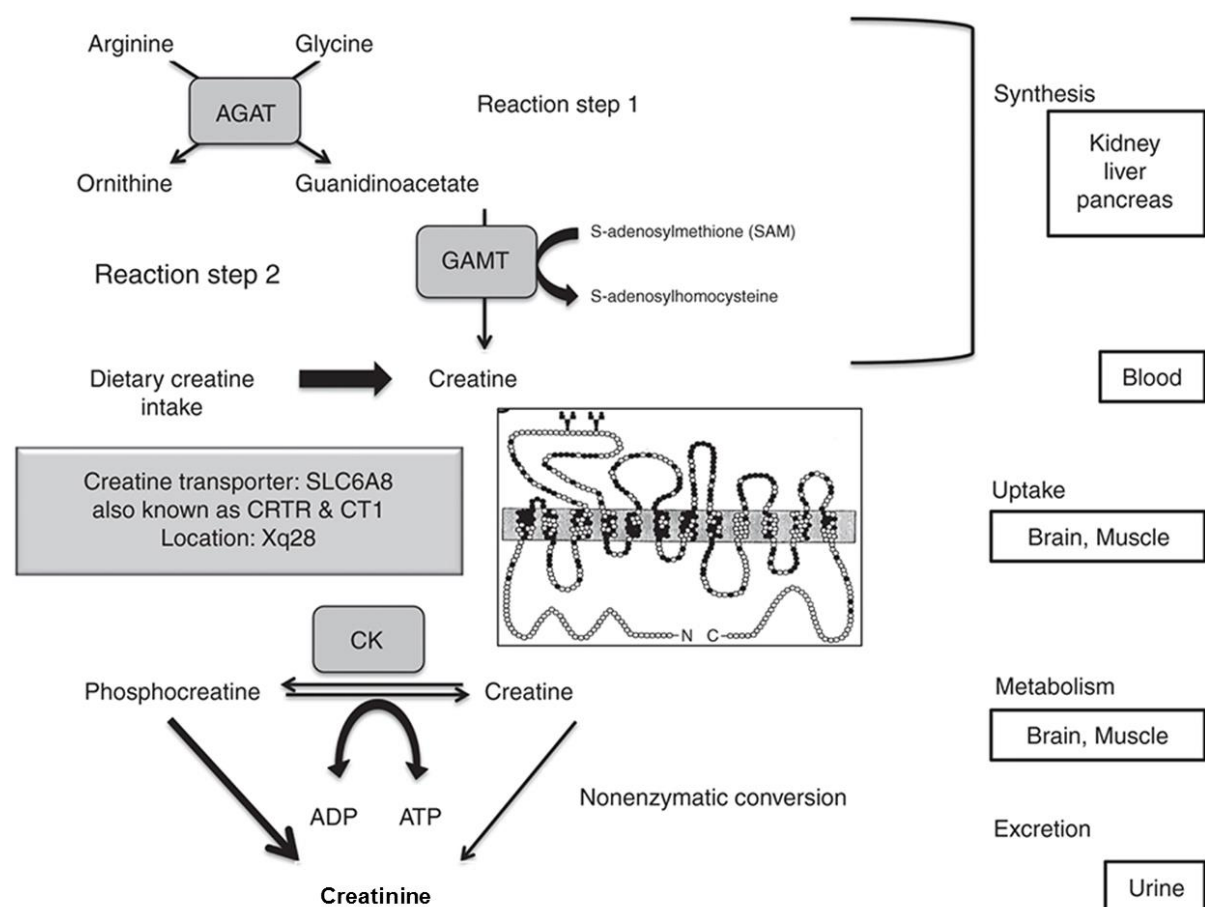


Figure 2.4: A schematic summary of creatine metabolism. The reactions required for creatine synthesis, uptake, metabolism and excretion with the organ or fluid sites involved noted on the right. Solute carrier family 6 member 8 (SLC6A8), creatine transporter; ADP, adenosine diphosphate; AGAT, arginine glycine acyl transferase; ATP, adenosine triphosphate; CK, creatine kinase; GAMT, guanidinoacetic acid methyl transferase. (Reproduced from Clark and Cecil 2015. *Pediatric Research*)

2.3.3 Creatine Supplementation

Basal muscle creatine concentrations in the adult male is about $120 \text{ mmol}\cdot\text{kg}^{-1}$ dry mass (dm) with an upper limit around $150\text{-}160 \text{ mmol}\cdot\text{kg}^{-1}$ dm (Harris, Soderlund, and Hultman 1992, Harris, Hultman, and Nordesjö 1974, Greenhaff 1996, Cooper et al. 2012). In healthy individuals, the turnover of creatine in the muscle is approximately $2 \text{ g}\cdot\text{d}^{-1}$ with excess creatine filtered in the kidney by simple diffusion and excreted in urine as creatinine (Walker 1979). Obtaining creatine from dietary sources is not essential for maintaining basal creatine levels because the body has all the necessary enzymes and substrates required for endogenous biosynthesis (Kraemer and Volek 1999). Meat products are a primary dietary source of creatine, ranging from trace amounts in prawn (shrimp) to $\sim 5 \text{ g}\cdot\text{kg}^{-1}$ in pork, beef and salmon (Balsom, Söderlund, and Ekblom 1994). The absence of creatine from the diet, however, has been shown to augment creatine retention during subsequent creatine supplementation (Delanghe et al. 1989). This is especially evident in individuals who do not regularly consume meat (i.e. vegetarians). Watt *et al.* (2004) showed that a 5-d creatine supplementation protocol ($0.4 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) resulted in a greater increase in total intramuscular creatine content in vegetarians with lower baseline creatine concentrations compared to meat eaters (Watt, Garnham, and Snow 2004). Hence, total body creatine concentrations are significantly affected by dietary creatine ingestion (Crim, Calloway, and Margen 1976). With regard to individuals who respond best to creatine, a review by Cooper *et al.* (2012) states that responders are those individuals with a lower initial level of total muscle creatine content and tend to have a greater population of type II fibres, thereby possessing a greater potential to improve performance in response to creatine supplementation (Cooper et al. 2012).

The most readily available and widely defined form of creatine recommended for dietary supplementation is creatine monohydrate which is creatine chemically bound to a

molecule of water (Jager et al. 2011). Harris *et al.* (1992) first showed that high-dose creatine monohydrate supplementation (20-30 g) for 5-d rapidly and significantly increases total intramuscular creatine content (Harris, Soderlund, and Hultman 1992). This supplementation protocol elicited an average between-subject rise in total intramuscular creatine concentration by 25 mmol·kg⁻¹ dm or ~10% above basal levels (Harris, Soderlund, and Hultman 1992). Furthermore, this protocol identified that 20% of the increased total creatine content was stored as PCr which was saturated after only 2-3 d of supplementation (Harris, Soderlund, and Hultman 1992). These results suggest that the pre-supplementation status of total muscle creatine is an important determining factor for subsequent accumulation of creatine (Harris, Soderlund, and Hultman 1992, Greenhaff 1997). Owing to the constant rate of turnover, it has been further suggested that following a period of increased creatine supplementation (~20 g·d⁻¹), it may take up to 3 wk for intramuscular creatine levels to return to baseline levels (Hultman et al. 1996).

Pioneering work by Hultman *et al.* (1996) established what is now deemed the “classic” model of creatine supplementation in humans, commonly known as creatine loading (Hultman et al. 1996). In their landmark study, participants were first supplemented with a loading dose of 20 g·d⁻¹ of creatine for 6 d after which individuals were instructed to either cease creatine ingestion or were given a smaller maintenance dose of 2 g·d⁻¹ for an additional 29 d (Hultman et al. 1996). Those participants who continued consuming the reduced dose of creatine maintained elevated intramuscular creatine levels, whereas creatine levels returned to baseline in individuals who ceased creatine supplementation (Hultman et al. 1996). The maintenance dose of 2-3 g of creatine was chosen because it was a slightly higher concentration than the rate at which creatine is turned over (Greenhaff 1997, Hultman et al. 1996) (Figure 2.5). Alternatively, a “slower loading” protocol in which the

maintenance dose is taken daily has also been shown to elicit a similar enhancement of body creatine stores, albeit only after ~4 wk (Hultman et al. 1996).

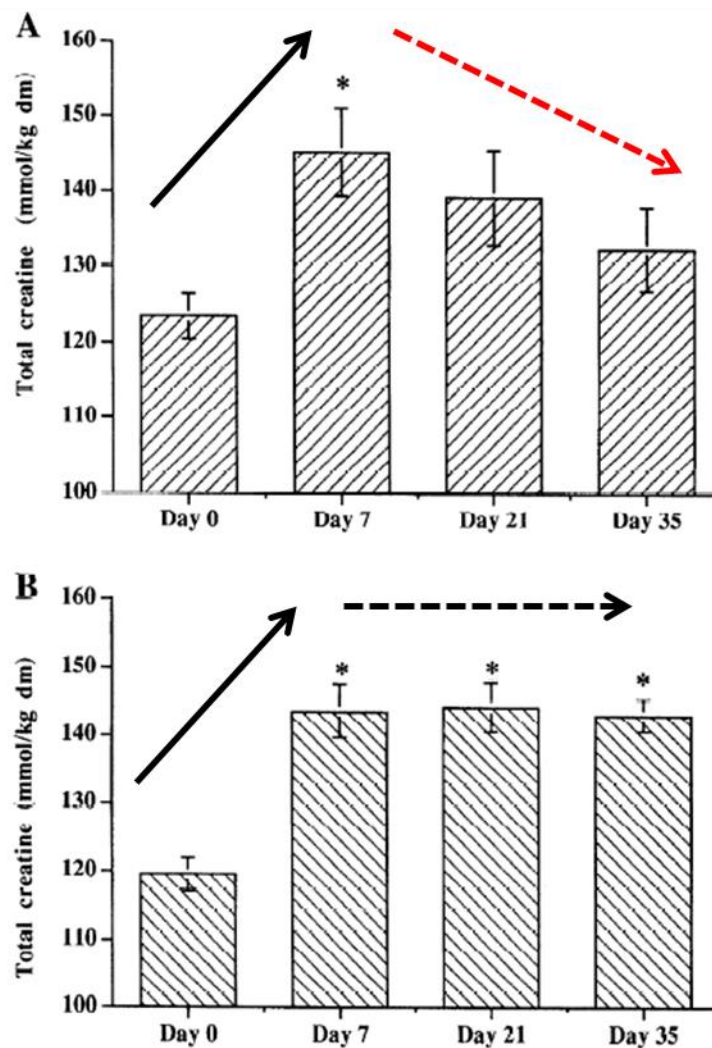


Figure 2.5: Total muscle creatine stores following creatine loading. This method consists of the ingestion of a high initial “loading” dose, traditionally 20 g/d for 3-5 d. Following an initial high dose of creatine, failure to continue supplementing (A) eventually brings creatine levels back to basal levels (red dotted line). However, (B) supplementing with a smaller 2-3 g “maintenance” dose is sufficient to maintain elevated levels of intramuscular creatine for a prolonged period of time (black dotted line). (Reproduced from Hultman *et al.* 1996. *Journal of Applied Physiology*)

This strategy has become the gold standard for creatine supplementation among trained individuals with most studies distributing the 20 g·d⁻¹ loading dose into lots of 4-5 g to be taken on multiple occasions throughout the day (Greenhaff 1997). Increased creatine ingestion has been shown to augment total intramuscular creatine levels toward a maximum “threshold” level of ~160 mmol·kg⁻¹ dm (Greenhaff 1997). It is, however, important to note that 20-30% of individuals do not respond to creatine supplementation. Greenhaff *et al.* (1994) observed a <10 mmol·kg⁻¹ dm increase in total muscle creatine accumulation in response to a similar (20 g·d⁻¹) supplementation regimen (Greenhaff et al. 1994). This variability is likely to be attributed to individual differences in the regulation of the creatine transporter and suggests that some participants may simply be low-responders to creatine supplementation (Schoch, Willoughby, and Greenwood 2006, Greenhaff et al. 1994).

2.3.4 Creatine and Carbohydrate Co-ingestion

Green *et al.* (1996) were the first to show that the co-ingestion of carbohydrate (370 g·d⁻¹ for 5 d from an 18.5% wt·vol⁻¹ glucose drink) with creatine (20 g·d⁻¹ for 5 d) resulted in increased intramuscular creatine uptake, specifically 60% above that of creatine supplementation alone (Green, Hultman, et al. 1996) (Figure 2.6). Subsequent studies confirmed that the consumption of carbohydrate (~1 g·kg⁻¹ BM) simultaneously with creatine (20 g) induced an insulin-mediated potentiation of intramuscular creatine which could, potentially, overcome the lack of responsiveness to creatine supplementation experienced by some individuals (Green, Simpson, et al. 1996, Robinson et al. 1999, Steenge et al. 1998). The combination of enhanced muscle PCr stores and resistance training leads to a greater increase in lean BM and strength than training alone (Juhn and Tarnopolsky

1998a), making the co-ingestion of carbohydrate and creatine a popular protocol for strength and power athletes.

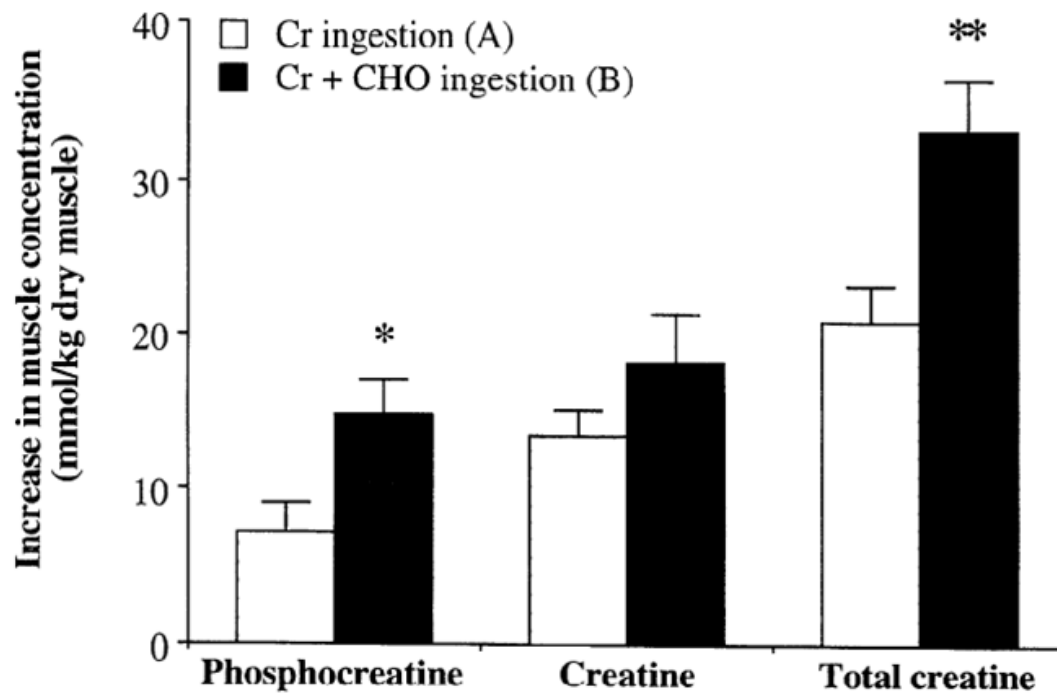


Figure 2.6: Muscle creatine concentrations following carbohydrate co-supplementation with creatine. The co-ingestion of carbohydrates ($370 \text{ g}\cdot\text{d}^{-1}$ for 5 d from an 18.5% wt·vol⁻¹ glucose drink) with creatine ($20 \text{ g}\cdot\text{d}^{-1}$ for 5 d) potentiates a reported insulin-mediated increase in creatine transport, maximizing intramuscular creatine stores by approximately 60%. (Reproduced from Green *et al.* 1996. *The American Journal of Physiology*)

2.4 Utilisation of Creatine as a Substrate for Exercise

Increases in cellular stores of creatine can be expected to have independent and interactive effects on energy metabolism. Despite the distinction among athletes for either

creatine or carbohydrate loading, there is accumulating evidence that the co-supplementation of creatine and carbohydrate may have interactive and beneficial effects for *both* resistance- and endurance-based training as well as performance.

2.4.1 *Creatine Utilisation during Exercise*

The exercise-induced decline in creatine, specifically PCr, concentrations has been associated with a gradual reduction in performance of high-intensity exercise, particularly when undertaken as repeated bouts of exercise with short recovery periods (Kreider 2003). Current dietary patterns do not appear to fully maximise creatine stores in the muscle, since it is now well known that supplemental creatine intake can increase muscle concentrations by an additional ~25% towards the upper limit of creatine storage ($150\text{--}160\text{ mmol}\cdot\text{kg}^{-1}\text{ dm}$). as previously described (Greenhaff 1997, Hultman et al. 1996, Harris, Soderlund, and Hultman 1992, Harris, Hultman, and Nordesjö 1974, Greenhaff 1996, Cooper et al. 2012). While elevated intramuscular creatine/PCr concentrations increase fatigue resistance and enhance the capacity for repeated bouts of high intensity exercise (Balsom et al. 1995, Volek et al. 1997, McNaughton, Dalton, and Tarr 1998), short term creatine supplementation has been shown to significantly increase force and power output (Balsom et al. 1995) and enhance muscular strength (Casey et al. 1996, Vandenberghe et al. 1997, Volek et al. 1997). A meta-analysis by Branch *et al.* (2003) on 96 studies revealed that creatine's most marked effects were observed in high intensity exercise bouts shorter than 30 sec with no significant effect on exercise lasting longer than 2 min (Branch 2003). Greenhaff *et al.* (1993) determined that 5 d of creatine supplementation ($20\text{ g}\cdot\text{d}^{-1}$) increased the peak muscle torque of repeated isokinetic contractions (Greenhaff et al. 1993). Furthermore, Volek *et al.* (1997) showed that supplementation of $25\text{ g}\cdot\text{d}^{-1}$ of creatine, administered in conjunction with a traditional resistance training program over several weeks significantly increased strength in squat and bench press maximum exercises (Volek et al. 1997). These findings highlight the

benefits of rapid ATP resynthesis by creatine for use during maximal intensity strength exercise (i.e. the rapid replenishment of ATP) (Kraemer and Volek 1999).

2.4.2 *Carbohydrate Utilization in Exercise*

Endurance athletes rely on carbohydrate-based fuels as the main source for muscle energetics during prolonged activities. Glycogen is a glucose polymer that provides the main form of carbohydrate storage in muscle, liver, and other insulin-sensitive tissues. The branched structure of glycogen provides an efficient energy store within the muscle cell, with a large surface area allowing rapid degradation to provide a fuel source while having a small impact on cellular osmolarity. Glycogen's role as an energy substrate has been well-known for more than 150 years, but recent interest has identified glycogen's role as a regulator of cellular signalling underpinning protein synthesis and training adaptation in the muscle (Philp, Hargreaves, and Baar 2012, Hawley et al. 2011). The present understanding of glycogen storage has identified specific cellular pools of glycogen in tissue. Two metabolically distinct forms of glycogen, pro- and macroglycogen, which vary in their sensitivities to glycogen synthesis and degradation, have been identified (Shearer and Graham 2002). Additionally, recent studies in both exhaustive (Marchand et al. 2007, Nielsen et al. 2011) and non-exhaustive, but maximal, training bouts (Fridén, Seger, and Ekblom 1985) showed that muscle glycogen is preferentially depleted (and subsequently replenished) from the inter-myofibrillar space when compared to intra-myofibrillar and subsarcolemmal glycogen. This decrease in inter-myofibrillar glycogen may be related to an impedance of Na^+ , K^+ -ATPase activity affecting sarcoplasmic reticulum calcium release and thereby leading to a decrease in overall force production (Ørtenblad, Westerblad, and Nielsen 2013).

Philp *et al.* (2012) highlight the importance of muscle glycogen not only as a substrate which determines exercise capacity but also as a substrate that directs the body's

response to exercise (i.e. regulating cellular signalling pathways associated with skeletal muscle metabolism) (Philp, Hargreaves, and Baar 2012). While trained individuals undergo a general increase in muscle glycogen stores with increased dietary carbohydrate intake, “super-compensation” above normal resting concentrations can be achieved by combining a carbohydrate intake of $> 8 \text{ g}\cdot\text{kg}^{-1} \text{ BM}$ for 24-48 h with an exercise taper (Bergström et al. 1967). This strategy, known as “carbohydrate loading” can double muscle glycogen concentrations from $\sim 400 \text{ mmol}\cdot\text{kg}^{-1} \text{ dry dm}$ to $\sim 800 \text{ mmol}\cdot\text{kg}^{-1} \text{ dm}$ (Hawley et al. 1997) and is associated with significant improvement in endurance capacity and performance, particularly in continuous activities lasting $> 90 \text{ min}$ (Hawley and Leckey 2015, Burke, van Loon, and Hawley 2016) (Figure 2.7). Sustained endurance exercise bouts of greater than 90 min can deplete muscle glycogen stores to levels as low as $100 \text{ mmol}\cdot\text{kg}^{-1} \text{ dm}$ (Hawley et al. 1997). It should be noted that elevated pre-exercise muscle glycogen content results in a higher rate of muscle glycogenolysis during exercise, leading to a greater dependence on carbohydrate-derived fuel sources for subsequent ATP production (Goforth, Bennett, and Law 1997).

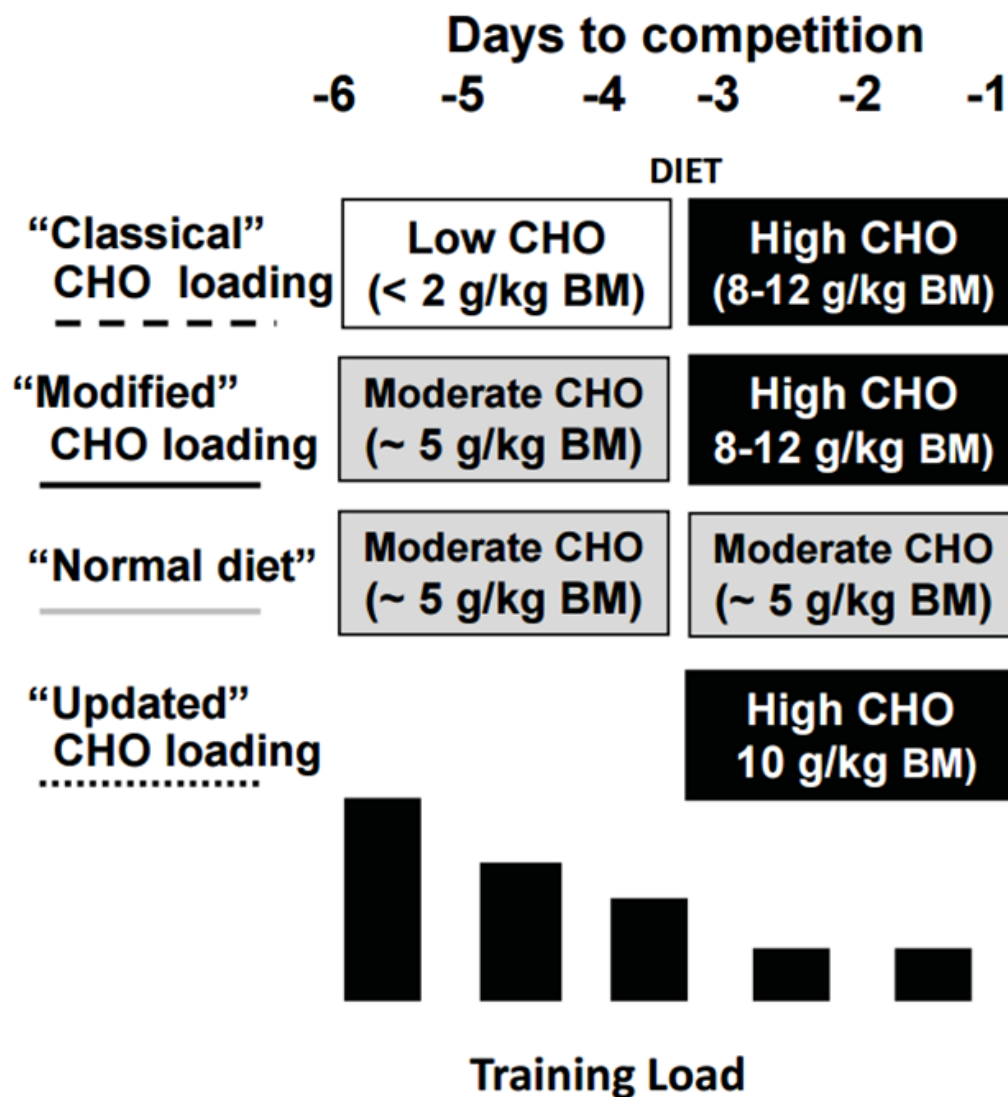


Figure 2.7: Protocols for carbohydrate loading illustrated by diet and training load prior to competition. The “Classical” loading protocol for glycogen supercompensation was developed by Bergstrom *et al* (1967) in untrained active individuals and involved an intake of $> 8 \text{ g} \cdot \text{kg}^{-1} \text{ BM}$ carbohydrate several days before an event concomitant with a reduction in activity. (Reproduced from Burke, van Loon and Hawley 2016, *Journal of Applied Physiology*)

2.4.3 Combined Creatine and Carbohydrate Utilization in Exercise

Changes in cellular substrates achieved by common practices in sports nutrition have been shown to cause unfavourable changes in BM. Balsom *et al.* (1993) conducted one of the first studies showing that subjects who followed a creatine loading protocol took significantly longer to complete a 6 km cross-country run than a placebo supplemented cohort (23.79 ± 0.85 vs. 23.36 ± 0.82 min; 1.8% Δ time) which was attributed to a 1.2% creatine-mediated increase in BM (74.4 ± 2.3 vs. 73.5 ± 2.3 kg) (Balsom et al. 1993). This has led to the current belief that creatine supplementation does not enhance endurance-based activities. Like creatine loading strategies, carbohydrate loading is associated with an increase in total BM. At a cellular level, ~3 g of water is bound to 1 g of glycogen in muscle (McBride, Guest, and Scott 1941). Early work by Olsson *et al.* (1970) found that glycogen was associated with the binding of 3-4 g of water, resulting in a 2.4 kg increase in BM in subjects who weighed ~75 kg, of which 2.2 L was attributed to an increase in total body water. (Olsson and Saltin 1970a). Few studies, however, have investigated how changes in creatine and carbohydrate loading strategies affect body composition and subsequent performance in athlete populations, largely as a result of weight gain.

The dietary ingestion of creatine (Kreider et al. 1998, Powers et al. 2003, Ziegenfuss, Lowery, and Lemon 1998, Francaux and Poortmans 1999) and carbohydrate (Olsson and Saltin 1970b, Baker and Jeukendrup 2014, Sherman et al. 1982) increase water retention resulting in osmotic swelling and augmented muscle cell volume. An increase in cell size as a consequence of creatine and/or carbohydrate-mediated water retention (Hultman et al. 1996, Kreider et al. 1998) facilitates greater glycogen storage in muscle than has been reported previously by creatine and/or carbohydrate loading alone (Nelson et al. 2001, van Loon et al. 2004, Odoom, Kemp, and Radda 1996). Osmotic swelling in rat skeletal myotubes promotes glycogen synthesis (Low, Rennie, and Taylor 1996) and storage

(Robinson et al. 1999, Nelson et al. 2001, van Loon et al. 2004) independent of changes in glucose uptake (Low, Rennie, and Taylor 1996). Robinson et al. (1999) investigated the effects of 5 d of creatine (15 g) and carbohydrate (18.5% wt·vol⁻¹ glucose drink) supplementation during recovery following one-legged submaximal cycling to exhaustion and observed increased glycogen storage in exercised muscle compared to carbohydrate supplementation alone (Robinson et al. 1999). Nelson et al. (2000) further reported that subjects who consumed a high carbohydrate diet following a traditional creatine supplementation strategy (20 g·d⁻¹ for 5 d) had a 53% increase in muscle glycogen content over high carbohydrate consumption alone (Nelson et al. 2001). These findings were corroborated by van Loon *et al.* (2004) who demonstrated that 7 wk of prolonged creatine supplementation (20 g·d⁻¹ for 5 d followed by 6 weeks of 2 g·d⁻¹) resulted in an 18% increase in muscle glycogen content (van Loon et al. 2004) (Figure 2.8).

While there is robust evidence of the enhanced glycogen storage capacity of the creatine-loaded muscle (van Loon et al. 2004, Nelson et al. 2001, Robinson et al. 1999), current studies have failed to systematically examine the effects of this co-supplementation strategy on endurance exercise capacity. The role of creatine for enhancing endurance performance is equivocal. However, there is some evidence showing that creatine supplementation may be of benefit in endurance events because of its effect on high-intensity efforts that occur during prolonged sports. It should be noted that these only make up a small proportion of the overall workload but often determine the outcome of events (i.e. breaking away from the peloton in cycling or sprint finishes during a marathon) (Jeukendrup, Craig, and Hawley 2000).

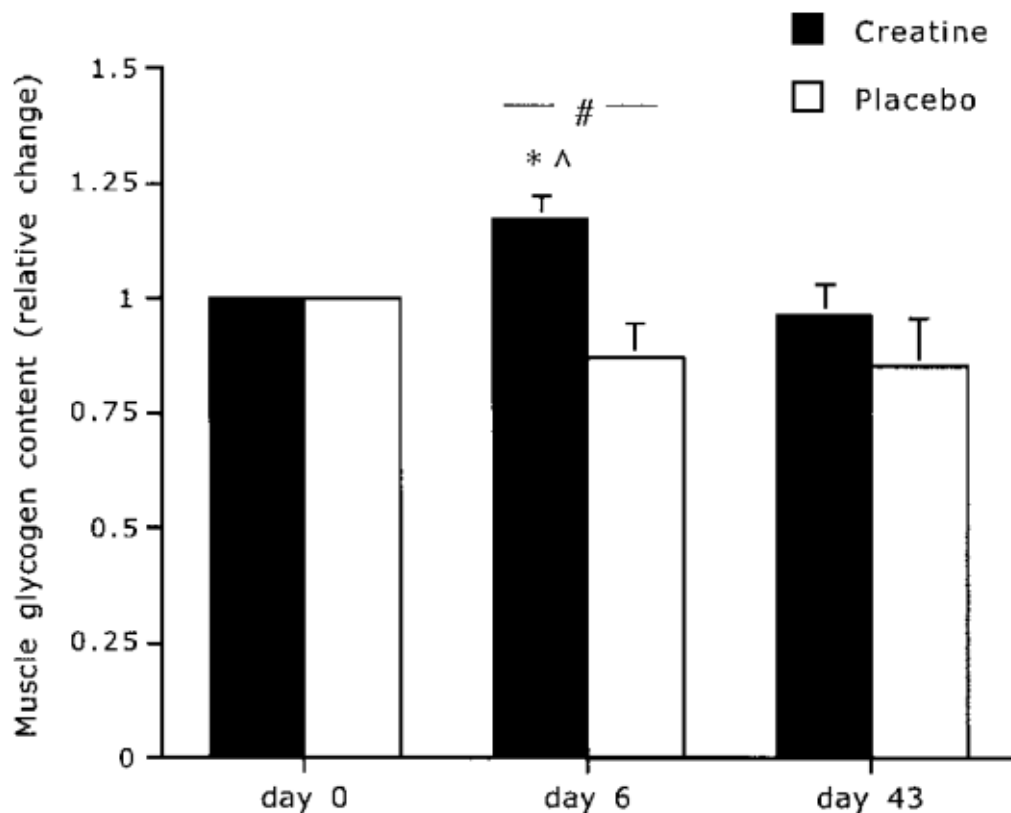


Figure 2.8: Muscle glycogen content after 6 weeks of creatine supplementation. Muscle biopsies from 20 male subjects with no prior history of exercise training were taken before and after 5 d of creatine loading ($20 \text{ g} \cdot \text{d}^{-1}$) and after 6 wk of continued supplementation ($2 \text{ g} \cdot \text{d}^{-1}$). Data were normalized to pre-supplementation values. * represents significant difference compared with values observed at day 0 within group; ^ represents significant difference compared with values observed at day 43 within groups; # represents significant difference between groups at day 6 ($P < 0.05$). (Reproduced from van Loon *et al.* 2004. *Clinical Science*)

Vandebuerie *et al.* (1998) reported that subjects who consumed creatine (25 g for 5 d) followed by carbohydrate ingestion ($60 \text{ g}\cdot\text{h}^{-1}$) exhibited an 8-9% increase in power output during a set of sprint efforts (5 x 10 s with 2 min recovery) which followed a 2.5 h time trial cycling simulation (Vandebuerie *et al.* 1998). Oliver *et al.* (2013) reported that 6 d of creatine supplementation ($20 \text{ g}\cdot\text{d}^{-1}$) and glucose ($60 \text{ g}\cdot\text{d}^{-1}$) resulted in significantly reduced lactate concentrations during exercise, with a trend toward increased maximum power output and total time to fatigue (Oliver *et al.* 2013). With regards to its application to endurance exercise, Tang *et al.* (2013) examined the metabolic changes associated with creatine supplementation ($12 \text{ g}\cdot\text{d}^{-1}$ for 15 d) on a 60 min running protocol (65-70% maximum heart rate) followed by a 100 m sprint (Tang, Chan, and Kuo 2013). They showed that the creatine-supplemented cohort displayed lower plasma lactate levels, suggestive of decreased muscle glycogen breakdown, as well as lower plasma purine metabolites, glutamine, urinary 3-methylhistidine, and urea nitrogen, indicative of decreased muscle protein degradation, following the 60 min bout of endurance exercise (Tang, Chan, and Kuo 2013). Other studies have also reported that creatine can attenuate protein breakdown during endurance exercise (Roschel *et al.* 2010, Gualano *et al.* 2008, Robinson *et al.* 1999). These findings (summarised in Table 2.1) provide evidence that enhanced creatine availability in the muscle may spare glycogen and reliance on anaerobic glycolysis. Further studies need to be conducted to determine whether this effect persists with regard to prolonged exercise conducted at intensities around the so-called lactate threshold (the intensity at which lactate production exceeds lactate clearance).

Creatine supplementation has been shown to lower muscle and blood lactate concentrations in individuals following high intensity (80-90% maximum heart rate) exercise, suggesting an alteration in the contribution of the non-oxidative pathways to the muscle's energy needs (Balsom *et al.* 1995). Balsom *et al.* (1995) reported that creatine

supplementation ($20 \text{ g}\cdot\text{d}^{-1}$ for 6 d) increased total muscle creatine stores and lowered muscle lactate levels (26.2 ± 5.5 vs. $44.3 \pm 9.9 \text{ mmol}\cdot\text{kg}^{-1} \text{ dm}$) following repeated bouts of fixed rate, high intensity cycling (Balsom et al. 1995). Additionally, total work and lactate threshold levels were improved following intravenous PCr injection 24 h and 30 min prior to incremental cycling bouts (Vorobiev et al. 1996).

Lower lactate levels with pre-exercise creatine supplementation have also been observed during recovery from high-intensity exercise, suggesting, but not proving, that carbohydrate utilization and glycogen degradation is decreased after endurance exercise with creatine supplementation (Tang, Chan, and Kuo 2013). As such, it has been suggested that creatine may have a positive effect on short term anaerobic efforts within prolonged bouts of endurance exercise, as seen in subsequent studies with increased power during bouts of cycling (Balsom et al. 1995, Balsom et al. 1993, Birch, Noble, and Greenhaff 1994, Casey et al. 1996) and running (Harris et al. 1993, Bosco et al. 1997, Earnest, Almada, and Mitchell 1997). It could be hypothesized that exercise capacity might be improved in events where glycogen availability is limiting, and can be further enhanced via creatine supplementation. Such findings would have practical significance for several scenarios in sports nutrition where greater glycogen availability is an important consideration for optimal performance. These include: 1) the optimization of glycogen storage capacity in individuals who are unable to obtain enough carbohydrate to maximize glycogen storage (e.g. athletes with low energy availability and sub-optimal intakes of carbohydrate due to physique management strategies (Mountjoy et al. 2014), or athletes with inadequate recovery time between glycogen-dependent events), 2) the further enhancement of glycogen super-compensation for prolonged events where glycogen depletion is likely, and/or 3) the reversal of the apparent inhibition on repeated attempts to carbohydrate load in a restricted time (i.e. 1 wk) (McInerney et al. 2005). Interestingly, a study by Sewell *et al.* (2008) demonstrated that

supplementation of a normal diet with additional carbohydrate (18.5% wt·vol⁻¹ glucose drink for 3 d prior to exercise) in addition to creatine supplementation, has no effect on muscle glycogen concentrations (Sewell, Robinson, and Greenhaff 2008). The authors suggested that creatine supplementation alone is insufficient to influence muscle glycogen synthesis and that a major factor underpinning creatine's role in increasing muscle glycogen is glycogen-depletion via exercise undertaken prior to creatine supplementation (Sewell, Robinson, and Greenhaff 2008). Further studies examining creatine's application in glycogen-limiting scenarios (i.e. endurance exercise) are therefore required.

Table 2.1: The effect of creatine supplementation on intramuscular glycogen storage

Study	Subjects	Exercise	Creatine	Carbohydrate	Effect
Robinson et al., 1999	14 healthy male (~23 y)	One-legged cycling bought (70 RPM) to exhaustion	20 g·d ⁻¹ Cr for 5 d	High carb diet (80% of daily energy intake)	Glycogen was increased above non-exercised concentrations in the exercised limb to a greater degree in the CHO + Cr group (p = 0.06) over CHO alone
Nelson et al., 2001	12 physically active but not structurally trained males (19-28 y)	Glycogen depleting one legged exercise	5 d of 20 g·d ⁻¹ before and after a high CHO diet period	High carb diet (80% of daily energy intake)	Post creatine load total glycogen content was significantly (P> 0.05) greater than pre-creatine load
Op't Eijnde et al., 2001	13 males and 9 females (20-23 y)	4x 12 unilateral knee-extensions at 60% max torque 3x a week for 10 weeks	20 g·d ⁻¹ for 2 wk of immobilization, 15 g·d ⁻¹ for first 3 weeks of rehab, 5 g·d ⁻¹ for following 7 wk	N/A	Muscle glycogen levels were higher in the creatine group after 3 weeks of rehab (P<0.05) but not after 10 weeks.
Derave et al., 2003	26 male and 7 female	6 weeks of resistance training following 2 weeks of leg immobilization	15 g·d ⁻¹ during immobilization, 2.5 g·d ⁻¹ during training	N/A	Increased muscle glycogen content and GLUT4 protein
Van Loon et al., 2004	20 fit, non-vegetarian males with no regular training (~20 y)	N/A	20 g·d ⁻¹ for 5 d (initial loading), 2 g·d ⁻¹ Cr for 37 d (maintenance)	15g glucose + 10 g maltodextrin	Creatine loading significantly increased (P< 0.05) muscle glycogen content, creatine maintenance dose did not maintain elevated glycogen levels
Sewell et al., 2007	6 healthy males with regular exercise of 1+ hr at least 3 d/wk (~26 y)	Ergometer set against 70% VO ₂ peak until cadence could not be maintained	20 g·d ⁻¹ Cr for 5 d prior to exercise trail	4-500ml servings of a 18.5% glucose drink	Creatine supplementation alone under controlled conditions and habitual diet had no effect on muscle glycogen content.
Safdar et al., 2007	12 healthy, non-smoking, non-obese males with reported exercise <2x a week (~26 y)	N/A	20 g·d ⁻¹ Cr for 3 d (loading); 5 g·d ⁻¹ Cr for 7 d (maintenance)	N/A (Placebo had 75g dextrose)	Cr supplementation significantly upregulated (P<0.05)the mRNA content of genes and proteins content of genes and protein content of kinases involved protein and glycogen synthesis regulation
Tang et al., 2014	12 males on a university track and field team (~20 y)	60 minute running exercise and a 100 M sprint running exercise	12 g·d ⁻¹ Cr for 15 d	N/A	Creatine supplementation tended to decrease muscle glycogen and protein degradation, especially after endurance exercise
Roberts et al. , 2016	14 recreationally active males	Cycling to fatigue @ 70% VO _{2peak}	20 g·d ⁻¹ Cr	37.5 kcal·kg ⁻¹ ·d ⁻¹ , >80% calories CHO	Augmented post-exercise muscle glycogen content (P<0.05); greatest difference during initial 24 h

2.4.4 Insulin Mediation of Creatine Transport

Insulin plays a vital metabolic role in increasing free fatty acid, amino acid and glucose uptake (Robergs and Roberts 2000). Glucose uptake has been extensively characterised by the insulin-stimulated translocation of glucose transporter 4 (GLUT4) to the cell membrane, thereby facilitating glucose transport from the bloodstream into muscle (Richter and Hargreaves 2013, Leto and Saltiel 2012, Oldham and Hafen 2003, Mora et al. 2004, Zierath 2002) (Figure 2.9). Furthermore, insulin, amongst a number of factors previously described (section 2.3.2), regulates creatine transport into skeletal muscle via the insulin-sensitive creatine transporter. Odoom and colleagues (1996) observed a two fold increase in intracellular creatine content after 48 h of insulin treatment in cultured G8 myotubes (Odoom, Kemp, and Radda 1996). This finding was confirmed by the *in vivo* work of Steenge *et al.* (1998) who found increased creatine uptake in subjects infused with varying rates of insulin concomitant with creatine consumption (Steenge et al. 1998). Subsequent studies have showed that insulin secretion in response to carbohydrate ingestion facilitates sodium-dependent creatine transport by increasing muscle Na^+/K^+ pump activity (Steenge et al. 1998, Loike, Somes, and Silverstein 1986, Odoom, Kemp, and Radda 1996), which results in increased total muscle creatine content (Koszalka and Andrew 1972, Haugland and Chang 1975, Green, Hultman, et al. 1996, Steenge, Simpson, and Greenhaff 2000, Steenge et al. 1998). In addition to the protein synthetic response elicited by creatine, the anabolic action of insulin *in vitro* has been shown to have mitogenic effects on stimulating muscle protein synthesis (subsequently discussed in section 1.6) (Reeds and Palmer 1983, Florini et al. 1986, Palmer et al. 1997).

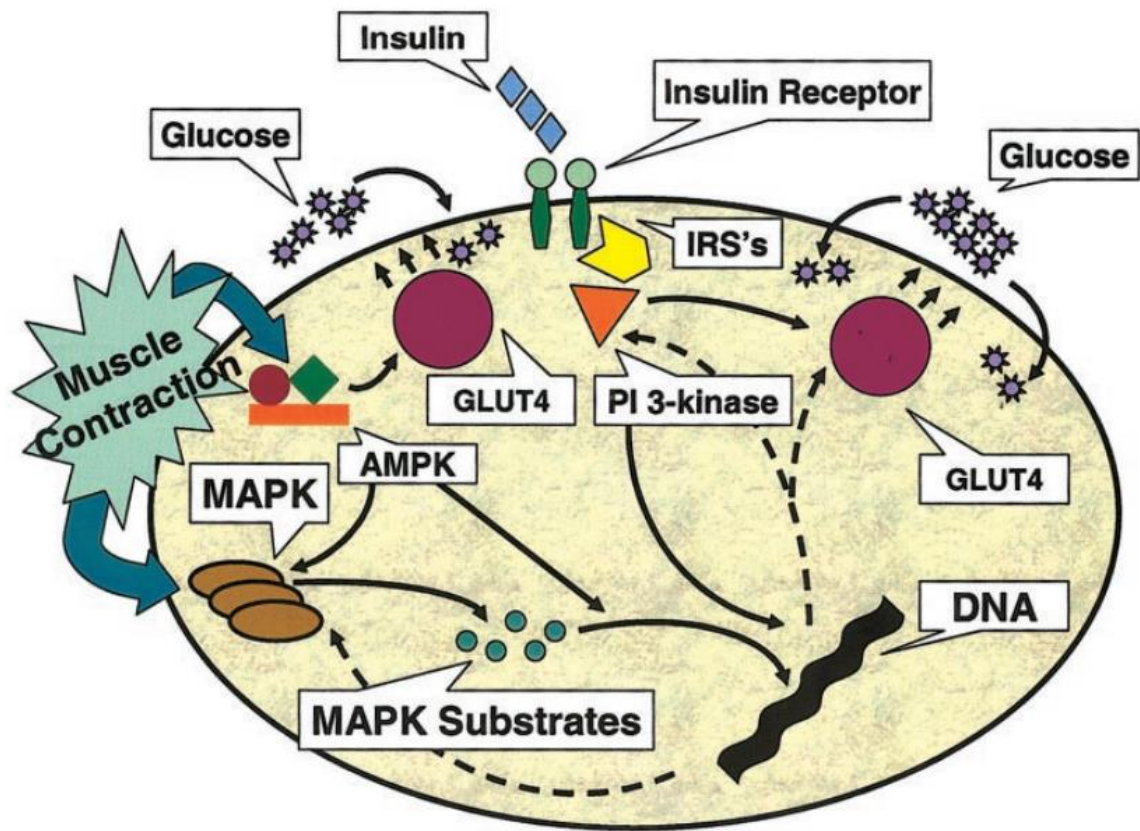


Figure 2.9: Schematic of insulin activity *in vivo*. Insulin signal transduction through the insulin receptor, insulin receptor substrate (IRS)-1/2 and phosphatidylinositol 3-kinase (PI3-kinase) is enhanced in skeletal muscle following ingestion of carbohydrates in non-diabetic individuals as well as in the hours after an exercise bout. These changes may enhance insulin sensitivity and promote glucose uptake via GLUT4, affect the uptake of creatine via the SLC6A8 creatine transporter and regulate the expression of several downstream signalling targets involved in protein synthesis and/or mitochondrial biogenesis. (Reproduced from Zierath 2002. *Journal of Applied Physiology*)

Given that increased creatine transport into skeletal muscle can be enhanced via an insulin-stimulated mechanism, it is possible that compounds which mimic insulin's effects may also be able to modulate substrate uptake. Several natural (Patel et al. 2012, Zaid 2013) and chemically-derived (Sakurai et al. 1998, Stapleton 2000, Orvig et al. 1995) products have been evaluated for their insulinotropic properties. For example, *Trigonella foenum-graecum* (commonly known as fenugreek), an herb widely used across the Indian subcontinent, has been used in alternative medicine practice as a therapeutic agent to control glucose levels in diabetics (Srinivasan 2006, Jaiswal et al. 2012, Vijayakumar et al. 2005, Sauvaire et al. 1998). Fenugreek, like insulin, has been found to stimulate glucose uptake by enhancing translocation of GLUT4 to the plasma membrane in rodent/rat skeletal muscle cells (Jaiswal et al. 2012).

A comprehensive examination of creatine's interactive role with insulin-mimicking compounds is presently lacking. Only one study to date had reported an ergogenic interaction between fenugreek and creatine. Taylor *et al.* (2011) found that, in a resistance trained population, supplementation with 3.5 g of creatine and 900 mg of fenugreek over 8 wk of periodised resistance training ($4 \text{ d} \cdot \text{wk}^{-1}$) resulted in significant increases in 1 repetition maximum (RM) strength along with an increasing lean BM (Taylor et al. 2011). These results were deemed to be as effective as supplementation with creatine and dextrose ($70 \text{ g} \cdot \text{d}^{-1}$) with regard to the improvements observed in upper (+9% dextrose; +6% fenugreek) and lower body (+19% dextrose; +17% fenugreek) strength. However, the authors reported only minimal differences between creatine-fenugreek and control creatine-carbohydrate supplemented cohorts with respect to strength and provided no direct measures of creatine content either via muscle biopsies or magnetic resonance spectroscopy. Thus, it is unclear to what degree fenugreek supplementation actually affected their main outcome variable (i.e. muscular strength) or by what mechanism creatine transport and uptake were promoted (if

any). Nevertheless, this study demonstrated a potential role for fenugreek in the augmentation of intramuscular creatine concentration, which may occur via a mechanism similar to previously reported insulin-induced modulations of the creatine transport system (Steenge et al. 1998).

2.5 Creatine's Role in Muscle Tissue Growth and Regeneration

A number of pathways involved in the regulation of glucose uptake, glycogen synthesis, and creatine transport have also been implicated in promoting cell growth and differentiation, muscle proteolysis, and regulation of hypertrophy (Glass 2003, Heszele and Price 2004, Sartorelli and Fulco 2004, Taniguchi, Emanuelli, and Kahn 2006). Before characterising the specifics of substrate-mediation of these mechanisms, a context for skeletal muscle myogenesis and hypertrophy (i.e. increase in myofibre size) will be provided.

2.5.1 Myogenesis

New skeletal muscle formation is a complex and highly regulated process that involves the proliferation of myoblasts and subsequent differentiation to multinucleated myotubes (Grabiec et al. 2014). Satellite cells, the progenitors for skeletal myotube formation, are the primary contributors to muscle growth via the provision of myonuclei for the production of new or repair of existing muscle (Wang and Rudnicki 2012). Post-natal muscle growth occurs via myofibrillar hypertrophy. Concurrent with increasing myofibril size, muscle cells also increase in nuclei number (Dangott, Schultz, and Mozdziak 2000, Olsen et al. 2006, Vierck et al. 2003). Given that post-embryonic muscle cells cannot undergo mitosis, new growth and development of skeletal muscle involves the emergence of satellite cells from a quiescent state into the cell cycle as active myoblasts where they either leave the cell cycle to the quiescent pool or to enter the differentiation pathway to become myotubes (Dumont, Wang, and Rudnicki 2015). Satellite cells, located between the

sarcolemma and the basal lamina surrounding muscle fibres become activated (i.e. following muscle injury or sheer stress from exercise) (Macpherson, Dennis, and Faulkner 1997, McCully and Faulkner 1985) and proliferate to provide additional myonuclei for the repair maintenance of the muscle milieu (Mauro 1961, Sestili et al. 2009, Olsen et al. 2006). During regeneration, the normally inactive satellite cells proliferate until molecular signalling brings mitotic activity to an end wherein differentiation and specialization to muscle precursor cells is initiated and promoted to the site of regeneration (Charge and Rudnicki 2004). The fusion of satellite cells to form myotubes is a morphological endpoint in the satellite cell's involvement in muscle hypertrophy (Vierck et al. 2003).

Various transcription factors along with a network of myogenic regulatory factors (MRFs) drive the progression of satellite cells toward the formation of new muscle (Collins et al. 2009). Paired-box transcription factor Pax7 plays important roles in developmental and regenerative myogenesis (Collins et al. 2009). Pax7 is specifically expressed in quiescent and newly activated satellite cell populations throughout adulthood (Seale et al. 2000, Kuang et al. 2006). Furthermore, it has been shown that constitutive expression of Pax7 by satellite cells increases the rate of proliferation (Collins et al. 2009). In muscle progenitor cells, Pax7 also function to maintain the timely expression of MRFs and promote population expansion (Dangott, Schultz, and Mozdziak 2000) (Figure 2.10).

MRFs are the primary regulators of satellite cell activation (Sestili et al. 2009, Louis et al. 2004). Myogenic differentiation factor (MyoD), myogenic factor 5 (Myf-5) and myogenin are among the most well-characterized MRFs responsible for governing the initiation of satellite cell proliferation and differentiation (Zammit et al. 2006, Zammit et al. 2004). These factors are members of family of basic helix-loop-helix proteins that act as transcriptional activators due to their properties as DNA-binding proteins (Burattini et al. 2009). DNA binding initiates transcription to regulate the expression level of muscle-

specific genes, myosin heavy and light chains, α -actin, and creatine kinase (Lowe, Lund, and Alway 1998). Activation of satellite cells in muscle which enter the cell cycle involves the rapid upregulation of MyoD just before proliferation begins which carries on through the differentiation phase while Myf-5 expression is a marker indicating satellite cell self-renewal (promotion of proliferation)(Kitzmann et al. 1998). Myogenin upregulation occurs as a marker of terminal satellite cell differentiation (Wang and Rudnicki 2012, Collins et al. 2005) (Figure 2.10).

In humans, the regulation of muscle hypertrophy and myogenesis via the activation of progenitor satellite cells is subject to the availability of nutrients, such as carbohydrates and amino acids, and hormones, such as insulin (Grabiec et al. 2014). Furthermore, molecular adaptations to exercise training are specific to the type of activity performed- with resistance exercise stimulating myofibrillar protein synthesis and hypertrophy (Fry 2004, Hawley 2009) while endurance training stimulates mitochondrial protein synthesis and aerobic metabolic adaptations (i.e. reduced rates of glycogen utilisation, increased fat oxidation, and lowered lactate production) (Hawley 2009, Holloszy and Coyle 1984). Therefore, an understanding of the effect nutrient availability on the signalling pathways is important to elucidate the role creatine plays in promoting adaptation that underpins improved exercise capacity/performance.

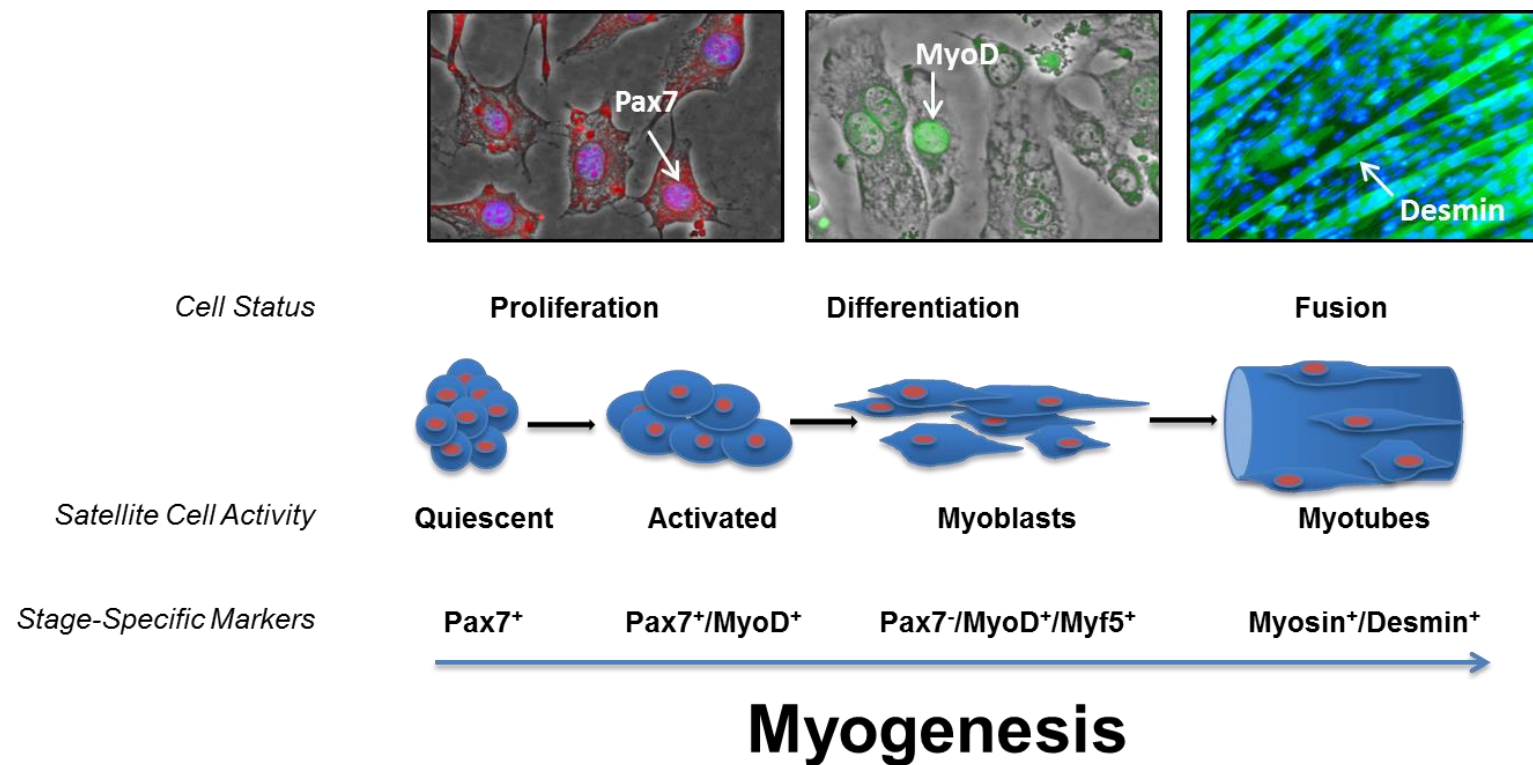


Figure 2.10: The activation and differentiation of skeletal muscle satellite cells. Satellite cells remain in a quiescent state in the sarcolemma of existing myofibrils. Mechanical damage (i.e. from resistance exercise) perturbs muscle fibres and signals the activation of satellite cells into a specialized lineage of myogenic cells and can fuse to existing fibres to repair muscle damage or fuse together to form new muscle fibre. Pax7 (stained red, image 1) is a transcription factor which regulates myogenesis via promotion of satellite cells from quiescence to early-stage proliferation. MyoD (stained green, image 2), an early marker of myogenic commitment to skeletal myoblast lineage, is expressed in the nuclei of activated satellite cells/myoblasts in response to muscle damage or exercise stimuli. Desmin (stained green, image 3) is a muscle-specific intermediate filament present in the lamina around mature muscle myotubes which is an early protein marker of muscle tissue that increases in expression as cells near terminal differentiation.

2.5.2 *Effects of Exercise on Muscle Repair*

Training-induced activation of satellite cells is essential for post-natal skeletal muscle hypertrophy. While satellite cells are essential for muscle regeneration, several studies contend that they are not necessary for post-natal muscle hypertrophy, particularly in animal models. Work by McCarthy and colleagues (2011) demonstrated that Pax7-DTA mice (conditional ablation of greater than 90% of satellite cells in mature skeletal muscle) which were subjected to 2 wk of mechanical overload showed the same increase in muscle mass (~2 fold) and fibre cross sectional area as those observed in a vehicle –treated group with the commonly observed increase in myonuclei with hypertrophy being absent in satellite cell-depleted muscle fibres (McCarthy et al. 2011). This suggests that skeletal muscle fibres are capable of mounting a robust hypertrophic response to mechanical overload that is not necessarily dependent on satellite cells per se. Fry and associates (2014) later established that, in this same mouse model, 8 wk of overload resulted in an accumulation of extracellular matrix and fibroblast expansion that resulting in reduced specific force of the plantaris muscle (Fry, Lee, et al. 2014). Furthermore, there was an attenuation of hypertrophy measured by both muscle wet weight and fibre cross-sectional area in satellite cell-depleted muscle (Fry, Lee, et al. 2014). *In vivo* work by Fry (2014) following 12 weeks of aerobic training performed on a cycle ergometer by sedentary adults resulted increase in satellite cell content, specifically in myosin heavy chain type I fibres with no change in satellite cell numbers in type II (Fry, Noehren, et al. 2014). These data therefore provide evidence for a fibre type-specific role for satellite cells in muscle adaptations following aerobic training.

Most commonly, resistance-based exercise increases the number of satellite cells and their fusion to myofibre, hence increasing myonuclei in muscle (Olsen et al. 2006, Kadi and Thornell 2000, Roth et al. 2001). It is currently accepted that a contraction stimulus is involved in all stages of muscle hypertrophy. Exercise induces satellite cell activation

(thereby maintaining an intramuscular pool following proliferation) promotes withdrawal from the cell cycle back to quiescence or towards differentiation, and allows formation of new myofibres via the donation of myonuclei (Kadi et al. 2005). As myonuclei in mature myofibres are unable to undergo further cell division, growth occurs via the addition of myonuclei added to a fibre by satellite cells. Each myonuclei in adult muscle fibres controls the production of mRNA and protein synthesis over a limited amount of cytoplasm (i.e. the myonuclear domain). In order for the mRNA abundance to increase, each myonuclei needs to increase the rate of transcription for a given gene or be incorporated into the parent fibre thereby providing additional myonuclei (Kadi et al. 2005). Cramer *et al.* (2004) demonstrated that the pool of satellite cells in muscle can be increased in as little as 4 d following an acute bout of intense resistance-based exercise (50 one-leg 'drop down' jumps, 8x10 maximal eccentric knee extensions and 8x10 maximal eccentric knee extensions; 30 sec rest between sets and 5 min rest between each exercise) (Cramer *et al.* 2004). Furthermore, this increase in satellite cell number was maintained with continual training (8 d), while a cessation of training gradually reduced the pool.

Resistance-based exercise, owing partly to the breakdown and repair of muscle fibres, is the primary modality which promotes satellite cell activation. A single bout of resistance-based contractile activity can result in increased MyoD and myogenin mRNA in both human and rodent skeletal muscle (Adams, Haddad, and Baldwin 1999, Haddad and Adams 2002, Yang et al. 2005). Roth *et al.* (2001) reported a 36% increase in the total number of satellite cells compared to undamaged muscle in individuals following a single bout of resistance exercise (Roth et al. 2001). Furthermore, Kosek *et al.* (2006) identified that sustained resistance training ($3 \text{ d} \cdot \text{wk}^{-1}$ for 16 wk) induced an elevated MyoD and myogenin response equal to that seen after a single acute bout of resistance exercise, implicating MRFs in actively promoting hypertrophy following strength training exercise

(Kosek et al. 2006).

The fusion of daughter cells arising from proliferating satellite cells to generate new myotubes has also been observed following endurance exercise, although to a lesser extent. Work by Kadi *et al.* (1999) has identified increased myosin heavy chain expression in about half of a cohort of young women undertaking endurance exercise (Kadi and Thornell 2000). While endurance activities like cycling and swimming are unlikely to induce substantial satellite cell activation (due to a lack of contractile-induced muscle perturbation), MyoD and myogenin's response to endurance exercise has been hypothesized to regulate pathway(s) which involves mitochondrial enzyme and/or transformations of muscle fibre type (Kadi et al. 2004, Siu et al. 2004, Vissing et al. 2005).

2.5.3 *Cellular Signalling Associated with Muscle Repair*

Hypertrophy is regulated by phosphorylation events which determine the rate of protein synthesis (Farrell et al. 2000, Richter and Sonenberg 2005). Translation initiation is an important step in the regulation of hypertrophy, particularly in response to external stimuli such as exercise or nutrient availability, and is generally considered a rate limiting step in protein synthesis (Richter and Sonenberg 2005). Generally, the initiation of protein synthesis is regulated via the phosphorylation of eukaryotic initiation factors (eIF) which mediate the binding of the 40S ribosomal subunit to mRNA- an event which is further mediated by the interaction of a number of eIFs and ribosomal S6 protein, the distal components of insulin-like growth factor -1 (IGF-1) and mitogen-activated protein kinase signalling cascades. The promotion of hypertrophy through translation initiation via insulin/IGF-1 binding and subsequent downstream signalling resulting in functional contractile protein, has been thoroughly reviewed (Coffey and Hawley 2007). Downstream targeting of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3-K) by IGF-1 promotes the activation of protein kinase B (also known as AKT), which not only plays a role in insulin signalling

(Mora et al. 2004) but is also responsible for the activation of a number of key signalling intermediates of the protein synthetic pathway including mechanistic target of rapamycin (mTOR) and p70 S6 kinase (p70S6K) (Baar and Esser 1999) which are critical for promoting hypertrophy in skeletal muscle (Figure 2.11).

Protein Kinase B/AKT

AKT represents the “crossroads” in insulin/IGF signalling wherein it is not only responsible for the regulation of GLUT4 translocation to the cell membrane but also for the maintenance of skeletal muscle cell size. Three isoforms of AKT exist, two of which are specifically expressed in skeletal muscle (AKT1 and AKT2) (Sumitani et al. 2002, Nader 2005). AKT1 has been associated with muscle hypertrophy whereas AKT2 has been associated with glucose transport (Taniguchi, Emanuelli, and Kahn 2006). For full activation, AKT which is co-localized to the cell membrane, is phosphorylated and subsequently activated by phosphorylation of the threonine 308 residue in the activation loop of pyruvate dehydrogenase kinase 1 (PDK1) and the serine 473 residue in the hydrophobic motif by mechanistic target of rapamycin complex 2 (mTORC2). The former is mostly associated with insulin stimulation (Kim et al. 1999, Vincent et al. 2011, McManus et al. 2004, Sarbassov et al. 2005).

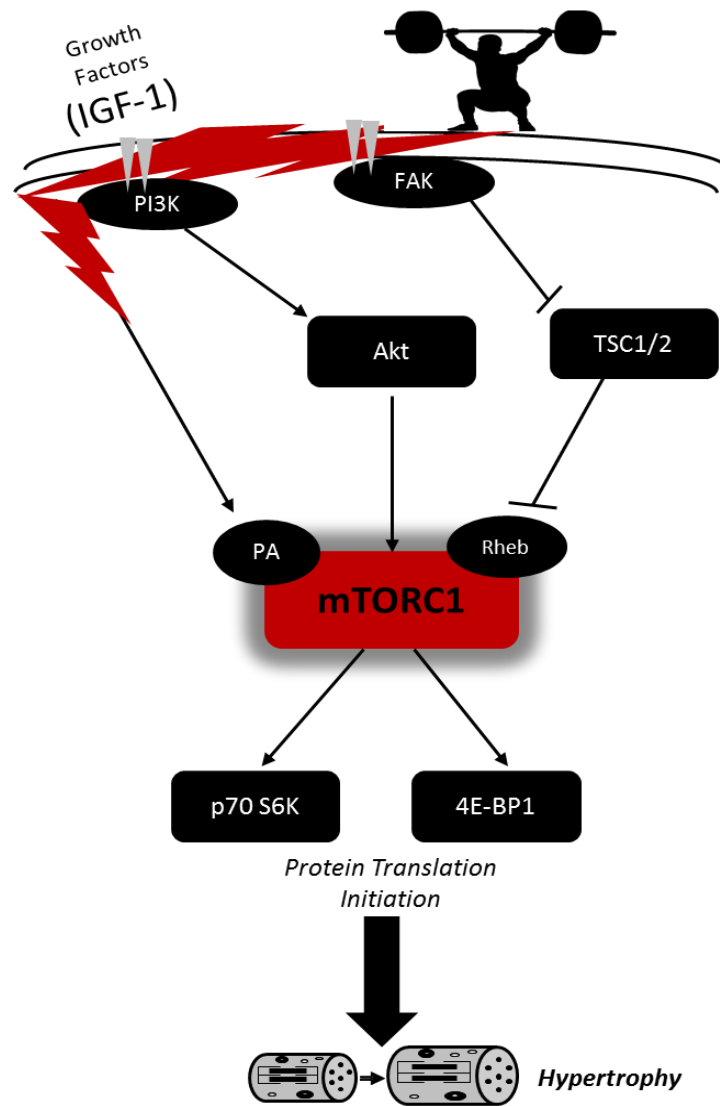


Figure 2.11: Schematic of intermediates involved in hypertrophy signalling. Combined resistance exercise and/or stimulation by nutritional/hormonal factors optimize protein translation initiation via the mammalian target of rapamycin complex 1 (mTORC1) by potentially enhancing the activity of its direct regulators phosphatidic acid (PA) and Ras homologue enriched in brain (Rheb) that are modulated upstream by diacylglycerol kinase ζ (DGK ζ) and tuberous sclerosis complex catalytic subunit 2 (TSC2), respectively. The mTORC1 kinase promotes protein synthesis by phosphorylating its substrates eIF4E binding protein 1 (4E-BP1) and p70 rpS6 kinase (p70S6K). (Adapted from Smiles, Hawley and Camera 2016. *Journal of Experimental Biology*)

The putative mechanism of AKT regulation of insulin-mediated glucose uptake is characterized by the binding and subsequent localization of the PI3-K subunit to the plasma membrane (Oldham and Hafen 2003). Phosphorylation of PI3-K causes the PDK1-driven recruitment of AKT to the plasma membrane for its subsequent activation (Mora et al. 2004). Insulin signalling can also proceed via activation of a divergent pathway involving the intermediate protein kinase C (PKC) (Vijayakumar et al. 2005). AKT directs activation of distal regulator, AS160 (GTP-ase activating protein), promoting insulin induced GLUT4 translocation to the cell membrane facilitating glucose transport (Richter and Hargreaves 2013, Leto and Saltiel 2012). In addition to regulating glucose uptake, AKT also regulates the process of glycogen synthesis via the phosphorylation and inhibition of glycogen synthase kinase 3 (GSK-3) which activates glycogen synthesis (Graham et al. 2010).

The phosphorylation and activation of AKT prevents the upregulation of pathways controlling muscle protein breakdown. Instead, AKT functions to activate downstream target mTOR for the promotion of protein synthesis via the activation of translation initiation and increased ribosomal protein content (Bodine et al. 2001, Nader 2005). Furthermore, AKT has been implicated in the regulation of the 5' AMP-activated protein kinase (AMPK), a “master switch” implicated in several metabolic systems including the cellular uptake of glucose, the β -oxidation of fatty acids and the biogenesis of mitochondria. (Rommel et al. 2001). AMPK phosphorylates tuberous sclerosis complex 2 (TSC2) and GSK3 β , with the former inhibiting the action of mTOR and the latter preventing the activation of eIF-2B (Hahn-Windgassen et al. 2005, Garami et al. 2003, Vyas et al. 2002). As such, AKT plays a role in enhancing translation initiation and protein synthesis through an inhibition of AMPK-mediated targets in addition to regulating the hypertrophic action of mTOR.

mTOR

AKT has the ability to phosphorylate downstream signalling kinase mTOR, which plays a key role in regulating translation of muscle protein synthesis. Two mTOR complexes exist wherein mTOR binds with a G-β-L protein and either rapamycin-sensitive (raptor, forming mTORC1) or rapamycin-insensitive (rictor, forming mTORC2) companion of mTOR protein. mTORC1 has been shown to be a positive regulator of cell growth and development whereas mTORC2 has been implicated in AKT activation and actin cytoskeleton regulation (Wang et al. 2005, Sarbassov et al. 2004, Park et al. 2005, Bodine et al. 2001).

Like AKT, mTOR is activated by a wide range of stimuli, both mechanical and metabolic (Miniaci et al. 2015). Amino acids play a role in the upregulation of mTOR, both *in vivo* and *in vitro* (Areta et al. 2014, Kim et al. 2008). Furthermore, it has been suggested that both PI3-K, an upstream effector of AKT, and mTOR are *both* required for insulin-specific induction of myogenic differentiation and furthermore myosin heavy chain (MHC) expression. (Sumitani et al. 2002). mTORC1 activity has also been shown to be related to levels of mitochondrial metabolism via its role in regulating mitochondrial-related gene expression (Cunningham et al. 2007). The primary role of mTOR, however, lies in the regulation of protein synthesis which is mediated primarily through the downstream activation of ribosomal protein S6 kinase (S6K) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1) (Van Wessel et al. 2010) (Figure 2.11).

p70S6K and 4E-BP1

Ribosomal protein S6 kinase (S6K) and 4E-BP1 are well-characterised downstream effectors of the AKT/mTOR hypertrophy signalling pathway. S6K has been shown to have two isoforms (S6K1 and S6K2). Knock-out mouse models have determined that S6K1 is the predominant regulator of cell size in skeletal muscle (Shima et al. 1998, Ohanna et al. 2005,

Ruvinsky and Meyuhas 2006). Subsequently, S6K also has dual cytosolic (p70S6K) and nuclear (P85 S6K) isoforms (Ruvinsky and Meyuhas 2006). S6K exerts its effect through several substrate targets and has been linked with a regulatory role in a number of cellular processes, with the most prominent being its central role in skeletal muscle hypertrophy (Ruvinsky and Meyuhas 2006, Baar and Esser 1999, Bodine et al. 2001). Furthermore, early studies have established that resistance training stimuli promotes the upregulation of p70S6K whereas endurance training does not (Nader and Esser 2001)

eIF4E binds to small phosphoproteins termed 4E-binding proteins of which 4E-BP1 is the best characterized (Wang and Proud 2006). 4E-BP1 is regulated and suppressed by mTORC1 via phosphorylation to depress 4E-BP1 inhibition of translation initiation cap binding protein eIF4E (Richter and Sonenberg 2005). 4E-BP1 emulates the function and activity of S6K1 in that there is an mTOR-mediated upregulation of 4E-BP1 phosphorylation that occurs under situations of mechanical stress (Bodine et al. 2001, Thomson and Gordon 2006). Resistance exercise increases the phosphorylation of 4E-BP1 with skeletal muscle overload decreasing the binding of 4E-BP1-eIF4E binding, allowing for greater rates of translation initiation (Bodine et al. 2001, Kubica et al. 2005). Atherton *et al.* (2005) demonstrated that in rats, endurance-like stimuli decreases 4E-BP1 which indicates a negative effect of endurance exercise on translational mechanisms and protein synthesis (Atherton et al. 2005).

p38 MAPK

The mitogen activated protein kinase (MAPK) pathway is stimulated by a number of factors and regulates a diverse array of cellular functions in mammalian cells. Of the five distinct MAPK groups: extracellular signal-regulated kinases (ERK) 1 and 2 and p38 isoforms alpha (α), beta (β), gamma (γ) and delta (δ), the p38 MAPK is the most responsive

to stress stimuli (Roux and Blenis 2004). Upon activation, both ERK 1/2 and p38 modulate gene expression and protein synthesis via phosphorylation of targets such as transcription factors and cytosolic proteins (Sakamoto and Goodyear 2002)

P38 MAPK is responsible for regulating numerous processes including myogenic and mitochondrial gene expression in addition to ubiquitin ligase activity (Lluís et al. 2006). p38 MAPK has been implicated in the activation of a number of transcription factors necessary for differentiation of myoblasts to myotubes (Lechner et al. 1996) which promote fusion of myoblasts and increase the expression of muscle specific genes, such as MHC (Deldicque et al. 2007). This process is primarily coordinated through p38's ability to act as a molecular switch for the activation of satellite cells (Lluís et al. 2006) Several studies have shown that p38 MAPK activity increases muscle differentiation and forced activation of this pathway could stimulate differentiation and promote myotube formation (Wu et al. 2000, Zetser, Gredinger, and Bengal 1999, Serra et al. 2007). Work by Gardner and colleagues (2015) found that blocking p38 MAPK expression, thereby promoting IGF-1 mediated signalling, results in a lack of C2 muscle cell fusion suggesting p38 MAPK is essential for the later stages of myotube differentiation resulting in multinucleated tubes (Gardner et al. 2015).

2.5.4 *Creatine's Effects on Muscle Repair*

Creatine supplementation can increase and prevent the inhibition of MRF expression in murine myoblasts (Louis et al. 2004, Sestili et al. 2009). *In vitro* studies have shown that creatine is able to enhance differentiation of myogenic C2C12 cells by activating both MAPK and AKT signalling pathways (Deldicque et al. 2007). In humans, creatine supplementation has been demonstrated to stimulate muscle hypertrophy during rehabilitation following muscle immobilization, an effect ascribed to increased Myogenic factor-4 (MRF-4) and myogenin expression (Hespel et al. 2001). Combining 12 weeks of creatine supplementation ($6 \text{ g}\cdot\text{d}^{-1}$) with resistance training also resulted in increased

myogenin and MRF-4 mRNA abundance and protein concentration (Willoughby and Rosene 2003). Dangott *et al.* (2000) reported that creatine supplementation in combination with compensatory hypertrophy (i.e. training stimulus) results in an increase in functional load and satellite cell proliferative activity in the skeletal muscle of Sprague-Dawley rats (Dangott, Schultz, and Mozdziak 2000). Furthermore, creatine increased satellite cell activity *only* when combined with the increased functional activity of the muscle (Dangott, Schultz, and Mozdziak 2000). This aligns with observations from previous work by Brannon *et al.* (1997) showing creatine's central role in hypertrophy is dependent on contractile stimuli (Brannon *et al.* 1997).

As discussed previously (section 2.4), creatine increases muscle mass and strength following resistance-based exercise (Cooper *et al.* 2012, Mesa *et al.* 2002, Hultman *et al.* 1996, Taylor *et al.* 2011, Harris, Soderlund, and Hultman 1992, Vierck *et al.* 2003) and also affects the rate of satellite cell proliferation (Vierck *et al.* 2003). An *in vitro* investigation by Vierck *et al.* (2003) determined the effects of creatine monohydrate, creatine pyruvate, L-glutamine, dehydroepiandrosterone (DHEA), androstenedione, *ephedra sinensis* extract, and *citrus aurantium* extract on ovine-derived satellite cells. It was found that following 96 h incubation, only creatine monohydrate was able to induce cell differentiation (Vierck *et al.* 2003). Notably, it was reported that with creatine incubation, insulin was deemed necessary for increasing fusion and muscle myotube number (Vierck *et al.* 2003). Skeletal muscle is one of the major targets for the anabolic action of insulin with *in vitro* studies having identified its mitogenic effects on cell metabolism as well as its role in stimulating muscle protein synthesis (Reeds and Palmer 1983, Florini *et al.* 1986, Palmer *et al.* 1997). Vierck *et al.* concluded that creatine-mediated hypertrophy occurs within a narrow concentration window via the stimulation of satellite cell proliferation and differentiation underpinned by insulin.

2.5.5 *Interactive Effects of Creatine, Carbohydrates and Insulin on Cell Signalling*

Both carbohydrate and creatine loading elicit a small but acute increase in BM (on average 1-2 kg), principally via the additional storage of intra-cellular water (Nygren et al. 2001, Ziegenfuss, Lowery, and Lemon 1998, Safdar et al. 2008). Creatine, especially, has been reported to increase intracellular water retention and alter cell volume (Francaux and Poortmans 1999, Juhn and Tarnopolsky 1998b, Powers et al. 2003, Kreider et al. 1998, Ziegenfuss, Lowery, and Lemon 1998). Augmented cell size may be the catalyst underlying increases in glycogen storage. Indeed, the cellular swelling caused by the storage of additional water within the muscle cell has been associated with the upregulation of a large number of genes involved in protein and glycogen synthesis, satellite cell proliferation and differentiation, and DNA replication and repair (Kreider et al. 1998, Powers et al. 2003, Ziegenfuss, Lowery, and Lemon 1998, Francaux and Poortmans 1999, Olsson and Saltin 1970b, Baker and Jeukendrup 2014, Sherman et al. 1982, Juhn and Tarnopolsky 1998b, Safdar et al. 2008).

Creatine supplementation alters various signalling pathways involved in myogenesis, mitochondrial biogenesis and protein synthesis. *In vitro* investigations by Delidicque *et al.* (2007) on anabolic signalling has shown that creatine incubation (5 mM) increases the expression of key hypertrophy-related proteins p38 MAPK, AKT and p70S6K in C2C12 mouse muscle cells (Deldicque et al. 2007). Additionally, the authors identified creatine stimulated myogenic differentiation via activation of the p38 MAPK signalling pathway, which subsequently up regulates MyoD, indicative of satellite cell activation (Deldicque et al. 2007). Interestingly, subsequent *in vivo* creatine supplementation (21 g for 5 d) investigation showed a reduction in the phosphorylation of AKT^{Thr308} and 4E-BP1 24 h post exercise with no effect on p38 MAPK signalling when creatine supplementation was coupled with resistance exercise (Deldicque et al. 2008). There was however, an increase in MHC

mRNA both at rest and post exercise, suggesting that the modification of genes associated with hypertrophy by creatine does not require the mechanical stimulus of exercise (Deldicque et al. 2008).

Carbohydrate-mediated insulin induction of myogenic differentiation has shown to require both PI3-K, an upstream effector of AKT, and mTOR for increasing functional hypertrophy, specifically increased MHC expression (Sumitani et al. 2002). Work by Sumitani *et al.* (2002) demonstrated that while insulin-stimulated proliferation occurs via both the activation of PI3-K and mTOR, transcription of myogenin, which is required for fusion of myogenic precursor cells to form new myofibres, requires PI3-K activation but not mTOR (Sumitani et al. 2002).

Chromatographic analysis of fenugreek seeds has revealed substantial quantities of (2S,3R,4S)-4-hydroxyisoleucine (4-HIL) (Narender et al. 2006, Sauvaire et al. 1998), a naturally-occurring plant amino acid that is unique to fenugreek and *bacillus thuringiensis* thought to be the major constituent responsible for the observed glucose-lowering effect (Ogawa et al. 2011). Broca *et al.* (2000) demonstrated that an acute dose of 4-HIL extracted from fenugreek seeds activated PI3-K in the liver and muscle cells of both diabetic and non-diabetic rats (Broca et al. 2004). AKT phosphorylation is widely associated with insulin stimulation (Kim et al. 1999). Vijayakumar *et al.* (2005), however, observed that fenugreek treatments had little effect on AKT expression in either hepatocytes or adipocytes, particularly at the serine 473 residue (Vijayakumar et al. 2005). The authors hypothesized that fenugreek signalling occurs via a divergent pathway involving protein kinase C (PKC) as an intermediate rather than AKT (Figure 2.12).

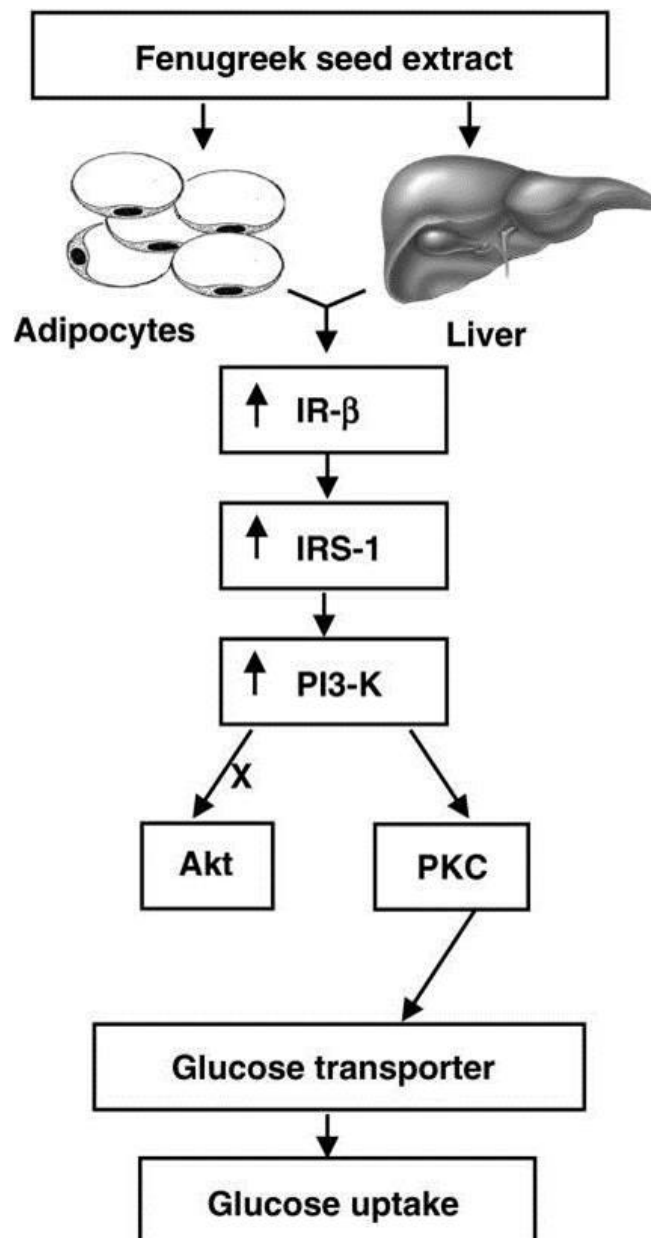


Figure 2.12: A proposed model for cellular signalling by fenugreek seed extracts. Previous work has suggested fenugreek effects glucose homeostasis via a protein kinase C (PKC) dependent pathway rather than protein kinase B (also known as AKT). (Reproduced from Vijayakumar *et al.* 2005. *British Journal of Pharmacology*)

In contrast, Jaiswal *et al.* (2012) examined the effect of 4-HIL on glucose uptake and translocation of GLUT4 to the plasma membrane in L6 skeletal muscle cells and showed that 4-HIL stimulated glucose uptake by enhancing translocation of GLUT4 to the cell surface in

a PI3-K/AKT-dependent mechanism (Jaiswal et al. 2012) Such discrepancies indicate that there may indeed be tissue-specific mechanisms which underpin fenugreek's reported therapeutic effects. These conflicting reports on substrate interactions on cellular level hypertrophy signalling pose an interesting conundrum that warrants further investigation.

Endurance athletes benefit from a greater mitochondrial capacity due to their increased requirement for oxygen-dependent energy turnover. Initially, creatine and PCr were thought to inhibit the activation of AMPK (Winder 2001). However, *in vitro* research by Ceddia *et al.* (2004) indicated that creatine supplementation (0.5 mM for 48 h) increases AMPK phosphorylation with a concurrent shift in basal glucose metabolism towards oxidation and a concomitant reduction in lactate production (Ceddia and Sweeney 2004). Lower lactate levels are indicative of reduced carbohydrate utilization and reflect the potential role of increased muscle creatine availability in sparing muscle glycogen use.

Taken collectively, creatine, carbohydrate and insulin's interactions suggest a synergistic, ergogenic relationship beneficial for both resistance-based hypertrophy and endurance-based mitochondrial biosynthesis (Figure 2.13). Carbohydrate-mediated insulin secretion increases creatine transporter activity, resulting in greater transport of extracellular creatine into the intracellular space (Green, Hultman, et al. 1996), thus also promoting greater hypertrophy via satellite cell activation. Augmented intracellular creatine content increases AMPK phosphorylation (Ceddia and Sweeney 2004), of which the 160 kDa AKT substrate (AS160), is a downstream target (Treebak et al. 2006).

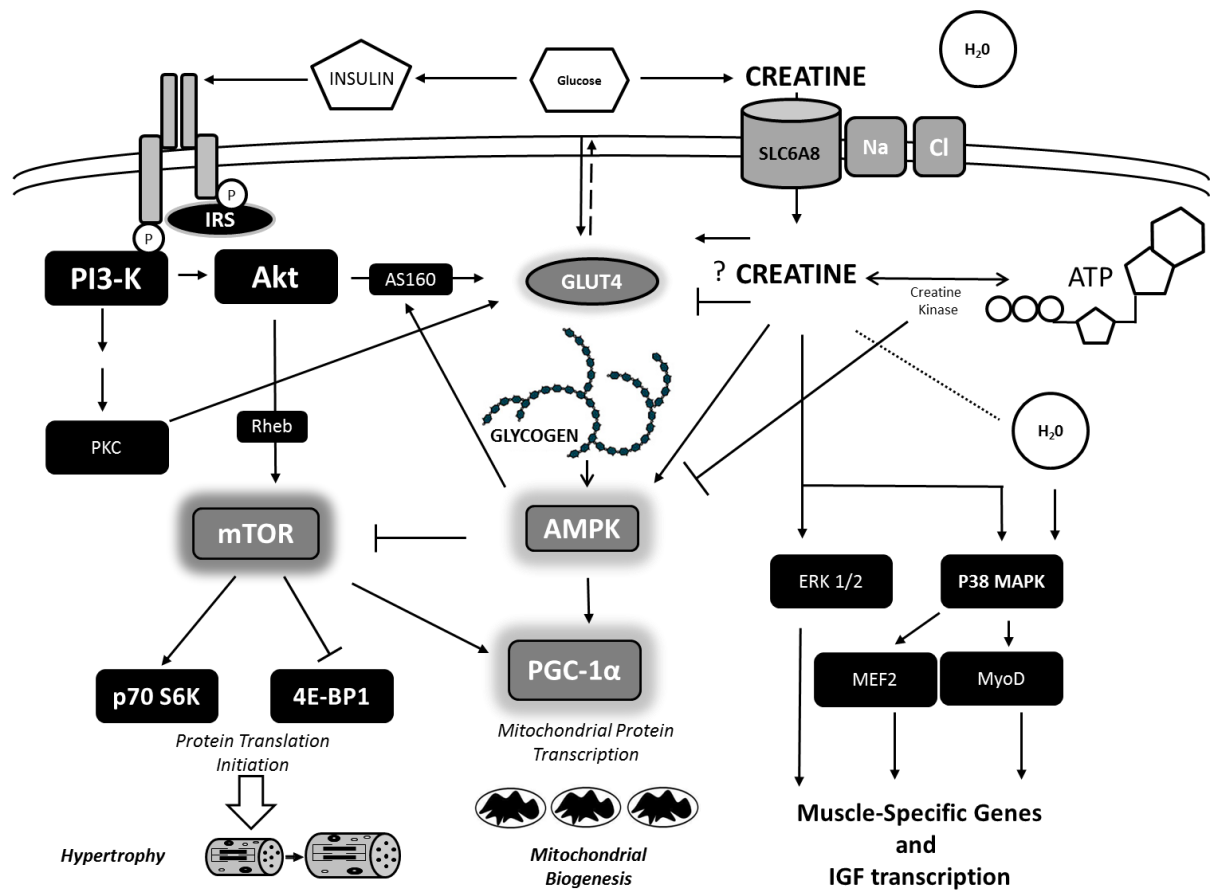


Figure 2.13: A theoretical schematic of signalling pathways involved in protein and mitochondrial synthesis as a result of creatine and carbohydrate supplementation. Glycogen concentration increases when creatine is co-ingested with carbohydrates as a result of creatine-mediated water retention promoting an increase in cell volume. Creatine supplementation per se does not alter insulin-stimulated glucose uptake via GLUT4 and glucose metabolism but has been shown to upregulate AMPK activity. A complex system of regulation between AMPK, creatine kinase, PCr and creatine, AMPK may play a role in regulating intramuscular creatine to PCr ratios. Arrows indicate activation; flat bars indicate inhibition of signalling.

The proposed schema outlined in Figure 2.13 suggests that creatine-mediated activation of AMPK phosphorylates AS160, leading to a greater degree of recruitment of GLUT4 to the cell membrane, thus allowing for additional glucose to enter the cell to be stored as glycogen. The co-transport of water along the concentration gradient produced by intracellular creatine (and, to a lesser extent, transported glucose) may increase the volume of the cell, further augmenting the capacity for glycogen storage. AMPK, which functions as a metabolic “fuel gauge” in response to glycogen levels and exercise stimuli, promotes downstream activation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), resulting in a rapid increase in mitochondrial biogenesis and subsequent aerobic capacity. However, the role creatine plays on GLUT4 translocation itself (Op't Eijnde et al. 2001, Ju et al. 2005, van Loon et al. 2004) as well as what occurs under endurance-based training scenarios are yet to be characterised.

2.6 Summary

Creatine has intrigued scientists and athletes with its capacity to augment muscle creatine stores and enhance performance. Strategies to alter intramuscular creatine stores not only influence the amount of energy available to undertake exercise, but also affect a variety of acute and chronic adaptations of muscle physiology to training. An abundance of research has been performed to rigorously evaluate the soundness of creatine supplementation strategies for enhanced performance, both through *in vivo* and *in vitro* testing. Through these testing models, it has been determined that creatine's ergogenic properties can be enhanced by a manipulation of co-supplemented substrates (i.e. carbohydrates, insulin mimetics) and, through these manipulations, the application of creatine has expanded beyond the scope of strength power training exercise. The aims of the studies described in the subsequent chapters were developed to elucidate the functional outcomes associated with various creatine co-supplementation strategies and protocols using both *in vitro* and *in vivo* models.

These studies determined physiological and mechanistic differences resulting from various creatine-based treatment strategies which provide a better understanding of creatine's role in metabolism and performance.

CHAPTER 3: Methodology and Design

As per university guidelines, methods utilised within each study presented in this thesis are described in their entirety in the section below. Chapter 4, 6 and 8 contain the specific methods used in each study presented according to the guidelines provided by the respective journals in which these studies were submitted for review.

3.1 Study 1 - Effects of Creatine and Carbohydrate Loading on Cycling Time Trial Performance

3.1.1 Subjects

Sample size was calculated on an expected difference in muscle phosphocreatine concentrations of $18 \text{ mmol}\cdot\text{kg}^{-1}$ between placebo and creatine + carbohydrate supplemented cohorts (van Loon et al. 2004, Roberts et al. 2016). With a standard deviation of $9 \text{ mmol}\cdot\text{kg}^{-1}$, a minimum number of 18 people (allowing for ~10% attrition) was calculated to be required to detect a difference (power = 90%, $\alpha = 0.05$). This calculation was performed based on the formula $n = f(\alpha/2, \beta) \times 2 \times \sigma^2 / (\mu_1 - \mu_2)^2$ with $f(\alpha, \beta) = [\Phi^{-1}(\alpha) + \Phi^{-1}(\beta)]^2$ (μ_1 and μ_2 are the mean outcomes in the control (placebo) and experimental (creatine) group of previous studies respectively, undertaken using creatine and carbohydrate supplementation strategies, α is the significance level, β is the power, σ is the standard deviation and Φ^{-1} is the cumulative distribution function of a standardized normal deviate. As such, eighteen endurance-trained male cyclists and triathletes with > 2 y racing history and currently cycling >250 km/wk commenced the study with age, body mass (BM), maximum oxygen uptake ($\text{VO}_{2\text{max}}$), and peak power output (PPO) (mean values \pm SD) of 31.2 ± 5.8 y, 78.2 ± 8.7 kg, $65.1 \pm 7.1 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, and 388 ± 42 W, respectively. Subjects who had taken any form of creatine supplement within 6 wk of the first performance trial (PT) or who had a history of abnormal bleeding, clotting, or history of seizure were excluded from the study. Experimental procedures and risks associated with the study were explained to all subjects who gave written informed consent prior to participating. The study was approved by the

Human Research Ethics Committees at the Australian Catholic University (Reference: 20140612) and the Australian Institute of Sport (Register Number: 2014 254N). The study was registered with the World Health Organization's International Clinical Trials Registry (UTN: U1111-1161-0890) and conducted in conformity with the policy statement regarding the use of human subjects according to the latest revision of the Declaration of Helsinki.

3.1.2 Study Overview

On separate days following familiarization (described subsequently), subjects completed three cycling PTs which consisted of a 120 km time trial (TT) ride on an electromagnetically-braked cycle ergometer immediately followed by a ride to volitional fatigue on an inclined treadmill set at a speed which elicited $\sim 90\%$ $\text{VO}_{2\text{max}}$. Following the first baseline (standardized $6 \text{ g}\cdot\text{kg}^{-1}$ BM CHO diet) PT, subjects were pair-matched into creatine (CR) or placebo (PLA) supplemented groups based on peak power output (PPO), performance measures from PT1 and DXA lean body mass measures in a double-blinded allocation. All subjects then undertook a randomized cross-over application of the carbohydrate intervention, consuming either a moderate- ($6 \text{ g}\cdot\text{kg}^{-1}$ BM/d; MOD) or CHO-loaded ($12 \text{ g}\cdot\text{kg}^{-1}$ BM/d; LOAD) diet 2 d before PT2 or PT3. A total of four muscle biopsies were obtained prior to PT1, 18 h following PT1 (i.e. glycogen depleted), and prior to PT2 and PT3 for biochemical analysis of intramuscular metabolites (as diagrammed subsequently in Figure 4.1)

3.1.3 Preliminary Testing

Upon arrival to the laboratory and after voiding, a nude measure of BM was obtained for $\text{VO}_{2\text{max}}$ and energy intake calculations. Subjects were subsequently weighed in full racing kit (e.g. jersey, cleats, knicks, socks, and helmet) and bicycle in order to calculate a relative treadmill speed for the hill climb simulation (described subsequently). The $\text{VO}_{2\text{max}}$ and PPO

of each subject were determined using an incremental test to volitional fatigue on an electromagnetically-braked cycle ergometer (Lode, Groningen, The Netherlands). The test protocol commenced at 150 watts (W) for 5 min and progressed to 250 W. Following 150 s, work load increased by 50 W with all subsequent work load increasing by 25 W every 150 s until volitional fatigue which was determined by the inability to maintain cadence >70 revolutions \cdot min $^{-1}$ (RPM). PPO was determined to be the power output of the highest stage completed plus the fraction of any uncompleted workload (W). Expired gases were collected into a calibrated customized Douglas bag gas analysis system, which incorporated an automated piston that allowed the concentrations of O₂ and CO₂ and the volume of air displaced to be quantified. VO_{2max} was calculated as the highest average O₂ consumption recorded over 60 s.

Following the VO_{2max} test, subjects undertook a 60 km familiarization time trial (TT) on a cycle ergometer (Velotron, Seattle, USA) with 1 km and 4 km sprint efforts (e.g. “as fast as possible”) alternating every 10 km during which time they consumed 60 g of CHO \cdot h $^{-1}$ in the form of a sports drink or gel. Upon the completion of the TT, subjects performed a timed ride to exhaustion (criteria subsequently defined) on a customized treadmill (Australian Institute of Sport, Canberra, Australia) set at an 8% gradient at a speed which elicited $\sim 90\%$ of a subject’s VO_{2max} as previously established (Ebert et al. 2007). This intensity was chosen to mimic hill climb durations (~ 10 -30 min) commonly observed in international road cycling races. The first study PT commenced within 7 d of familiarization.

3.1.4 Study Diet and Exercise Protocol

Dietary control was implemented for the 2 d prior to each PT using a pre-packaged standardized diet protocol. An individualised menu was constructed for each subject using *FoodWorks Professional Edition, Version 7.0* (Xyris Software, Brisbane, Australia) based on their BM and food preferences. Subjects received a moderate-CHO (MOD) diet providing 6

$\text{g}\cdot\text{kg}^{-1}$ BM/d CHO; $1.5 \text{ g}\cdot\text{kg}^{-1}$ BM/d protein; $1.5 \text{ g}\cdot\text{kg}^{-1}$ BM/d fat, with a total energy of $\sim 215 \text{ kJ}\cdot\text{kg}^{-1}$ BM/d 2 d prior to undertaking their first baseline PT. Subjects refrained from any intake of alcohol during the dietary standardisation period. Caffeine intake was allowed *ad libitum* up to 2 d prior to each PT and up to 2 standard servings (e.g. 1 cup of coffee or 1 can caffeinated soft drink) the day before the experimental trial. Subjects recorded their caffeine intake and this was repeated during the dietary standardisation period of subsequent PTs. Subjects were provided with all food and drinks in their standardised menu in portion-controlled packages and were given verbal and written instructions on how to follow the diet. Checklists were used to record each menu item as it was consumed and to note any deviations from the menu. On arrival at the laboratory for each PT, this checklist was cross-checked for compliance with study requirements. Hydration status was assessed using the specific gravity test (UG-a, Atago Refractometer, Japan) on an “on waking” urine sample.

On the morning of each PT, subjects reported to the laboratory at the same time (0700-0800 h) following an overnight fast. Following 20 min of rest in a supine position, a muscle biopsy was obtained under local anaesthetic (2-3 mL of 1% Xylocaine) from the *vastus lateralis* using a 5 mm Bergstrom needle modified for manual suction (Bergstrom 1975). Muscle samples were immediately snap-frozen in liquid N_2 and stored at -80°C until later analysis. A standardized “pre-race” meal providing $2 \text{ g}\cdot\text{kg}^{-1}$ BM CHO was consumed 2 h prior to the start of each PT. Subjects were asked to void prior to the start of the PT in order to obtaining a starting weight and began the PT exactly 2 h post breakfast.

PTs consisted of a 120 km self-paced cycling TT on a cycle ergometer during which subjects performed maximal intermittent high-intensity sprint efforts alternating between 1 and 4 km every 10 km. A fan that maintained air circulation ($15\text{-}17 \text{ m}\cdot\text{s}^{-1}$) and cooling was positioned 3 m away from the subject for all trials. Heart rate (HR) readings (RS300, Polar Electro, Kempele, Finland) were obtained both before and after the completion of each

sprint. Rating of perceived exertion (RPE) was obtained at the completion of each sprint effort. Subjects were required to follow a standardized hydration and CHO intake ($60 \text{ g}\cdot\text{h}^{-1}$) plan which was recorded and repeated in subsequent PTs. Upon the completion of the TT, subjects quickly dismounted the ergometer where they voided, towelled off and were reweighed. Subjects then rode their own bicycle on an inclined treadmill set at an 8% gradient at a speed which elicited $\sim 90\%$ of a subject's $\text{VO}_{2\text{max}}$. Subjects rode until volitional fatigue which was determined to be three verbal warnings that they had drifted back beyond a predetermined safety zone on the treadmill or when subjects grabbed onto the side of the treadmill safety bar. The transition from ergometer to treadmill was $<5 \text{ min}$. Subjects received similar encouragement through all PTs and did not receive any feedback on their performance until the completion of the study.

Following the completion of the first PT, subjects were fed a pre-packaged standardised low-CHO diet ($<1 \text{ g}\cdot\text{kg}^{-1} \text{ BM}$) for the remainder of the day to minimise resynthesis of muscle glycogen stores (Camera et al. 2012). Participants then reported to the laboratory in a fasted state the next morning ("glycogen depleted") where a second muscle biopsy was taken under rested and fasted conditions. Following the biopsy, subjects were randomised into either creatine loaded ($20 \text{ g}\cdot\text{d}^{-1}$ for 5 d followed by $3 \text{ g}\cdot\text{d}^{-1}$ until the end of the trial; CR) or placebo (PLA) cohorts using a pair-matched design based on PPO ($\text{W}\cdot\text{kg}^{-1}$), results of the first PT, and DXA lean mass estimates. In the 2 d prior to each of the two additional PTs (commenced 1 wk apart), subjects receive either a repeat of the MOD diet or a CHO loaded (LOAD) diet ($12 \text{ g}\cdot\text{kg}^{-1} \text{ BM/d}$) in a cross-over allocation (Figure 1). These dietary treatments were implemented using a PLA-controlled design whereby the overall menu for the day was kept constant, but key items were provided either as a low-energy/low-CHO option or an indistinguishable high-energy/CHO-enriched form. Protein and fat intake each remained constant at $1.5 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ in these diets but energy intake was increased in the

CHO LOAD diet ($\sim 320 \text{ kJ}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$). Subjects completed all subsequent biopsy and PTs as described, however no dietary restriction or follow up biopsy was given after the second and third PT. Analysis of all the diets consumed by participants was undertaken on completion of the study by a registered dietitian.

3.1.5 Determination of Muscle Creatine Content

For the measurement of muscle creatine content, 20-30 mg wet weight muscle tissue was freeze dried, weighed and extracted with 1M perchloric acid. Samples were analysed in duplicate for free-creatine, PCr, and ATP using fluorimeter. Total creatine was measured as a sum of free creatine and PCr.

3.1.6 Determination of Muscle Glycogen Content

For the measurement of muscle glycogen content, ~ 20 mg muscle tissue was freeze-dried and powdered with all blood and connective tissue removed. The freeze dried samples were extracted with 500 μL of 2M HCl, heated to 100°C for 2 h to hydrolyse the glycogen to glycosyl units, and then neutralized with 1.5 mL of 0.67 M sodium hydroxide. Glycogen concentrations were determined via enzymatic analysis with fluorometric detection (Jasco FP-750 spectrofluorometer, Easton, MD) at excitation 365 nm/emission 455 nm.

3.1.7 SDS-PAGE and Immunoblotting Analysis

Quantification of protein expression was carried out as previously described (Camera et al. 2012). Approximately 20 mg of skeletal muscle was homogenized using a motorized pellet pestle in an ice-cold buffer containing 50 mM TrisHCl (pH 7.5), 1 mM EDTA, 1mM EGTA, 10% glycerol, 1% Triton X-100, 50 mM NaF, 5 mM sodium pyruvate, 1 mM DTT, 10 $\mu\text{g}/\text{mL}$ trypsin inhibitor, 20 $\mu\text{g}/\text{mL}$ aprotinin, 1mM benzimidine, and 1 mM PMSF. Samples were spun at 16,000 g for 30 min at 4°C with supernatant collected for analysis. Total protein concentration was determined using a BCA protein assay (Pierce

Biotechnology, Rockford, IL) with lysate re-suspended in Laemmli sample buffer, separated by SDS-PAGE, and transferred to polyvinylidene fluoride membranes blocked with 5 % non-fat milk, washed with 10 mM Tris-HCl, 100 mM NaCl, and 0.02 % Tween 20, and incubated with primary antibody (1:1000) overnight at 4°C on a shaker. Membranes were incubated with secondary antibody (1:2000), and proteins were detected via enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK; Pierce Biotechnology) and quantified by densitometry (Chemidoc, BioRad, Gladesville, Australia). All samples for each individual were run on the same gel. Antibodies for total expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α ; no. 2187), mechanistic target of rapamycin (mTOR; no. 2972) 5' AMP-activated protein kinase (AMPK; no. 2532) were from Cell Signaling Technology (Danvers, MA, USA). SLC6A8 (no. AV42248) creatine transporter was from Sigma-Aldrich (St. Louis, MO, USA). For all proteins, volume density of each target band was normalized to the total protein loaded into each lane using stain-free technology (Gürtler et al. 2013).

3.1.8 Statistical Analysis

Normal distribution and equal variance of the data were tested using Shapiro-Wilk and F tests, respectively. All data (except for creatine concentrations by time point) were analysed using two-way repeated measures ANOVA where the independent, between subject factor was Treatment (Placebo or Creatine) and the within subject, repeated measure was Carbohydrate conditions (baseline, depleted, moderate-CHO diet or CHO-loaded diet). Time (P1, Post-PT1, P2 and PT3) was not a factor in these analyses because CHO-diet order was randomly assigned. Data comparing creatine content (irrespective of CHO-treatment) were also analysed using a two-factor ANOVA where the independent, between subject factor was Treatment (Placebo or Creatine), and the within subject, repeated measures factor was Time (PT1, Post-PT1, PT2 and PT3). Where there were significant main

effect differences for treatments or time, pre-planned Student-Newman-Keuls post-hoc analysis were used to locate these differences. All data were analysed using Sigma Plot (version 3.1). Statistical significance was set at $P < 0.05$ with data being represented as mean \pm standard deviation (SD).

3.2 Study 2 – The *in vitro* Effects of Creatine and Insulin on Skeletal Muscle

Myotubes Cultured in Different Glucose Concentrations

3.2.1 Cell Culture

C2C12 murine satellite cells (LONZA, Mount Waverley, VIC, AUS) were cultured on 2% gelatine- (Life Technologies, Mulgrave, VIC, AUS) coated T-75 flasks (Thermo Fisher, Scoresby, VIC, AUS) in $4.5 \text{ g}\cdot\text{L}^{-1}$ glucose Dulbecco's Modified Eagle Medium (DMEM; Life Technologies) supplemented with 10% foetal bovine serum (FBS; Life Technologies) and 0.5% penicillin/streptomycin (PenStrep; Life Technologies) and maintained at 37°C in a humidified incubator at 5% CO_2 .

At 70-90% confluence cultures were treated with serum free trypsin-EDTA (Life Technologies) at 37°C for 3-5 min before DMEM + 10% FBS were added to stop the reaction. Dissociated cells were collected and spun down (3 min at 3,000 rpm), resuspended in proliferation medium composed of DMEM + 10% FBS and seeded onto gelatine-coated 6-well culture dishes (Thermo Fisher) at a density of 10,000 cells/mL. At 70% confluence, the proliferation medium was replaced with differentiation medium composed of $4.5 \text{ g}\cdot\text{L}^{-1}$ glucose DMEM + 2% horse serum (HS; Sigma Aldrich, Castle Hill, NSW, Australia) and PenStrep for 168 h to allow myotube formation. Differentiation solution was replaced every 48 h. At 168 h ("baseline"), differentiation medium was replaced with either $1 \text{ g}\cdot\text{L}^{-1}$ glucose ("low"; LG) or $4.5 \text{ g}\cdot\text{L}^{-1}$ glucose ("high"; HG) DMEM containing 2% HS and 0.5% PenStrep supplemented with either 0.5 mM creatine (CR; Sigma Aldrich), 100 nM insulin

(INS; Sigma Aldrich) or 0.5 mM CR + 100 nM INS (CR+INS) for an additional 2 wk. In total 8 groups were established: control no treatment (NT) creatine treatment (CR), insulin treatment (INS) and creatine + insulin treatment (CR+INS) in either LG or HG concentrations. Treatment medium was changed every 24 h for the following 2 wk (Figure 3.1).

3.2.2 *Myotube Size and Fusion Index*

Myotubes were measured at baseline, 1 and 2 wk from treatment using the AVOS imaging system (Life Technologies). Pictographs were taken under 10x magnification (400 μ m scale) with 5 image fields (one in the centre of the well and four others around the centre) taken from each treatment at baseline, 1 and 2 wk of culture time. Images were taken from the same culture wells at each of the time points. A total of 10 myotubes per field were chosen at random with myotube width measured in μ m at the centre of each myotube (total of 50 myotubes per treatment per time point). This was carried out in two separate biological replicate experiments. Measurements at 1 and 2 wk were normalized to the average size at baseline.

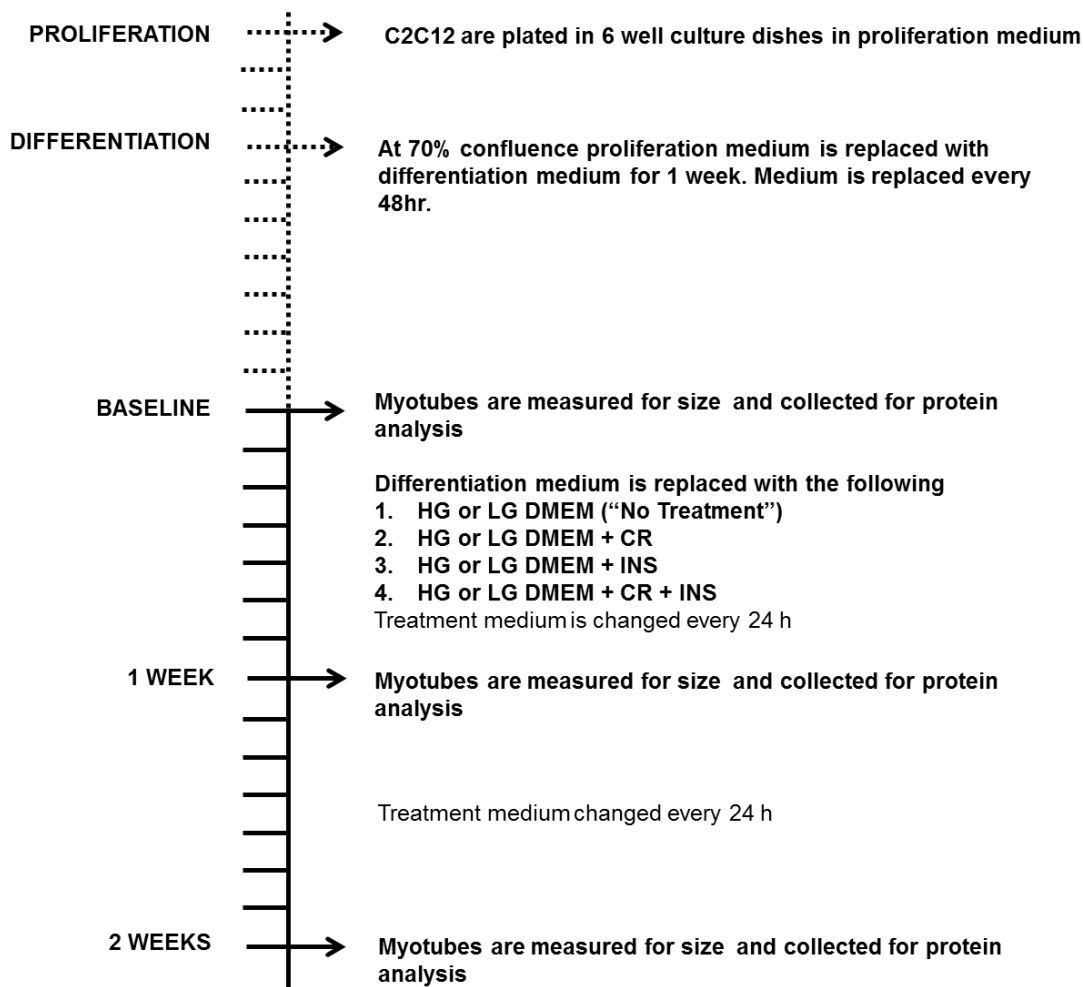


Figure 3.1: Timeline of Study II C2C12 cell culture protocol.

Based on the myotube diameter results, the number of fused nuclei to myotubes treated with CR and CR+INS in HG cultures were examined using immunofluorescence methods as detailed previously (Saiti and Lacham-Kaplan 2007). Cultures were fixed and incubated with anti-mouse Desmin. Following a series of washes in phosphate buffer (Life Technologies), cultures were incubated with anti-mouse-GFP secondary antibody. Stained cultures were visualized under the AVOS imaging system following co-staining with DAPI for nuclear staining. Antibodies and DAPI were purchased from Life Technologies. A total of 20 myotubes were randomly selected from four microscopic fields from two repeats and the number of nuclei within each myotube was recorded.

3.2.3 SDS-PAGE and Immunoblotting Analysis

Cultures were rinsed twice with 1X PBS (Life Technologies) before 1x cell lysis buffer (Cell Signalling Technology, Danvers, MA) supplemented with 1 mM PMSF was added to cultures. To ensure cell lysis, cells were collected and passed through an 18-gauge needle 10 times. Samples were centrifuged at 13,000 rpm for 10 min at 4°C and the supernatant collected for total protein analyses using a BCA protein assay (Pierce, Rockford, IL, USA). Western blotting was carried out as previously described (Tomcik et al. 2016). Briefly, lysates were re-suspended in Laemmli sample buffer, separated by SDS-PAGE, and transferred to polyvinylidene fluoride membranes blocked with 5% non-fat milk, washed with 10 mM Tris·HCl, 100 mM NaCl, and 0.02% Tween 20, and incubated with primary antibody (1:1,000) overnight at 4°C. Membranes were incubated with secondary antibody (1:2,000), and proteins were detected via enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK; Pierce Biotechnology) and quantified by densitometry (Chemidoc, BioRad, Gladesville, Australia). For all proteins, volume density of each target band was normalized to the total protein loaded into each lane using stain-free technology (Gürtler et al. 2013). Each treatment was run on the same gel according to time point.

The total and phosphorylated forms of the following proteins with putative roles in muscle growth were determined: mechanistic target of rapamycin (mTOR), ribosomal protein S6 kinase beta-1 (p70S6K), protein kinase B (AKT) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). Additionally, Solute Carrier Family 6 Member 8 (SLC6A8) creatine transporter, Myosin Heavy Chain (MHC), and the total and phosphorylated form of the osmotic stress related *p38* mitogen-activated protein kinase (p38 MAPK) were measured. Antibodies against p-mTOR^{Ser2448} (no. 2971), total mTOR (no. 2972), p-p70S6K^{Thr389} (no. 9205), total p70S6K (no. 9202), p-AKT^{Thr308} (no. 9275), total AKT (no. 9272), p-4E-BP1^{Thr37/46} (no. 2855) and total 4E-BP1 (no. 2452), p-p38 MAPK

Th180/Ty182 (no. 9211) and total p38 MAPK (no. 9212) were obtained from Cell Signaling Technology (Danvers, MA, USA). MHC antibody (no. PA5-31466) was obtained from Life Technologies. SLC6A8 antibody (no. AV42248) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

3.2.4 Statistical Analysis

Data were analysed using two-way repeated measures ANOVA (treatment x time) and student t-test for paired analyses. Myotube size and protein relative expression were compared between treatment groups (No treatment, Creatine, Insulin, or Creatine + Insulin within high and low glucose) and time (at 1 and 2 wk) following relative adjustment to baseline measures. Where there were significant main effect differences for treatment or time, pre-planned LSD Fisher post-hoc analysis was used to locate these differences. All data were analysed using Sigma Plot (version 3.1). Normal distribution and equal variance of the data were tested using Shapiro-Wilk and F tests, respectively. Statistical significance was set at $P < 0.05$ with data being represented as mean \pm standard error of the mean (SEM).

3.3 Study 3- Fenugreek Increases Insulin-Stimulated Creatine Content in L6C11 Muscle Myotubes

3.3.1 Preparation of Fenugreek Extracts

Raw fenugreek seeds were obtained locally (origin: India) and dried at 105°C for 3 h before being ground into a fine powder. The ground seeds were placed into a sample cell and loaded into an ASE TM 100 System (Dionex, Sunnyvale, CA) and extracted as per manufacturer's instructions. Prepared samples were extracted using 50% ethanol. Upon the completion of the extraction process, ethanol and water were evaporated and any residual

solution removed via overnight freeze drying. Fenugreek extract materials were stored in a dry environment until treatment.

3.3.2 *Fenugreek Extract Analysis*

Known amounts of fenugreek seed extract samples and a commercially available extract (Sigma Aldrich, St Louis, MO USA) were dissolved in 1mL of AR methanol and sonicated for 5min to extract 4-HIL. Standards of 4-HIL (Sigma Aldrich) (Isostd mg/mL) were run on an Electrospray Ionization (ESI) mass spectrometer (Micromass Platform II) to determine peak areas of the standards (Mass 148 was monitored). All samples and standards were made up in acetonitrile: water 50:50 with 0.1% formic acid. A calibration curve was produced using four standard concentrations of 4-HIL. The fenugreek sample areas were read from the curve to determine the concentrations of 4-HIL (recorded as % weight) in each extract.

3.3.3 *Cell Culture*

L6C11 myoblasts were maintained in high glucose Dulbecco's modified Eagle medium (DMEM) (Life Technologies, CA) supplemented with 10% foetal bovine serum (FBS) (Life Technologies), 0.5% Fungizone (Life Technologies) and penicillin streptomycin (Life Technologies). Cells were cultured in T-75 flasks and maintained at 37°C in a humidified incubator at 5% CO₂. Culture medium was changed every 2-3 d and cells were sub-cultured when >80% confluent. All experiments were performed in passages 6-11.

3.3.4 *Cell Viability*

Cell viability was measured using the CellTiter Blue® Cell Viability Assay Kit (Promega, Madison, WI). Cells were cultured at a density of 2.5×10^3 cells per well in flat-bottomed 96-well plates and equilibrated for 24 h prior to the addition of varying doses (0-25 µg/mL) of fenugreek extract. Following treatment, CellTiter Blue® reagent was added to

each well according to manufacturer's instructions. Cell viability was assessed by measuring the fluorescence excited at 550 nm and read at 600 nm using a SpectraMax Paradigm plate reader (Molecular Devices, Sunnyvale, CA). All assays were performed in quadruplicate and independently repeated twice.

3.3.5 *Determination of Creatine Content*

Total cellular creatine content was determined using modified versions of fluorometric determination methods. Myoblasts were cultured at a density of 12×10^3 cells per well into 24-well plates. When 80-90% confluent, differentiation of L6C11 myoblasts to myotubes was initiated by replacing the mitogen-rich media with DMEM supplemented with 2% horse serum (Life Technologies), 0.5% Fungizone and penicillin streptomycin for 4 d. Myotubes underwent treatments made with control DMEM (CON), 0.5 mM creatine (CR), CR and 20 μ g/mL fenugreek extract (CR+FEN), CR and 100nM insulin (CR+INS), and CR+INS+FEN for up to 24 h (n=6; obtained from 2 independent experiments). Myotubes were rinsed then lysed with 0.1 M NaOH. Lysates were hydrolysed with 1 M HCl and water at 60°C for 40 min. Freshly prepared 1% ninhydrin and 10% KOH (in EtOH) were subsequently added. Following 8 min incubation at room temperature, fluorescence was excited at 410 nm and read at 525 nm using a SpectraMax Paradigm plate reader. Total creatine content was calculated from a creatine standard curve and further normalized to total cellular protein, as determined using the bicinchoninic acid (BCA)-based protein assay kit (Pierce Biotechnology, IL USA) according to manufacturer's instructions. Absorbance was read at 562 nm.

3.3.6 *SDS-PAGE and Immunoblotting Analysis*

Myotubes were rinsed twice with 1X PBS before lysis with cell lysis buffer (Cell Signaling Technology, Danvers, MA) supplemented with 1 mM PMSF. Extracts were incubated with continuous rotation at 4°C for 1 h then centrifuged at 13,000 rpm at 4°C for

15 min to pellet cell debris. The supernatant was analysed for total protein and sample protein concentration using a BCA protein assay (Pierce, Rockford, IL, USA). Cell lysate was re-suspended in Laemmli sample buffer, separated by SDS-PAGE, and transferred to polyvinylidene fluoride membranes blocked with 5% non-fat milk, washed with 10 mM Tris·HCl, 100 mM NaCl, and 0.02% Tween 20, and incubated with primary antibody (1:1,000) overnight at 4°C on a shaker. Membranes were incubated with secondary antibody (1:2,000), and proteins were detected via enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK; Pierce Biotechnology) and quantified by densitometry (Chemidoc, BioRad, Gladesville, Australia). All samples for each treatment group were run on the same gel. Phospho-AKT^{Thr308} (no. 4056), total AKT (no. 9272), PKC ζ / λ (no. 9378), and GLUT4 (no. 9001) were from Cell Signaling Technology (Danvers, MA, USA). SLC6A8 (no. AV42248) was from Sigma-Aldrich (St. Louis, MO, USA). Data are expressed relative to α -tubulin (no. 3873; Cell Signaling Technology) in arbitrary units.

3.3.7 Statistical Analysis

All data were analysed by one- or two-way ANOVA comparing factors of Treatment (Creatine, Creatine + Fenugreek, Creatine + Insulin, and Creatine + Insulin + Fenugreek) made relative to a control baseline and Time (at 1, 4, and 24 h post treatment) and validated for equal variance by Mauchly's sphericity test. Where there were significant main differences between treatment and time, pre-planned Tukey's post hoc analysis was used to locate the difference. Statistical significance was established when $P < 0.05$. All data are expressed as arbitrary units \pm standard error of the mean (SEM).

CHAPTER 4:
**The Effects of Creatine and Carbohydrate Loading on Cycling Time Trial
Performance**

This chapter is comprised of the following paper which has been submitted and is under
second review in *Medicine & Science in Sport & Exercise (MSSE)*:

Effects of Creatine and Carbohydrate Loading on Cycling Time Trial Performance

Tomcik KA, Camera DM, Bone JL, Ross ML, Jeacocke NA, Tachtsis B, Senden J, van
Loon LJC, Hawley JA, Burke LM.

4.1 Abstract

Introduction

Creatine- and carbohydrate-loading are dietary strategies used to enhance exercise capacity. Creatine loading may increase muscle glycogen storage and delay fatigue during prolonged exercise. This study examined the effect of combined creatine and CHO-loading on cycling performance, associated changes in muscle metabolites, and select anabolic signalling markers.

Methods

Eighteen well-trained males completed three performance trials (PT) comprising a 120-km cycling TT with 1- and 4-km sprints followed by an inclined ride to fatigue (~90% $\text{VO}_{2\text{max}}$). Following the completion of PT1, subjects were pair-matched into creatine-loaded ($20 \text{ g}\cdot\text{d}^{-1}$ for 5 d + $3 \text{ g}\cdot\text{d}^{-1}$ for 9 d; CR) or placebo (PLA) groups ($n=9$). All subjects undertook a cross-over application of the carbohydrate interventions, consuming either moderate carbohydrate ($6 \text{ g}\cdot\text{kg}^{-1} \text{ BM/d}$; MOD) or carbohydrate-loaded ($12 \text{ g}\cdot\text{kg}^{-1} \text{ BM/d}$; LOAD) diets before the remaining PTs. Muscle biopsies were taken prior to PT1, 18 h post-PT1 and prior to PT2 and PT3..

Results

Power output with CR was greater than PLA during 1-km (357 ± 73 vs 330 ± 45 W; $P<0.001$) and 4-km (294 ± 54 vs 279 ± 42 W; $P<0.01$) sprints, with a significant increase above baseline observed with CR in the last 4-km sprint. No differences in PT times were observed. CR increased BM compared to PLA ($+1.54\%$ vs $+0.99\%$ from baseline; $P<0.05$). LOAD induced greater muscle glycogen concentrations than baseline and MOD (704 ± 111 vs 584 ± 135 vs $579\pm 123 \text{ mmol}\cdot\text{kg}^{-1} \text{ dry wt.}$, respectively; $P<0.01$) and greater total creatine

concentrations compared to PLA ($P=0.053$). Mechanistic target of rapamycin (mTOR) decreased from baseline following glycogen depletion ($\sim 30\%$; $P < 0.05$).

Conclusion

Power output in sprints undertaken within a prolonged cycling time trial, particularly in the late stages of a race, are affected by creatine independent of muscle glycogen content. Therefore, in spite of a creatine-mediated increase in weight, creatine and carbohydrate co-supplementation may be beneficial for intense bursts which occur during breakaway moments in endurance events.

4.2 Introduction

The bioenergetics of athletic training and competition require readily available pools of energy-generating substrates to support the demands of skeletal muscle metabolism. Athletes involved in endurance-type sports (e.g. stage cycling, marathons, triathlons) commonly maximize carbohydrate (CHO) availability through CHO loading to increase glycogen stores, while athletes involved in brief or intermittent high-intensity events (e.g. weightlifting, sprinting) target strategies to increase their capacity to utilize the phosphagen pathway, through creatine supplementation.

Adenosine triphosphate (ATP) is the metabolic intermediary in the energy flow from stored energy substrates (e.g. creatine, glycogen) to muscular contraction as well as numerous other cellular processes (Holloszy and Coyle 1984). The primary role of intramuscular creatine is to rapidly re-phosphorylate adenosine diphosphate (ADP) to ATP for energy. Both dietary and endogenous creatine enters muscle cells via the insulin-sensitive SLC6A8 creatine transporter where ~60% is bound to a phosphate group and stored as phosphocreatine (PCr). The concentration of PCr in muscle is 3-4 fold greater than that of ATP but can be exhausted quickly during high intensity resistance and sprint exercise. The co-ingestion of CHO with creatine potentiates an insulin-mediated increase in creatine transport, maximizing intramuscular creatine stores (Green, Hultman, et al. 1996) which, when combined with resistance-type exercise training, has been shown to result in greater lean body mass (BM) and strength.

There is evidence that creatine loading ($20 \text{ g}\cdot\text{d}^{-1}$ for 5 d) in conjunction with a moderate-CHO diet ($\sim 6 \text{ g}\cdot\text{kg}^{-1}$ CHO for 3 d) results in a substantial (53%) increase in muscle glycogen content when compared to CHO consumption alone (Nelson et al. 2001). Recent studies have also determined that enhanced glycogen capacity resulting from creatine supplementation (20 g/d) peaks within 24 h following exhaustive ($70\% \text{ VO}_{2\text{peak}}$) exercise

(Roberts et al. 2016). This response is thought to be the result of increased cell size due to creatine- and CHO-induced water retention (Kreider et al. 1998) and is associated with the upregulation of signalling pathways mediating glycogen and protein synthesis, namely 5' AMP-activated protein kinase (AMPK)- and mechanistic target of rapamycin (mTOR)-mediated signalling (Kreider et al. 1998, Safdar et al. 2008). However, it is presently unknown whether concomitant creatine and CHO loading strategies can enhance endurance exercise performance outcomes during the course of, rather than following, glycogen limiting events (e.g. time trial cycling). Hence, the aim of this study was to investigate whether glycogen 'super-compensation' with creatine and high-CHO intake leads to improved performance in well trained cyclists over a laboratory protocol involving a 120 km time trial and a simulated hill climb, with the latter element being included to investigate the effect of the increased body mass (BM) expected as a result of the loading strategies. In light of previous research reporting water retention/changes in cell size as a result of creatine and CHO supplementation altering signalling pathways associated with protein and glycogen synthesis, the present study investigated proteins with putative roles in creatine and/or CHO metabolism to provide further insight into the molecular adaptations associated with the present exercise and dietary intervention.

4.3 Methods

4.3.1 Subjects

Eighteen endurance-trained male cyclists and triathletes with > 2 y racing history and currently cycling >250 km/wk commenced the study with age, body mass (BM), maximum oxygen uptake ($\text{VO}_{2\text{max}}$), and peak power output (PPO) (mean values \pm SD) of 31.2 ± 5.8 y, 78.2 ± 8.7 kg, 65.1 ± 7.1 mL \cdot kg $^{-1}\cdot$ min $^{-1}$, and 388 ± 42 W, respectively (Table 4.1). Subjects who had taken any form of creatine supplement within 6 wk of the first performance trial (PT) or who had a history of abnormal bleeding, clotting, or history of seizure were excluded

from the study. Experimental procedures and risks associated with the study were explained to all subjects who gave written informed consent prior to participating. The study was approved by the Human Research Ethics Committees at the Australian Catholic University (Reference: 20140612) and the Australian Institute of Sport (Register Number: 2014 254N). The study was registered with the World Health Organization's International Clinical Trials Registry (UTN: U1111-1161-0890) and conducted in conformity with the policy statement regarding the use of human subjects according to the latest revision of the Declaration of Helsinki.

Table 4.1: Subject characteristics

	Creatine	Placebo	TOTAL
Participants	9	9	18
Age (years)	30.0 ± 5.8	32.3 ± 6.0	31.2 ± 5.8
Body Mass (kg)	76.3 ± 8.5	80.1 ± 9.1	78.2 ± 8.7
VO₂max (ml·kg⁻¹)	66.8 ± 7.5	63.4 ± 6.6	65.1 ± 7.1
PPO (W)	394.2 ± 44.8	381.7 ± 39.9	387.9 ± 41.7
PPO (W·kg⁻¹)	5.2 ± 0.6	4.8 ± 0.5	5.0 ± 0.6

4.3.2 Study Overview

On separate days following familiarization (described subsequently), subjects completed three cycling PTs which consisted of a 120 km time trial (TT) ride on an electromagnetically-braked cycle ergometer immediately followed by a ride to volitional

fatigue on an inclined treadmill set at a speed which elicited $\sim 90\%$ $\text{VO}_{2\text{max}}$. Following the first baseline (standardized $6 \text{ g}\cdot\text{kg}^{-1}$ BM CHO diet) PT, subjects were pair-matched into creatine (CR) or placebo (PLA) supplemented groups based on peak power output (PPO), performance measures from PT1 and DXA lean body mass measures in a double-blinded allocation. All subjects then undertook a randomized cross-over application of the carbohydrate intervention, consuming either a moderate- ($6 \text{ g}\cdot\text{kg}^{-1}$ BM/d; MOD) or CHO-loaded ($12 \text{ g}\cdot\text{kg}^{-1}$ BM/d; LOAD) diet 2 d before PT2 or PT3. A total of four muscle biopsies were obtained prior to PT1, 18 h following PT1 (i.e. glycogen depleted), and prior to PT2 and PT3 for biochemical analysis of intramuscular metabolites (Figure 4.1).

4.3.3 Preliminary Testing

Upon arrival to the laboratory and after voiding, a nude measure of BM was obtained for $\text{VO}_{2\text{max}}$ and energy intake calculations. Subjects were subsequently weighed in full racing kit (e.g. jersey, cleats, knicks, socks, and helmet) and bicycle in order to calculate a relative treadmill speed for the hill climb simulation (described subsequently). The $\text{VO}_{2\text{max}}$ and PPO of each subject were determined using an incremental test to volitional fatigue on an electromagnetically-braked cycle ergometer (Lode, Groningen, The Netherlands). The test protocol commenced at 150 watts (W) for 5 min and progressed to 250 W. Following 150 s, work load increased by 50 W with all subsequent work load increasing by 25 W every 150 s until volitional fatigue which was determined by the inability to maintain cadence >70 revolutions $\cdot\text{min}^{-1}$ (RPM). PPO was determined to be the power output of the highest stage completed plus the fraction of any uncompleted workload (W). Expired gases were collected into a calibrated customized Douglas bag gas analysis system, which incorporated an automated piston that allowed the concentrations of O_2 and CO_2 and the volume of air displaced to be quantified. $\text{VO}_{2\text{max}}$ was calculated as the highest average O_2 consumption recorded over 60 s.

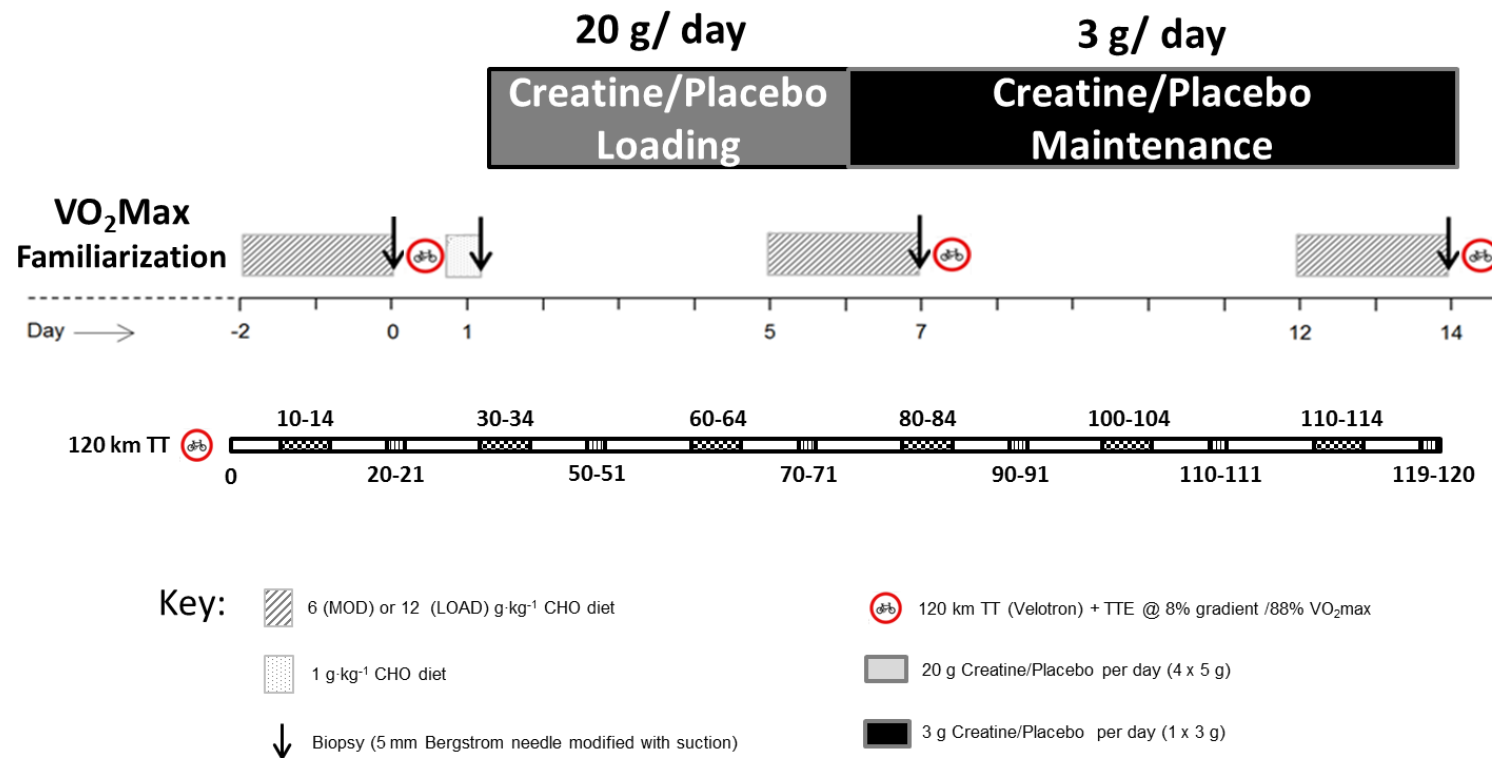


Figure 4.1: Study I cycling and diet intervention schematic. Subjects completed three performance trials (PT) on a cycle ergometer comprising a 120 km TT with alternating 4 km and 1 km sprints undertaken every 10 km. Immediately following, subjects cycled to volitional fatigue on an inclined treadmill set at speeds eliciting ~90% of individual VO₂max. Two days prior to each PT, participants in each group consumed either a moderate (6 g·kg⁻¹ body mass (BM)/d; MOD) or CHO-loaded (12 g·kg⁻¹ BM/d; LOAD) diet. Muscle biopsies were taken prior to each PT as well as 18 h following the first PT while glycogen depleted. Following the glycogen depleted biopsy, subjects were randomly allocated into either creatine loaded or placebo cohorts. Groups were pair matched based on weight, peak power output, and VO₂max.

Following the $\text{VO}_{2\text{max}}$ test, subjects undertook a 60 km familiarization time trial (TT) on a cycle ergometer (Velotron, Seattle, USA) with 1 km and 4 km sprint efforts (e.g. “as fast as possible”) alternating every 10 km during which time they consumed 60 g of $\text{CHO}\cdot\text{h}^{-1}$ in the form of a sports drink or gel. Upon the completion of the TT, subjects performed a timed ride to exhaustion (criteria subsequently defined) on a customized treadmill (Australian Institute of Sport, Canberra, Australia) set at an 8% gradient at a speed which elicited $\sim 90\%$ of a subject’s $\text{VO}_{2\text{max}}$ as previously established (Ebert et al. 2007). This intensity was chosen to mimic hill climb durations ($\sim 10\text{-}30$ min) commonly observed in international road cycling races. The first study PT commenced within 7 d of familiarization.

4.3.4 Study Diet and Exercise Protocol

Dietary control was implemented for the 2 d prior to each PT using a pre-packaged standardized diet protocol. An individualised menu was constructed for each subject using *FoodWorks Professional Edition, Version 7.0* (Xyris Software, Brisbane, Australia) based on their BM and food preferences. Subjects received a moderate-CHO (MOD) diet providing $6\text{ g}\cdot\text{kg}^{-1}\text{ BM/d CHO}$; $1.5\text{ g}\cdot\text{kg}^{-1}\text{ BM/d protein}$; $1.5\text{ g}\cdot\text{kg}^{-1}\text{ BM/d fat}$, with a total energy of $\sim 215\text{ kJ}\cdot\text{kg}^{-1}\text{ BM/d}$ 2 d prior to undertaking their first baseline PT (Figure 4.1). Subjects refrained from any intake of alcohol during the dietary standardisation period. Caffeine intake was allowed *ad libitum* up to 2 d prior to each PT and up to 2 standard servings (e.g. 1 cup of coffee or 1 can caffeinated soft drink) the day before the experimental trial. Subjects recorded their caffeine intake and this was repeated during the dietary standardisation period of subsequent PTs. Subjects were provided with all food and drinks in their standardised menu in portion-controlled packages and were given verbal and written instructions on how to follow the diet. Checklists were used to record each menu item as it was consumed and to note any deviations from the menu. On arrival at the laboratory for each PT, this checklist was cross-checked for compliance with study requirements. Hydration status was assessed

using the specific gravity test (UG-a, Atago Refractometer, Japan) on an “on waking” urine sample.

On the morning of each PT, subjects reported to the laboratory at the same time (0700-0800 h) following an overnight fast. Following 20 min of rest in a supine position, a muscle biopsy was obtained under local anaesthetic (2-3 mL of 1% Xylocaine) from the *vastus lateralis* using a 5 mm Bergstrom needle modified for manual suction (Bergstrom 1975). Muscle samples were immediately snap-frozen in liquid N₂ and stored at -80°C until later analysis. A standardized “pre-race” meal providing 2 g·kg⁻¹ BM CHO was consumed 2 h prior to the start of each PT. Subjects were asked to void prior to the start of the PT in order to obtaining a starting weight and began the PT exactly 2 h post breakfast.

PTs consisted of a 120 km self-paced cycling TT on a cycle ergometer during which subjects performed maximal intermittent high-intensity sprint efforts alternating between 1 and 4 km every 10 km (Figure 4.1). A fan that maintained air circulation (15-17 m·s⁻¹) and cooling was positioned 3 m away from the subject for all trials. Heart rate (HR) readings (RS300, Polar Electro, Kempele, Finland) were obtained both before and after the completion of each sprint. Rating of perceived exertion (RPE) was obtained at the completion of each sprint effort. Subjects were required to follow a standardized hydration and CHO intake (60 g·h⁻¹) plan which was recorded and repeated in subsequent PTs. Upon the completion of the TT, subjects quickly dismounted the ergometer where they voided, towelled off and were reweighed. Subjects then rode their own bicycle on an inclined treadmill set at an 8% gradient at a speed which elicited ~90% of a subject's VO_{2max}. Subjects rode until volitional fatigue which was determined to be three verbal warnings that they had drifted back beyond a predetermined safety zone on the treadmill or when subjects grabbed onto the side of the treadmill safety bar. The transition from ergometer to treadmill

was <5 min. Subjects received similar encouragement through all PTs and did not receive any feedback on their performance until the completion of the study.

Following the completion of the first PT, subjects were fed a pre-packaged standardised low-CHO diet ($<1 \text{ g}\cdot\text{kg}^{-1} \text{ BM}$) for the remainder of the day to minimise resynthesis of muscle glycogen stores (Camera et al. 2012). Participants then reported to the laboratory in a fasted state the next morning (“glycogen depleted”) where a second muscle biopsy was taken under rested and fasted conditions. Following the biopsy, subjects were randomised into either creatine loaded ($20 \text{ g}\cdot\text{d}^{-1}$ for 5 d followed by $3 \text{ g}\cdot\text{d}^{-1}$ until the end of the trial; CR) or placebo (PLA) cohorts (Figure 4.1) using a pair-matched design based on PPO ($\text{W}\cdot\text{kg}^{-1}$), results of the first PT, and DXA lean mass estimates. In the 2 d prior to each of the two additional PTs (commenced 1 wk apart), subjects receive either a repeat of the MOD diet or a CHO loaded (LOAD) diet ($12 \text{ g}\cdot\text{kg}^{-1} \text{ BM/d}$) in a cross-over allocation (Figure 4.1). These dietary treatments were implemented using a PLA-controlled design whereby the overall menu for the day was kept constant, but key items were provided either as a low-energy/low-CHO option or an indistinguishable high-energy/CHO-enriched form. Protein and fat intake each remained constant at $1.5 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ in these diets but energy intake was increased in the CHO LOAD diet ($\sim 320 \text{ kJ}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$). Subjects completed all subsequent biopsy and PTs as described, however no dietary restriction or follow up biopsy was given after the second and third PT. Analysis of all the diets consumed by participants was undertaken on completion of the study by a registered dietitian.

4.3.5 *Determination of Creatine Content*

Muscle creatine content was measured as described previously (Harris, Hultman, and Nordesjö 1974). Briefly, 20-30 mg wet weight muscle tissue was freeze dried, weighed and

extracted with 1M perchloric acid. Samples were analysed in duplicate for free-creatine, PCr, and ATP using fluorimetry. Total creatine was measured as a sum of free creatine and PCr.

4.3.6 Determination of Glycogen Content

Muscle glycogen content was measured as described previously (Camera et al. 2012). Briefly, ~20 mg muscle tissue was freeze-dried and powdered with all blood and connective tissue removed. The freeze dried samples were extracted with 500 μ L of 2M HCl, heated to 100°C for 2 h to hydrolyse the glycogen to glycosyl units, and then neutralized with 1.5 mL of 0.67 M sodium hydroxide. Glycogen concentrations were determined via enzymatic analysis with fluorometric detection (Jasco FP-750 spectrofluorometer, Easton, MD) at excitation 365 nm/emission 455 nm.

4.3.7 SDS-PAGE and Immunoblotting Analysis

Quantification of protein expression was carried out as previously described (Camera et al. 2012). Approximately 20 mg of skeletal muscle was homogenized using a motorized pellet pestle in an ice-cold buffer containing 50 mM TrisHCl (pH 7.5), 1 mM EDTA, 1mM EGTA, 10% glycerol, 1% Triton X-100, 50 mM NaF, 5 mM sodium pyruvate, 1 mM DTT, 10 μ g/mL trypsin inhibitor, 20 μ g/ml aprotinin, 1mM benzmidine, and 1 mM PMSF. Samples were spun at 16,000 g for 30 min at 4°C with supernatant collected for analysis. Total protein concentration was determined using a BCA protein assay (Pierce Biotechnology, Rockford, IL) with lysate re-suspended in Laemmli sample buffer, separated by SDS-PAGE, and transferred to polyvinylidene fluoride membranes blocked with 5 % non-fat milk, washed with 10 mM Tris-HCl, 100 mM NaCl, and 0.02 % Tween 20, and incubated with primary antibody (1:1000) overnight at 4°C on a shaker. Membranes were incubated with secondary antibody (1:2000), and proteins were detected via enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK; Pierce Biotechnology) and quantified by densitometry (Chemidoc, BioRad, Gladesville, Australia). All samples for

each individual were run on the same gel. Antibodies for total expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α ; no. 2187), mechanistic target of rapamycin (mTOR; no. 2972) 5' AMP-activated protein kinase (AMPK; no. 2532) were from Cell Signaling Technology (Danvers, MA, USA). SLC6A8 (no. AV42248) creatine transporter was from Sigma-Aldrich (St. Louis, MO, USA). For all proteins, volume density of each target band was normalized to the total protein loaded into each lane using stain-free technology (Gürtler et al. 2013).

4.3.8 Statistical analysis

All data were compared using two-way repeated measures ANOVA (treatment x time). Where there were significant main effect differences for treatment or time, pre-planned Student-Newman-Keuls post-hoc analysis were used to locate these differences. All data were analysed using Sigma Plot (version 3.1). Statistical significance was set at $P < 0.05$ with data being represented as mean \pm standard deviation (SD).

4.4 Results

4.4.1 Dietary Compliance

Assessment of individual records revealed universal (100%) compliance with the dietary protocols. Cross-checked intakes of energy, CHO, protein and fat intakes were similar to the prescribed diets and there were no differences in nutrient intakes for each dietary protocol between intervention groups or between dietary protocols that were repeated within the same group (e.g. MOD diet, or pre-race diet) (Table 4.2).

Table 4.2: Dietary intake for each of the dietary standardisation protocols

Nutrient	Moderate CHO diet (n=72 days; 18 subjects x 2 days x 2 trials)	CHO loading diet (n=36; 18 subjects x 2 days x 1 trial)	Pre-race diet (n=54; 18 subjects x 3 trials)	Post-race CHO restriction (n=18; 18 subjects x 1 day)
Energy (kJ·kg ⁻¹ ·d ⁻¹)	216 ± 2	317 ± 2	n/a	n/a
CHO (g·kg ⁻¹ ·d ⁻¹)	6.0 ± 0	12.0 ± 0	2.0 ± 0	0.9 ± 0.1
Protein (g·kg ⁻¹ ·d ⁻¹)	2.0 ± 0	2.1 ± 0	n/a	n/a
Fat (g·kg ⁻¹ ·d ⁻¹)	2.0 ± 0	2.0 ± 0	n/a	n/a
Caffeine (serves)	1.3 ± 1.2	1.3 ± 1.2	0	0.3 ± 0.7

4.4.2 Changes in Body Mass

In weights obtained prior to the start of the TT portion of the PT (i.e. at rest), irrespective of the CHO diet, CR significantly increased BM compared to PLA (+1.54% vs +0.99% from baseline; $P < 0.05$). CR resulted in an increase in BM compare to PLA with both MOD (+1.13% vs +0.21%; $P < 0.05$) and LOAD (+2.49% vs +1.76%; $P < 0.05$) diets. Within the CR cohort, BM increased with LOAD above baseline (+1.49%; $P < 0.001$) and MOD (+1.36%; $P < 0.001$). Within the PLA group, BM was reduced below baseline when subjects consumed MOD (-0.78%; $P < 0.05$) but increased above baseline when consuming LOAD (+1.54%; $P < 0.001$). LOAD also increased BM above MOD (+1.36%; $P < 0.05$) in the PLA group. LOAD resulted in an overall increase in BM above both baseline (+1.12%; $P < 0.001$) and MOD (+1.45%; $P < 0.001$) diets irrespective of CR/PLA treatments (Figure 4.2A).

In body mass obtained just prior to commencement of the hill climb portion of the PT, differences between CR and PLA groups were no longer significant (Figure 4.2B). Within the CR cohort, BM increased with LOAD above baseline (+1.49%; $P < 0.001$) and MOD (+1.34%; $P < 0.001$). Within the PLA group, BM increased with the LOAD diet above baseline (+0.72%; $P < 0.05$) and MOD (+1.40%; $P < 0.001$) diets. LOAD resulted in an overall increase in BM above both baseline (+1.11; $P < 0.001$) and MOD (+1.37%; $P < 0.001$)

diets irrespective of CR/PLA treatments (Figure 4.2B). There was no significant difference in total weight lost between pre-TT and pre-hill climb weights (i.e. sweat loss) between the different trial dates or treatment interventions.

4.4.3 *Performance Trial Outcomes*

There were no differences in 120 km TT time or between treadmill cycling time to exhaustion between trials (CHO diets or supplement) (Table 4.3).

4.4.4 *Power Output and Aerobic Capacity*

There were no differences in mean power output or percentage of maximal aerobic power (MAP) between the supplemented cohorts or CHO diet interventions compared to baseline measures (Table 4.3). There was a significant treatment effect of creatine supplementation on overall 1 km (357 ± 72 vs 330 ± 45 W; $P < 0.001$) (Figure 4.3A) and 4 km (294 ± 54 vs 279 ± 42 W; $P < 0.01$) (Figure 4.3B) sprint power compared to PLA with a significant effect of LOAD ($P < 0.01$) observed on 1 km sprint efforts (Table 4.3). Power generated during the 4 km ($P < 0.05$) and 1 km ($P < 0.05$) sprint bouts was significantly greater with CR than PLA across all sprint efforts (Table 4.3). An increase in MAP was observed with CR ($P < 0.05$) above PLA across all PTs. Within the PLA cohort, greater power and MAP was achieved with both MOD ($P < 0.05$) and LOAD ($P < 0.05$) significantly increasing above baseline (Table 4.3). Within the 1 km sprint efforts, LOAD improved MAP above baseline ($P < 0.05$) during the final sprint effort in the PLA treated group (Figure 4.3C). Within the 4 km sprint efforts, CR with both MOD and LOAD significantly increased ($P < 0.05$) MAP above baseline during the final sprint effort (Figure 4.3D).

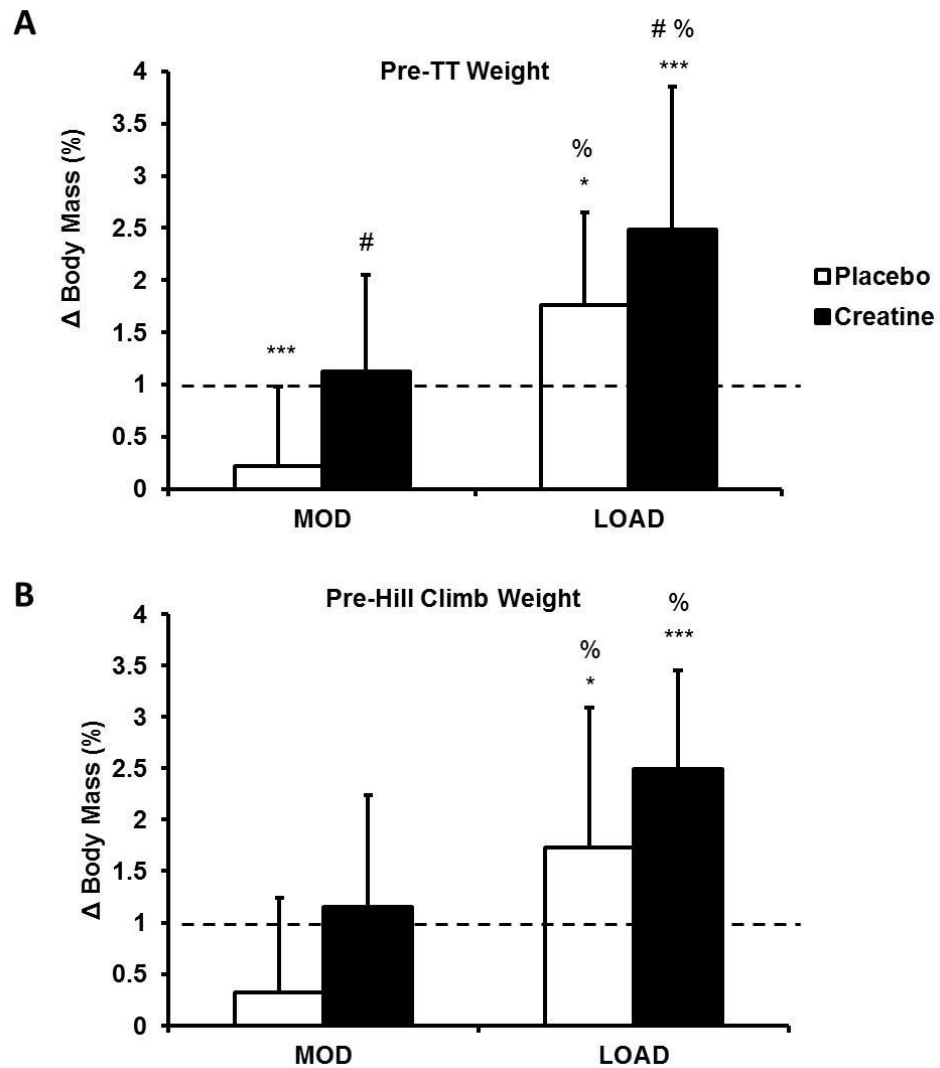


Figure 4.2: Change in body mass between treatments. **(A)** Change in body mass relative to baseline measures for all dietary intervention groups prior to the commencement of the TT portion of the performance trial (i.e. prior to any exercise). **(B)** Change in body mass relative to baseline measures for all dietary intervention groups prior to the commencement of the hill climb portion of the performance trial. Values are expressed as percent change relative to baseline (dotted line) \pm SD. * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$) denotes significant difference to baseline; # ($P < 0.05$) denotes significant difference between supplement (PLA vs CR) under that CHO diet, % ($P < 0.001$) denotes significant difference to CHO treatment (MOD vs LOAD) within the supplement.

Table 4.3: Summary of cycling time trial and treadmill performance associate measures. Time values are reported in hh:mm:ss.oo, power output is reported in watts (W), and maximal aerobic power (MAP) is reported in percentage (%). All values presented as a mean \pm SD using two-way ANOVA analysis for supplement and CHO condition. * denotes significant difference ($P < 0.05$) to placebo within the specified CHO intervention (i.e. creatine + baseline, MOD or LOAD compared to placebo baseline, MOD or LOAD, respectively). † denotes significant difference ($P < 0.05$) to baseline within the specified supplement s (i.e. within creatine/placebo MOD or LOAD to baseline only).

Intervention	120 km TT time (h:mm:ss.oo)	Treadmill Time (h:mm:ss.oo)	POWER Overall		4 km Sprint		1 km Sprint	
			W	% MAP	W	% MAP	W	% MAP
Creatine								
Baseline	3:09:40.96 ± 0:14:01.52	0:05:04.15 ± 0:04:01.81	238 ± 44.1	60.3 ± 5.0	289 ± 52.7*	73.2 ± 8.6	350 ± 66.9*	89.1 ± 15.6*
MOD	3:12:33.94 ± 0:15:15.51	0:06:00.25 ± 0:03:05.88	232 ± 43.9	58.9 ± 5.4	295 ± 50.2*	74.7 ± 8.8	360 ± 72.7*	90.9 ± 13.3*
LOAD	3:11:10.51 ± 0:17:36.33	0:07:17.58 ± 0:04:59.17	235 ± 48.6	59.4 ± 6.1	296 ± 60.5*	74.7 ± 10.8	360 ± 79.6*	91.0 ± 16.1*
Placebo								
Baseline	3:11:33.71 ± 0:10:36.45	0:05:00.78 ± 0:02:34.10	224 ± 35.5	58.6 ± 5.3	278 ± 42.7	72.7 ± 7.5	318 ± 44.6	83.4 ± 10.1
MOD	3:10:08.14 ± 0:10:49.02	0:06:25.81 ± 0:03:07.85	231 ± 35.1	60.2 ± 4.6	277 ± 44.3	72.4 ± 7.5	336 ± 47.4 †	88.4 ± 11.9 †
LOAD	3:11:32.18 ± 0:09:23.49	0:06:35.29 ± 0:04:16.93	227 ± 29.3	59.6 ± 4.4	282 ± 39.7	73.8 ± 6.3	337 ± 41.2 †	88.7 ± 11.0 †
Combined								
Baseline	3:10:37.33 ± 0:12:06.11	0:05:02.46 ± 0:03:16.71	231 ± 39.4	59.4 ± 5.1	284 ± 48.1	73.0 ± 8.0	334 ± 58.9	86.3 ± 13.4
MOD	3:11:21.04 ± 0:12:53.48	0:06:13.03 ± 0:03:01.76	231 ± 38.6	59.6 ± 4.9	286 ± 48.0	73.6 ± 8.2	348 ± 62.2	89.7 ± 12.6
LOAD	3:11:21.35 ± 0:13:41.37	0:06:56.44 ± 0:04:31.40	231 ± 39.1	59.5 ± 5.1	289 ± 51.4	74.3 ± 8.8	349 ± 64.2	89.8 ± 13.7

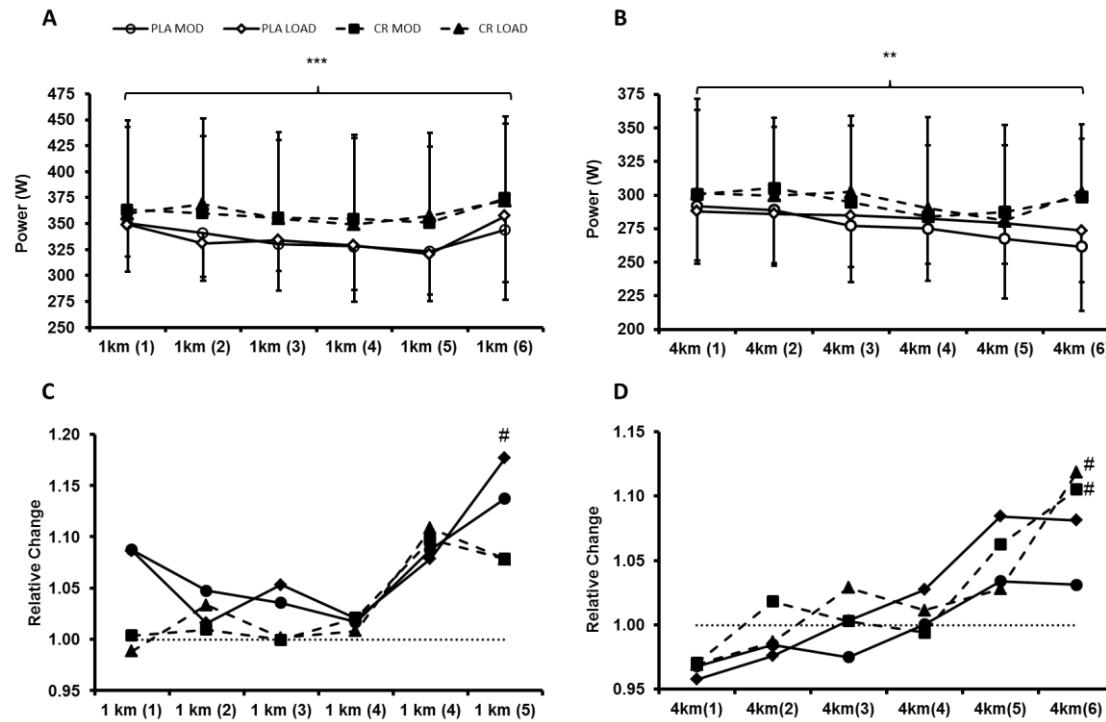


Figure 4.3: Power output during time trial sprint efforts. (A) 1 km and (B) 4 km sprint effort power output (W) between treatments and relative to baseline (C and D for 1 and 4 km sprints, respectively) Data are presented as mean \pm SD using two-way ANOVA analysis for supplement and CHO condition. *** ($P < 0.001$), ** ($P < 0.01$) denote significant overall treatment effect between supplement groups (CR vs PLA only). # ($P < 0.05$) denotes significance relative to baseline at that specific sprint interval.

4.4.5 Rating of Perceived Exertion

There was a significant interaction between CHO diets and supplementation on RPE ($P < 0.001$). CR elicited a greater overall perceived exertion (16 vs 15; $P < 0.001$) than PLA. There was also a significant difference in perceived exertion between LOAD and baseline ($P < 0.001$). LOAD decreased RPE ($P < 0.001$) from baseline with CR. CR subjects reported significantly greater RPE when on both MOD ($P < 0.001$) and LOAD ($P < 0.001$).

4.4.6 Muscle Glycogen

Muscle glycogen concentrations were significantly decreased ($P < 0.05$) following the first PT (“depleted”; $264 \pm 102 \text{ mmol} \cdot \text{kg}^{-1} \text{ dm}$) compared to baseline ($584 \pm 135 \text{ mmol} \cdot \text{kg}^{-1} \text{ dm}$), MOD ($579 \pm 123 \text{ mmol} \cdot \text{kg}^{-1} \text{ dm}$) and LOAD ($704 \pm 111 \text{ mmol} \cdot \text{kg}^{-1} \text{ dm}$) irrespective of supplemented cohorts. The ingestion of LOAD resulted in greater muscle glycogen concentrations than baseline, depleted and MOD ($P < 0.05$). There were no differences in glycogen contents between baseline and MOD. There were also no significant differences in muscle glycogen concentrations between CR and PLA conditions (Figure 4.4A).

4.4.7 Muscle Creatine and ATP Concentrations

CR supplementation resulted in a near significant increase in total Cr ($P = 0.059$) above placebo by Day 7 but this was not sustained by the maintenance dose at Day 14 (Figure 4.4B). CR loaded subjects had a near significant increase in total Cr above PLA when CHO loaded ($P = 0.053$; Table 4.4). Subsequently, values for Total Cr and PCr at MOD and LOAD in the Cr group were higher than at baseline for the CR supplemented group, and higher than the placebo group, with a significant change from baseline observed in the CR loaded cohort when on the LOAD diet ($P < 0.05$; Table 4.4).

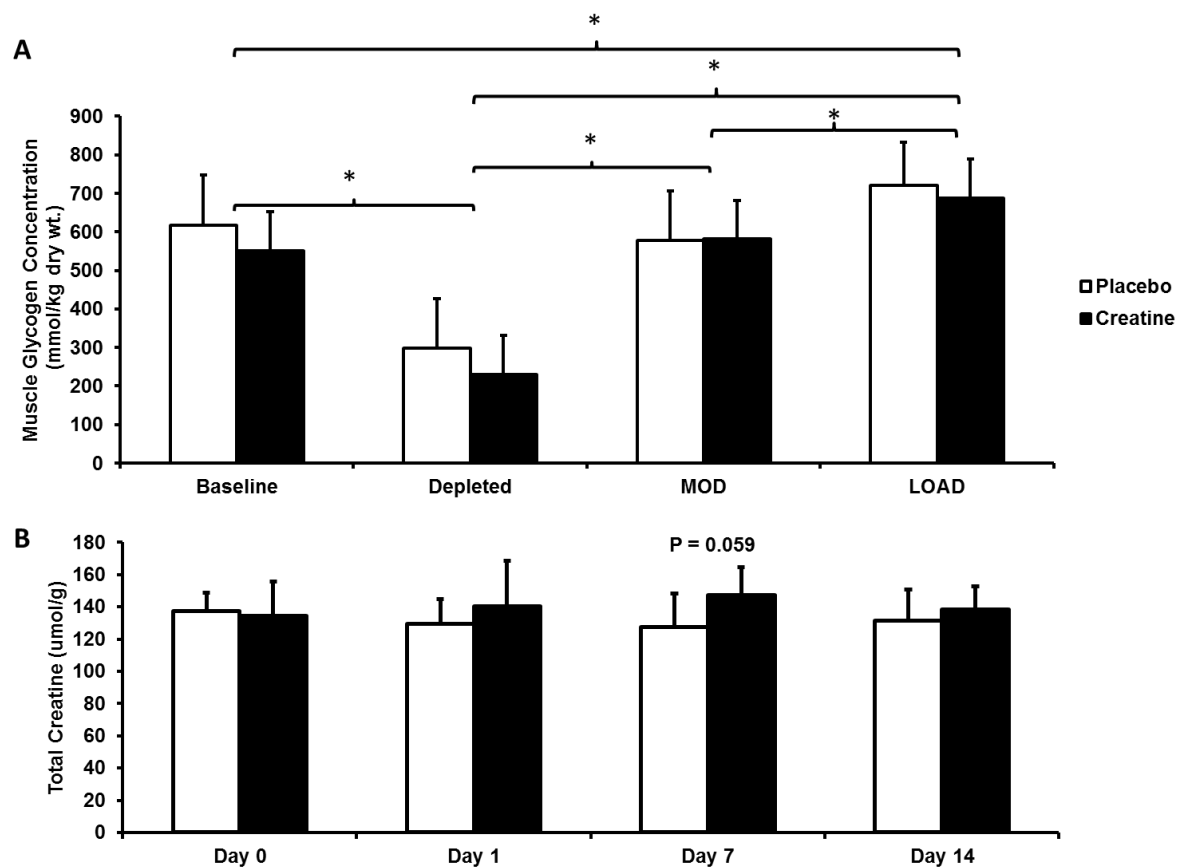


Figure 4.4: Analysis of muscle glycogen and total creatine. **(A)** Skeletal muscle glycogen concentrations at Baseline (CHO = 6 g·kg⁻¹ BM·d⁻¹), after PT1 (Depleted), and following Moderate CHO intake (6 g·kg⁻¹ BM·d⁻¹) and CHO Loading (12 g·kg⁻¹ BM·d⁻¹) in groups who took undertook Creatine loading or Placebo. Values are presented as a mean ± SD using two-way ANOVA analysis for supplement and CHO condition. * denotes significant difference (*P* < 0.05) CHO treatment irrespective of supplement (Baseline, Depleted, MOD CHO or CHO LOAD). **(B)** Total creatine content in muscle at Baseline (Day 0), after PT1 (Day 1), creatine loading (5 d x 20 g·d⁻¹ and 2 d x 3 g·d⁻¹; Day 7) and creatine maintenance (5 more d x 3 g·d⁻¹; Day 14). Values are presented as a mean ± SD using two-way ANOVA analysis for supplement and CHO condition.

Table 4.4: Free and total creatine, phosphocreatine, and ATP data at Baseline and following moderate (MOD) CHO intake and CHO loaded (LOAD), noting that MOD and LOAD days were crossed over between Day 7 and Day 14. Values are presented as a mean \pm SD using two-way ANOVA analysis for supplement and CHO condition. * denotes significant difference ($P < 0.05$) from baseline within supplement group (i.e. within CR or PLA), # denotes significant difference ($P < 0.05$) between supplement groups at the CHO intervention (i.e. CR vs PLA).

	Placebo (n=9)			Creatine (n=9)		
	Baseline	MOD	LOAD	Baseline	MOD	LOAD
Free Creatine	49.4 \pm 8.4	48.7 \pm 11.2	45.2 \pm 11.5	50.5 \pm 8.7	51.9 \pm 8.5	50.3 \pm 7.3
Phosphocreatine	87.9 \pm 7.2	84.3 \pm 13.3	80.7 \pm 10.8	83.7 \pm 15.8	89.5 \pm 10.0	95.4 \pm 9.8
Total Creatine	137.3 \pm 11.4	133.0 \pm 21.1	125.9 \pm 18.3	134.3 \pm 21.2	141.4 \pm 17.1	144.1 \pm 16.2 # *
ATP	23.1 \pm 1.9	23.1 \pm 2.3	21.2 \pm 2.4	22.9 \pm 1.9	22.2 \pm 2.1	21.9 \pm 2.1

4.4.8 Protein Expression

The expression of total mTOR significantly decreased following glycogen depletion compared to baseline (~30%; $P < 0.05$) (Figure 4.5A). No significant changes were observed with total AMPK, although a trend toward reduced expression was observed following glycogen depletion with CR relative to baseline (Figure 4.5B). No changes were observed in PGC-1 α (Figure 4.5C) and SLC6A8 (Figure 4.5D) total protein expression.

4.5 Discussion

CHO loading is a common tactic utilised by endurance athletes as a means of increasing intramuscular glycogen stores with research indicating that creatine and CHO co-ingestion can increase muscle glycogen storage which may enhanced endurance-based exercise tasks (Nelson et al. 2001, van Loon et al. 2004). The present study reports for the first time greater power output during repeated high intensity sprint efforts undertaken in the late-stages of a simulated 120 km TT when cyclists combined creatine and CHO loading. This response was independent of muscle glycogen concentrations and total mTOR expression. These outcomes suggest that creatine, when co-ingested with CHO, may have a beneficial effect on specific in-competition aspects of endurance cycling performance which mimicked the demands typical observed in multi-stage tours (i.e., multiple sprints and hill climbing) and typically results in glycogen depletion (Arkinstall et al. 2004). The changes in power output observed during sprints are likely to have a major practical impact on the final outcome of a race, as both cycling and running events are often won by the athlete who can either stay with the leading pack during breakaways or sprint to the finish line in the latter stages of a race. Although a significant difference in overall performance times as a result of either creatine and/or CHO loading was not observed, an increase in BM as a result of these strategies did not negatively impact the weight-sensitive “hill climbing” simulation included

at the end of the performance trial, suggesting that an avoidance of creatine use in endurance cycling may be unfounded.

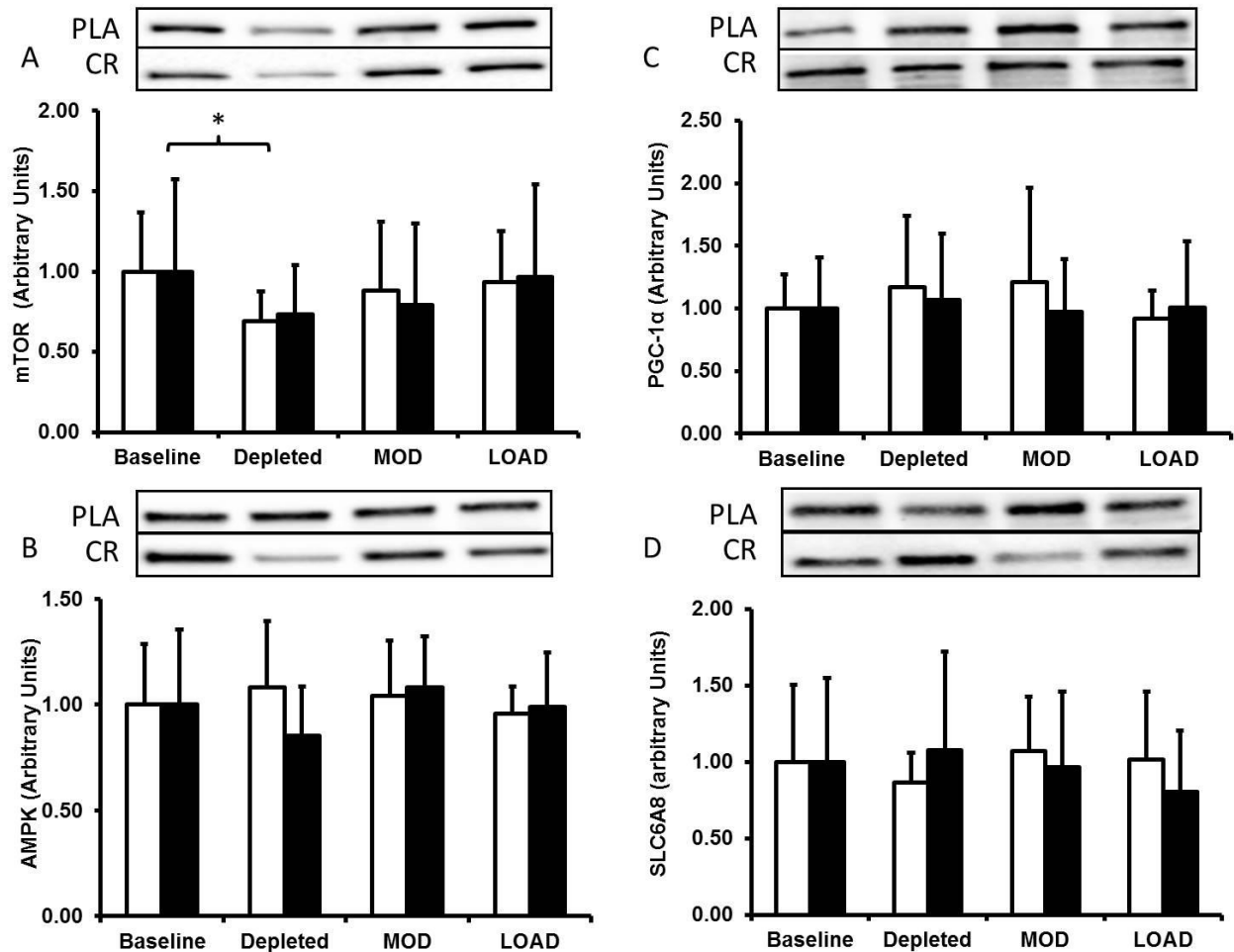


Figure 4.5: Expression of select signalling proteins prior to cycling performance trials and following glycogen depletion. **(A)** Total mechanistic target of rapamycin (mTOR), **(B)** total 5'adenosine monophosphate-activated protein kinase (AMPK), **(C)** total peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), and **(D)** SLC6A8 creatine transporter protein in skeletal muscle taken prior to time trials completed under baseline, glycogen depleted, moderate-CHO (MOD), or CHO-loaded (LOAD) diets with or without creatine supplementation. Images are representative blots and values are expressed relative to total protein and presented in arbitrary units \pm SD (n=9 per supplement group). * denotes significant difference ($P < 0.05$) between CHO treatments (baseline, depleted, MOD or LOAD), irrespective of CR or PLA supplement.

The estimates of muscle creatine concentrations in this study suggest that the loading protocol was effective in increasing muscle creatine above baseline values; however, there is some uncertainty about the efficacy of the maintenance dose in sustaining these elevations in muscle creatine content. This may be an artefact of the variability of the technique (biopsy sampling plus the co-efficient of variation of the creatine assays) or an indication that a creatine dose of $3 \text{ g}\cdot\text{d}^{-1}$ is insufficient, at least in the short period following high creatine doses, or maintaining such elevations (van Loon et al. 2003). An inability to maintain elevated creatine concentrations may explain a lack of change in glycogen content between CR and PLA groups and furthermore be a factor contributing to a lack of change in performance times. Studies have showed that creatine transporter activity can be regulated/enhanced by extracellular creatine concentrations (Loike et al. 1988) and the presence of insulin (Tomcik et al. 2016, Steenge et al. 1998). Little further difference between groups in terms SLC6A8 protein expression was observed; however, this is in line with previous studies on both short and long term creatine supplementation ($0.125 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ either 2 or 4 months) which showed no change in SLC6A8 mRNA content when combined with a standardised cycle exercise program (Tarnopolsky et al. 2003). It is also possible that the exogenous loading dose downregulated endogenous creatine production to such a large extent that there was an undershoot in total muscle creatine in the phase immediately following the cessation of this dose (van Loon et al. 2003, Snow and Murphy 2001). Regardless, since the present study followed a protocol that is evidence-based and showed numerical increases in muscle creatine content at both loading ($P = 0.059$) and maintenance phases (LOAD day; $P = 0.053$), there is confidence that the creatine supplement group achieved muscle creatine content at both the MOD and LOAD performance trials that was functionally higher than in their baseline trial, and in comparison with the placebo group.

Creatine's ergogenic benefits stem from its ability to act as a "reservoir" for substrates which are required for the rapid regeneration of energy during brief periods of high intensity, maximal effort. In contrast to previous work which showed that creatine did not increase power output during short (200 m) sprint efforts during a 25.2 km cycling sprint trial (Levesque, Kenefick, and Quinn 2007), the present work reports that creatine loading increases power output during the late-stages of maximal sprint efforts performed during a 120 km cycling time trial lasting ~3 h. Work by Vandebuerie and colleagues reported a ~9% increase in power output in sprint efforts (5 x 10 s) of creatine loaded ($25 \text{ g}\cdot\text{d}^{-1}$ for 5 d) elite cyclists only after a 2.5 h TT cycling protocol (Vandebuerie et al. 1998). Oliver and colleagues subsequently demonstrated that a creatine loading protocol ($20 \text{ g}\cdot\text{d}^{-1}$ for 6 d) further supplemented with glucose ($15 \text{ g}\cdot\text{d}^{-1}$) was able to reduce lactate concentrations during exercise but with no difference in maximal power output or total time to fatigue during incremental cycling (Oliver et al. 2013). This study is the first to show increased sprint power with creatine during the course of, rather than following, a prolonged endurance cycling bout. Although the creatine and/or carbohydrate loading did not provide a detectable benefit to overall performance of the 120 km cycling time trial, this study noted that the increase in BM associated with the single and combined use of these strategies did not impair capacity for a weight-sensitive exercise element in the present cycling protocol. This latter finding is of practical significance since many endurance athletes are disinclined to use creatine as an performance supplement due to fears that the associated BM gain will impair performance in events where power to mass ratios are important.

While it is likely that the observed increase in power output is due to the ability of creatine to rapidly re-phosphorylate ATP for rapid energy generation, recent evidence indicates that this response may also be a result of decreased muscle glycogen utilization and muscle protein degradation (Tang, Chan, and Kuo 2013). Specifically, Tang and colleagues

observed consistent decreased lactate levels indicative of decreased muscle glycogenolysis in subjects supplemented with creatine ($12 \text{ g}\cdot\text{d}^{-1}$ for 15 d) following an endurance exercise session involving a 60 min run followed by 100 m sprint efforts (Tang, Chan, and Kuo 2013). Lactate measures obtained from subjects after the sprint bouts tended to be lowered in the creatine loaded cohort (data not shown) which may suggest “glycogen sparing” but direct measures would be needed to confirm such a notion.

Seminal work by Gollnick, Piehl, and Saltin (1974) observed selective glycogen depletion patterns in human muscle fibres after varying intensities of exercise. Glycogen depletion dramatically increases ~2-7 time with increasing exercise intensities of ~60-85% $\text{VO}_{2\text{max}}$ (Gollnick, Piehl, and Saltin 1974). Type I fibres are the first to become depleted of glycogen at all workloads below $\text{VO}_{2\text{max}}$ with further depletion occurring in type II fibres as work continues (Gollnick, Piehl, and Saltin 1974). At workloads exceeding MAP however, initial depletion of glycogen occurs in both fibre types. Overall, there is a primary reliance on slow twitch fibres during submaximal endurance exercise with fast twitch fibres being recruited after slow twitch fibres are depleted. The rate of glycogen resynthesis, which has a higher initial rate in slow twitch fibres, may attribute to fibre-type differences in glucose uptake and disposal (Casey et al. 1995).

In humans, while total creatine and phosphocreatine content has been shown to be greater in fast twitch muscle (Meyer, Brown, and Kushmerick 1985, Casey et al. 1996), creatine supplementation leads to higher expression of creatine transporter protein in slow twitch muscle fibres (Murphy et al. 2003). Willott *et al.* (1999) suggest that the rate of creatine loss from fast twitch fibres is lower than slow twitch fibres. Hence, although the rate of creatine entry into type II fibres is lower than type I, type II fibres have greater creatine content than type I (Willott et al. 1999) and are therefore able generate more explosive power during exercise. In the present study, mixed muscle glycogen concentrations were measured.

Muscle fibre composition, therefore, could contribute to variations between glycogen content and MAP. Furthermore, pacing strategies may have been adopted following baseline trials which may explain increased MAP with MOD and in the PLA group above baseline measures.

The addition of CHO to a traditional creatine loading strategy ($20 \text{ g}\cdot\text{d}^{-1}$ for 3-5 d) has previously been reported to increase muscle glycogen stores (Nelson et al. 2001, van Loon et al. 2004). The basis for this response has centred around the idea that greater intracellular water retention is associated with increased creatine transport which subsequently increases cell volume, promoting a greater capacity for glycogen storage (Safdar et al. 2008, van Loon et al. 2004, Powers et al. 2003, Kreider et al. 1998, Ziegenfuss, Lowery, and Lemon 1998). Nelson and colleagues (2001) showed that subjects who consumed a moderate CHO ($\sim 6.6 \text{ g}\cdot\text{kg}^{-1} \text{ BM}$) diet following traditional creatine loading increased muscle glycogen content by 53% above those consuming high CHO alone (Nelson et al. 2001). Similarly, Van Loon and colleagues demonstrated that 7 wk of prolonged creatine supplementation ($20 \text{ g}\cdot\text{d}^{-1}$ for 5 d followed by 6 wk of $2 \text{ g}\cdot\text{d}^{-1}$) resulted in an 18% increase in muscle glycogen content (van Loon et al. 2004). Neither of these studies, however, involved any exercise modality to test the implications or benefits of elevated glycogen stores on training and performance. The present study showed that CHO-loading increased muscle glycogen by $\sim 17\%$ above baseline, but observed no further increase in muscle glycogen contents or performance time when CHO loading was combined with creatine loading. Previous studies had failed to observe a change in a similar cycling protocol (100 km with 1 and 4-km sprint efforts) following carbohydrate loading alone, suggesting that CHO consumed during exercise, such the subjects of the present study did, offsets any detrimental performance on lower pre-exercise glycogen concentrations (Burke et al. 2000). This may further be explained by recent findings indicating that the optimal window for increasing glycogen stores using a

concomitant creatine and CHO supplementation strategy occurs within a narrow timeframe (Roberts et al. 2016). Roberts and colleagues reported that 5 d of creatine supplementation ($20 \text{ g}\cdot\text{d}^{-1}$) and high CHO ($37.5 \text{ kcal}\cdot\text{kg}^{-1} \text{ BM/d}$) intake following an exhaustive bout of exercise ($70\% \text{ VO}_{2\text{max}}$) resulted in augmented muscle glycogen contents within 24 h ($\uparrow\sim 82\%$ compared to placebo) with no subsequent changes in glycogen contents observed between creatine and placebo cohorts (Roberts et al. 2016). These data, however, represent the potent effects of creatine and CHO loading on recovery rather than its effects on performance.

Creatine induced a significant increase in BM compared to PLA, indicating that the current ingestion protocol promoted additional intra-cellular water storage. Cell swelling caused by creatine and CHO loading via the storage of additional water within the muscle cells has been associated with the upregulation of a large number of signalling markers involved in protein and glycogen synthesis (Kreider et al. 1998, Powers et al. 2003, Ziegenfuss, Lowery, and Lemon 1998, Safdar et al. 2008). It has been hypothesised that creatine-mediated cell swelling may further result in the increased expression of key proteins involved in hypertrophy-related signal transduction (Fry et al. 2010), namely mechanistic target of rapamycin (mTOR) (Loenneke et al. 2012). However, no studies have directly examined cell signalling responses when performing glycogen-depleting exercise (i.e. TT cycling/sprints to exhaustion) in concert with concomitant creatine and CHO loading.

Several lines of evidence indicate little involvement of creatine in myofibrillar and sarcoplasmic protein turnover both following resistance type exercise and at rest (Louis, Poortmans, Francaux, Berré, et al. 2003, Louis, Poortmans, Francaux, Hultman, et al. 2003). A decrease in total mTOR abundance in biopsies taken 18 h following a glycogen-depleting TT and exhaustive hill climb was observed in the current study. However, in contrast to the original hypothesis, mTOR expression was similar irrespective to whether participants were creatine and/or carbohydrate loaded (Figure 4.5). While this indicates creatine

supplementation exerts no further beneficial effect on mTOR signalling, this finding further supports the glycogen data between conditions by demonstrating creatine was unable to increase cell size through mTOR-mediated mechanisms to enhance glycogen storage capacity. mTOR has also been implicated in stimulating SLC6A8 creatine transporter via the serum and glucocorticoid-inducible kinase (SGK1) which is also involved in the regulation of cell volume and activity of various Na⁺/K⁺ carriers such as SLC6A8 (Shojaiefard, Christie, and Lang 2006). It is plausible that the decreased mTOR expression observed in this study attenuated activation of SGK1 resulting in submaximal transport of creatine into muscle and therefore negating any increase in cell volume and glycogen storage.

Endurance athletes benefit from a greater mitochondrial capacity due to their increased requirement for available energy. The activation of 5'adenosine monophosphate-activated protein kinase (AMPK) monitors metabolic and energetic states in muscle which is affected by exercise stimuli (Winder 2001) and is furthermore modulated by the PCr:creatine ratio (Ponticos et al. 1998). The data from the present study showed no change in AMPK status despite observing a slight (~5%) drop in the PCr:creatine ratio following a glycogen depleting PT. Other studies have reported that muscle with augmented glycogen content tended to have lower resting levels of AMPK and were also less sensitive to activation in response to stimuli such as exercise (Richter et al. 2001, Wojtaszewski et al. 2002). A failure to detect changes in AMPK expression may be because subjects had adequate CHO (6 or 12 g·kg⁻¹ BM CHO·d⁻¹) in their diet two days prior to the completion of the first (and subsequent) PT as well as the fact that they consumed 60 g·h⁻¹ of CHO during the ride itself. Furthermore, no change in PGC-1 α , a signalling target of AMPK, was observed. A limitation to the present study was the time at which biopsies were taken which may have been too late to observe any changes in PGC-1 α signalling as a result of nutrient or exercise interventions (Pilegaard et al. 2005). Future studies should investigate long-term creatine and

CHO loading strategies with muscle samples obtained both pre and immediately post bouts of exercise to further elucidate any beneficial adaptations.

In summary, the present study presents novel data to demonstrate that the combination of creatine and CHO loading can increase power output during repeated high intensity sprint efforts undertaken during late stages of prolonged simulated time trial cycling which mimicked the physiological demands typically observed in multi-stage cycle tours (i.e., multiple sprints and hill climbing). The increased power outputs were independent of muscle glycogen content. Even though creatine loading increased BM at the start of the performance trial, these differences became less significant over the course of the time trial and, despite being heavier, did not interfere with performance of the weight-sensitive part of the TT (the hill climb). In fact, if anything, the performances were longer (better endurance) with the loaded conditions. As cycling and running events are often won by the athlete who can either stay with the leading pack during breakaways or sprint to the finish line in the latter stages of a race, it is likely that the higher power outputs observed during these late intense sprints would have a major impact on the final outcome of such races.

CHAPTER 5:

The *in vitro* Effects of Creatine and Insulin on Skeletal Myotubes Cultured in Different Glucose Concentrations

This chapter is comprised of the following paper which has been submitted and is under review in the *Journal of Nutritional Biochemistry*.

The *in vitro* Effects of Creatine and Insulin on Skeletal Myotubes Cultured in Different Glucose Concentrations

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Preface to Chapter Five

Carbohydrate ingestion stimulates the release of insulin, which not only regulates both glucose and creatine uptake into muscle, but also induces anabolic signalling resulting in hypertrophy. Both creatine (Kreider et al. 1998, Powers et al. 2003, Ziegenfuss, Lowery, and Lemon 1998, Francaux and Poortmans 1999) and carbohydrate (Olsson and Saltin 1970b, Baker and Jeukendrup 2014, Sherman et al. 1982) augment intracellular water retention, resulting in an increase in muscle cell size. Early studies by Low *et al.* (1996) first reported that osmotic swelling in rat myotubes modulates glycogen synthesis independent of changes in glucose uptake (Low, Rennie, and Taylor 1996). Short-term creatine supplementation, *in vitro* and *in vivo*, also alters cell osmolarity which upregulates signalling pathways associated with hypertrophy (Safdar et al. 2008, Deldicque et al. 2007). The majority of research investigating the metabolic effects of creatine has been undertaken *in vivo*. Few studies have tested the cellular effects of novel *in vivo* creatine supplementation strategies, such as those investigated in Chapter Four, using *in vitro* models to elucidate potential mechanistic changes as a result of dietary intervention. Therefore, to further investigate the interplay between creatine, carbohydrates (glucose) and insulin, cell culture models were implemented in the following studies.

In general, muscle-specific primary cells (i.e. isolated directly from tissue) known as satellite cells are able to proliferate in growth medium for several days and differentiate into mono-nucleated muscle precursor cells known as *myoblasts* (Spurway and Wackerhage 2006). Changes in the nutrient content of growth medium or external stimuli drive cell development toward terminal differentiation in which myoblasts fuse to become mature, multi-nucleated muscle fibres known as *myotubes*. Most primary cells can normally only divide a limited number of times before the ability to proliferate is lost. Some cell lines, known as *immortalized* cell lines, contain a genetic mutation which allows myoblasts to

divide indefinitely. These secondary cells (i.e. derived, or passaged, from established primary cells) are able to be suspended in liquid nitrogen for extended time periods, thawed and used to initiate new cultures, thus requiring no further muscle samples to be obtained (Spurway and Wackerhage 2006). Each passage of these cells results in a genetically homogeneous population that allows for controlled testing of various conditions or treatments in growth medium using a number of biological techniques.

A number of different cell lines have been validated for use in investigating skeletal muscle growth and regeneration, with *C2C12* and *L6* muscle cells being the most widely utilized immortalized skeletal muscle cell lines. Mouse-derived C2C12 and rat-derived L6 cells display a number of characteristics analogous to differentiated skeletal muscle (Pittner et al. 1996). Both cell types are able to fuse to form multinucleated myotubes which are subsequently able to express key metabolic enzymes, including creatine kinase, myokinase and a number of glycogen-metabolizing enzymes (Yaffe 1968, Shainberg, Yagil, and Yaffe 1971, Kubo 1991).

Derived from mouse satellite cells, C2C12 cells differentiate rapidly upon serum removal and form contractile myotubes (formation appearing within 3-5 d) which produce characteristic muscle proteins (Burattini et al. 2004). Undifferentiated C2C12 cells are flat, star-shaped mono-nucleated cells which become elongated once they are ready to fuse with other activated cells to form young myotubes which mature during culture (Burattini et al. 2004). Mature C2C12 myotubes express sarcomeric and filamentous actin as well as skeletal myosin (Kataoka et al. 2003), the main components of contractile function (Salamon et al. 2003). Hence, C2C12 cells appear to be better suited for studies of contractile ability as a result of better sarcomere development during differentiation (Nedachi, Fujita, and Kanzaki 2008).

Rat-derived L6 cells are also able to undergo a high degree of differentiation to myotubes. The L6 line naturally expresses GLUT4 glucose transporter protein and, as such, exhibit a significant insulin-stimulated glucose uptake response. Compared to C2C12 cells, L6 cells also contain a higher ratio of GLUT1:GLUT4, making them an excellent model for testing glucose transport and the mechanisms associated with the progression of diabetes (Sarabia et al. 1992). The major limitation when using cells to identify distinct stages of growth and development is the alteration of growth characteristics over time. Both L6 and C2C12 cell lines decrease their proliferation rate and ability to form myotubes the more they are subcultured (i.e. phenotype drift) (Table 5.1) Therefore, the cells used in the present study were limited to passages 3-5.

C2C12 cells have previously been used to investigate the role of creatine on differentiation (Deldicque et al. 2007, Sestili et al. 2009), identify the osmotic effects of creatine supplementation on creatine transport (Alfieri et al. 2006), elucidate the role of insulin on cell proliferation and differentiation (Xiong et al. 2013) and to expound upon the effects of extracellular glucose and the role of insulin in promoting transport and signalling alterations (Nedachi et al. 2008, Sumitani et al. 2002). Thus, the C2C12 cell line provides a simple and effective platform for further examination of creatine's interaction with other substrates. The work presented in Chapter Five determined the magnitude by which creatine, insulin and glucose, independently or combined, affect muscle morphology and hypertrophy signalling.

Table 5.1: Select differences between L6 and C2C12 cell lines

Study	Cell Line	
	L6	C2C12
Kubo 1991	Display infrequent ATP response Partial fibronectin expression	ATP response shown before differentiation induction; lost after differentiation Intense fibronectin expression pre differentiation
Sarabia <i>et al.</i> 1992	Normally expresses GLUT4 glucose transporter protein Higher ratio of GLUT1/GLUT4 transporters	Detectable levels of GLUT 1
Portier <i>et al.</i> 1999	Creatine kinase MM already near maximum expression in myoblast stage 5 fold increase in acetylcholine receptor	Higher % of creatine kinase-muscle isoenzyme with differentiation Greater creatine kinase activity1 9 fold increase in acetylcholine receptor Forms a continuous basement membrane
Nedachi <i>et al.</i> 2008		Better sarcomere development Greater contractile activity

5.1 Abstract

Introduction

Muscle cell proliferation, differentiation and size are augmented by creatine and glucose availability with substrate transport mediated by insulin. The present study aimed to determine the magnitude by which creatine, insulin and glucose, independently or combined, affect muscle growth.

Methods

C2C12 myotubes were cultured in low ($1\text{g}\cdot\text{L}^{-1}$; LG) or high ($4.5\text{g}\cdot\text{L}^{-1}$; HG) glucose DMEM supplemented with 0.5 mM creatine (CR), CR + 100 nM INS (CR+INS) or INS alone for 2 wk. Myotube size (i.e. diameter), fusion and expression of putative signalling proteins associated with muscle growth were measured following 1 and 2 wk.

Results

Myotube size was highest in HG cultures compared to baseline at 1 wk ($P < 0.001$). Only HG+CR potentiated myotube growth (1.2 to 1.5 fold change from 1 to 2 wk, respectively; $P < 0.001$). Myosin Heavy Chain (MHC) expression increased under both LG and HG concentrations with CR+INS (10 and 6 fold increase, respectively; $P < 0.001$) by 2 wk. Differences in MHC expression correlated with p38 MAPK expression but not mTOR, p70S6K or 4E-BP1.

Conclusion

Creatine's anabolic role *in vitro* is more dependent on the prevailing insulin than glucose concentration. Creatine treatment resulted in larger myotubes, while insulin treatment resulted in small size myotubes but increased MHC protein expression, which may be a result of upregulation of the p38 MAPK pathway.

5.2 Introduction

The development of skeletal muscle involves the exit of satellite cells, the progenitors for skeletal myotube formation, from the cell cycle into the myogenic differentiation pathway (Dumont, Wang, and Rudnicki 2015). Both dietary creatine and glucose availability have been associated with satellite cell proliferation and differentiation, protein synthesis and DNA replication and repair (Juhn and Tarnopolsky 1998b, Safdar et al. 2008, Collins et al. 2009). Creatine acts as an ‘anabolic agent’ which can directly increase the expression of key intermediates involved in protein synthesis, such as myosin heavy chain (MHC) and mechanistic target of rapamycin (mTOR) both *in vivo* and *in vitro* (Loenneke et al. 2012, Deldicque et al. 2007).

Creatine’s effects on cell signalling (Deldicque et al. 2008) and muscle fibre adaptations (Volek et al. 1999) have largely been studied *in vivo* and concomitant with resistance exercise. Few investigations have examined the nutrient-specific effects of creatine on mature myotubes *in vitro* and the potential mechanisms associated with the greater creatine-induced muscle accretion. Furthermore, insulin, which activates signalling pathways that stimulate glucose and creatine uptake (Odoom, Kemp, and Radda 1996, Steenge et al. 1998, Elkalaf, Anděl, and Trnka 2013, Tomcik et al. 2016), has also been associated with the proliferation and differentiation of skeletal muscle cells (Ball and Sanwal 1980, Kumegawa et al. 1980). Investigations into the effects of creatine and insulin on skeletal muscle proliferation and differentiation have been undertaken over short-term treatment periods (hours to days) (Tsuka et al. 2015). Furthermore, there is presently a lack of insight on the treatment of already established myotubes, a model which more closely resembles *in vivo* skeletal muscle fibres.

The present study determined the effects of creatine and insulin treatments with varying glucose concentrations on established myotube morphology and putative cellular

signalling responses associated with myogenic growth. It was hypothesised that a 2 wk treatment with creatine and insulin combined with high glucose culture medium would result in increased myotube size compared to creatine treatment alone. Furthermore, this would be reflected in the upregulation of putative signaling proteins associated with hypertrophy.

5.3 Methods

5.3.1 Cell Culture

C2C12 murine satellite cells (LONZA, Mount Waverley, VIC, AUS) were cultured on 2% gelatine- (Life Technologies, Mulgrave, VIC, AUS) coated T-75 flasks (Thermo Fisher, Scoresby, VIC, AUS) in 4.5 g·L⁻¹ glucose Dulbecco's Modified Eagle Medium (DMEM; Life Technologies) supplemented with 10% foetal bovine serum (FBS; Life Technologies) and 0.5% penicillin/streptomycin (PenStrep; Life Technologies) and maintained at 37°C in a humidified incubator at 5% CO₂.

At 70-90% confluence cultures were treated with serum free trypsin-EDTA (Life Technologies) at 37°C for 3-5 min before DMEM + 10% FBS were added to stop the reaction. Dissociated cells were collected and spun down (3 min at 3,000 rpm), resuspended in proliferation medium composed of DMEM + 10% FBS and seeded onto gelatine-coated 6-well culture dishes (Thermo Fisher) at a density of 10,000 cells/mL. At 70% confluence, the proliferation medium was replaced with differentiation medium composed of 4.5 g·L⁻¹ glucose DMEM + 2% horse serum (HS; Sigma Aldrich, Castle Hill, NSW, Australia) and PenStrep for 168 h to allow myotube formation. Differentiation solution was replaced every 48 h. At 168 h ("baseline"), differentiation medium was replaced with either 1 g·L⁻¹ glucose ("low"; LG) or 4.5 g·L⁻¹ glucose ("high"; HG) DMEM containing 2% HS and 0.5% PenStrep supplemented with either 0.5 mM creatine (CR; Sigma Aldrich), 100 nM insulin (INS; Sigma Aldrich) or 0.5 mM CR + 100 nM INS (CR+INS) for an additional 2 wk. In total 8 groups were established: control no treatment (NT) creatine treatment (CR), insulin

treatment (INS) and creatine + insulin treatment (CR+INS) in either LG or HG concentrations. Treatment medium was changed every 24 h for the following 2 wk.

5.3.2 *Myotube Size and Fusion Index*

Myotubes were measured at baseline, 1 and 2 wk from treatment using the AVOS imaging system (Life Technologies). Pictographs were taken under 10x magnification (400 μ m scale) with 5 image fields (one in the centre of the well and four others around the centre) taken from each treatment at baseline, 1 and 2 wk of culture time. Images were taken from the same culture wells at each of the time points. A total of 10 myotubes per field were chosen at random with myotube width measured in μ m at the centre of each myotube (total of 50 myotubes per treatment per time point). This was carried out in two separate biological replicate experiments. Measurements at 1 and 2 wk were normalized to the average size at baseline.

Based on the myotube diameter results, the present study examined the number of fused nuclei to myotubes treated with CR and CR+INS in HG cultures using immunofluorescence methods as detailed previously (Saiti and Lacham-Kaplan 2007). Cultures were fixed and incubated with anti-mouse Desmin. Following a series of washes in phosphate buffer (Life Technologies), cultures were incubated with anti-mouse-GFP secondary antibody. Stained cultures were visualized under the AVOS imaging system following co-staining with DAPI for nuclear staining. Antibodies and DAPI were purchased from Life Technologies. A total of 20 myotubes were randomly selected from four microscopic fields from two repeats and the number of nuclei within each myotube was recorded.

5.3.3 SDS-PAGE and Immunoblotting Analysis

Cultures were rinsed twice with 1X PBS (Life Technologies) before 1x cell lysis buffer (Cell Signalling Technology, Danvers, MA) supplemented with 1 mM PMSF was added to cultures. To ensure cell lysis, cells were collected and passed through an 18-gauge needle 10 times. Samples were centrifuged at 13,000 rpm for 10 min at 4°C and the supernatant collected for total protein analyses using a BCA protein assay (Pierce, Rockford, IL, USA). Western blotting was carried out as previously described (Tomcik et al. 2016). Briefly, lysates were re-suspended in Laemmli sample buffer, separated by SDS-PAGE, and transferred to polyvinylidene fluoride membranes blocked with 5% non-fat milk, washed with 10 mM Tris·HCl, 100 mM NaCl, and 0.02% Tween 20, and incubated with primary antibody (1:1,000) overnight at 4°C. Membranes were incubated with secondary antibody (1:2,000), and proteins were detected via enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK; Pierce Biotechnology) and quantified by densitometry (Chemidoc, BioRad, Gladesville, Australia). For all proteins, volume density of each target band was normalized to the total protein loaded into each lane using stain-free technology (Gürtler et al. 2013). Each treatment was run on the same gel according to time point.

The total and phosphorylated forms of the following proteins with putative roles in muscle growth were determined: mechanistic target of rapamycin (mTOR), ribosomal protein S6 kinase beta-1 (p70S6K), protein kinase B (AKT) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). Additionally, Solute Carrier Family 6 Member 8 (SLC6A8) creatine transporter, Myosin Heavy Chain (MHC), and the total and phosphorylated form of the osmotic stress related *p38* mitogen-activated protein kinase (p38 MAPK) were measured. Antibodies against p-mTOR^{Ser2448} (no. 2971), total mTOR (no. 2972), p-p70S6K^{Thr389} (no. 9205), total p70S6K (no. 9202), p-AKT^{Thr308} (no. 9275), total AKT (no. 9272), p-4E-BP1^{Thr37/46} (no. 2855) and total 4E-BP1 (no. 2452), p-

p38MAPK^{Th180/Ty182} (no. 9211) and total p38 MAPK (no. 9212) were obtained from Cell Signaling Technology (Danvers, MA, USA). MHC antibody (no. PA5-31466) was obtained from Life Technologies. SLC6A8 antibody (no. AV42248) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

5.3.4 Statistical Analysis

Data were analysed using two-way repeated measures ANOVA (treatment x time) and student t-test for paired analyses. Where there were significant main effect differences for treatment or time, pre-planned LSD Fisher post-hoc analysis was used to locate these differences. All data were analysed using Sigma Plot (version 3.1). Statistical significance was set at $P < 0.05$ with data being represented as mean \pm standard error of the mean (SEM).

5.4 Results

5.4.1 Cultures

Myotubes began forming in all groups at 72 h post-differentiation with fully differentiated myotubes present by 168 h. Myotubes became gradually more crowded over time with the greatest qualitative density observed in CR+INS and INS alone in HG cultures (Figure 5.1A). Myotubes in cultures that contained only CR were less crowded at 1 and 2 wk for both LG and HG concentrations (Figure 5.1A and 5.1B). Branching of myotubes was qualitatively more prevalent in CR+INS treated cultures in HG (Figure 5.1C) and to a lesser degree in LG concentrations. Also, more incidences of contracting myotubes were observed in cultures treated with CR+INS in HG concentrations.

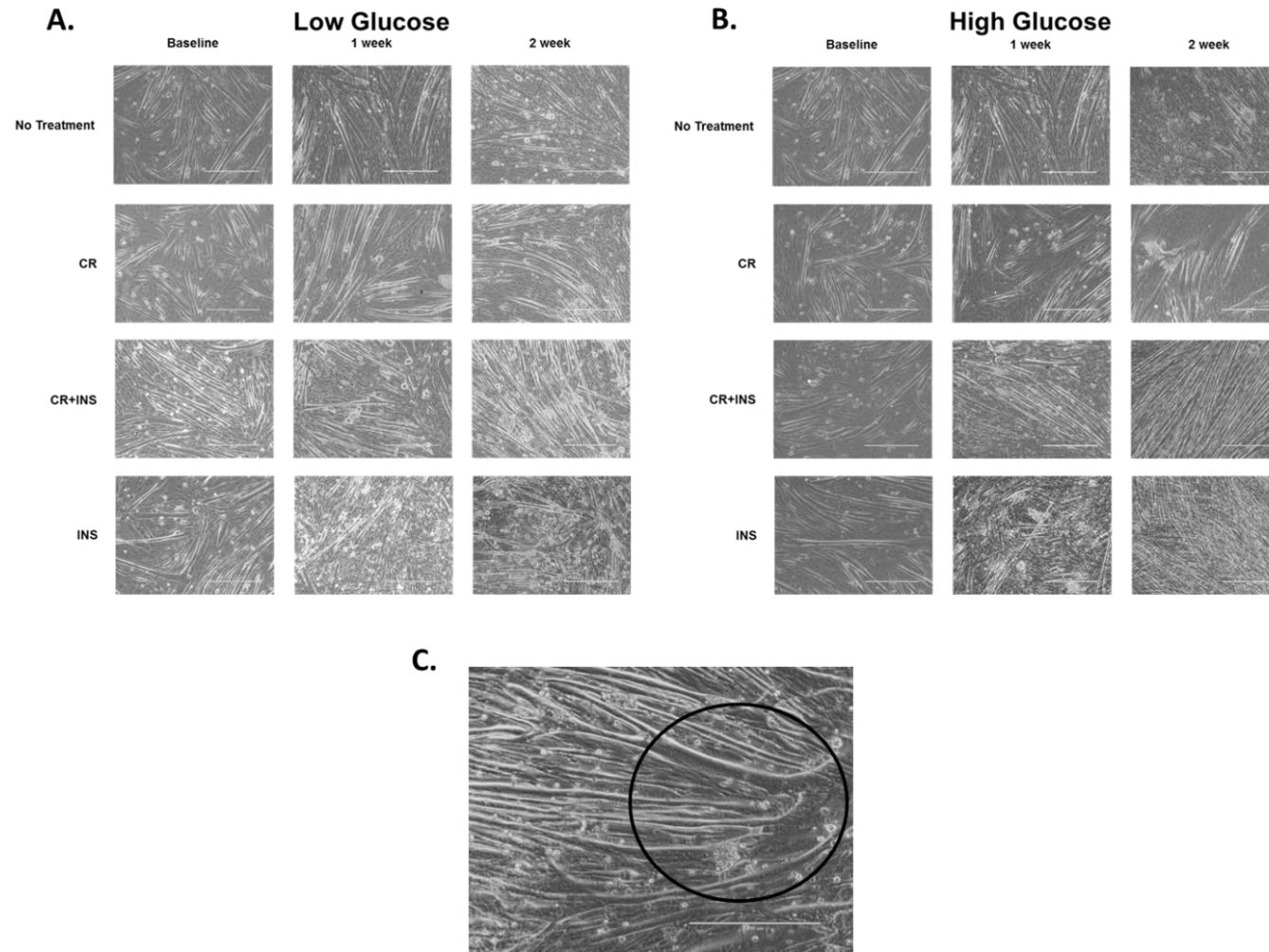


Figure 5.1: Representative myotube culture images at baseline, 1 week and 2 weeks of treatment. Differentiated cells were treated with (A) low (LG) or (B) high glucose (HG) no-treatment groups (NT) supplemented with creatine (CR), creatine and insulin (CR+INS), or insulin (INS) for up to 2 weeks. (C) Myotube branching in CR+INS culture treatment (x10 magnification; Scale bar = 400 μ m).

5.4.2 *Myotube Size*

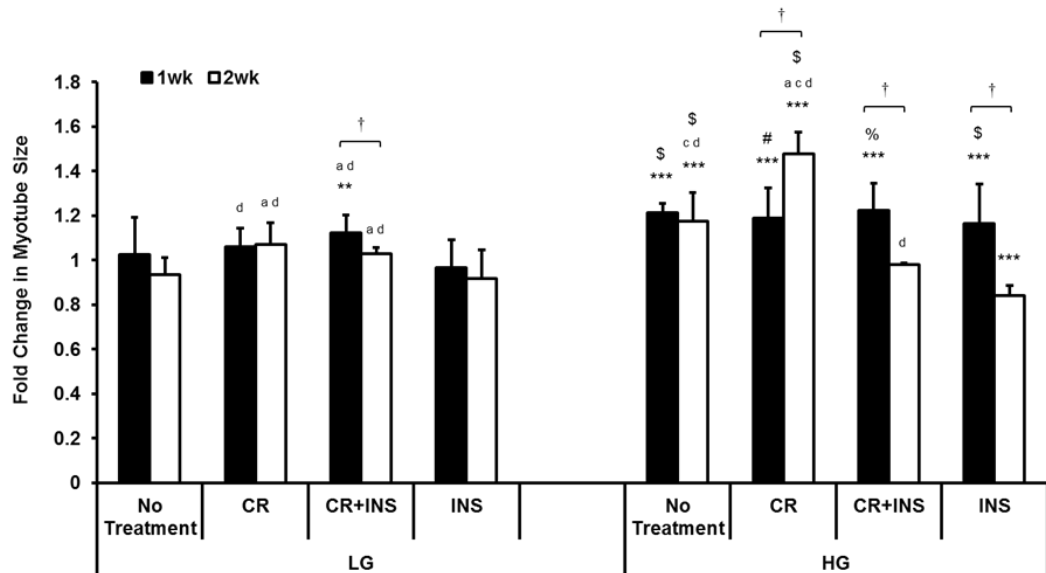
There was a significant interaction between treatment and time for myotube size ($P < 0.001$). When comparing treatments within the same glucose concentration, the relative size of myotubes cultured with LG showed no change in size except for CR+INS which had the greatest myotube diameter at 1 wk compared to baseline and 2 wk ($P < 0.05$; Figure 5.2A). There was a significant difference in myotube size with time in all HG treated cultures by 1 wk compared to baseline ($P < 0.001$). Significant differences in size were observed between 1 and 2 wk with CR, CR+INS and INS treatments ($P < 0.05$) with only CR-treated myotubes increasing in size from 1 to 2 wk (from 1 to 1.5 fold; $P < 0.001$; Figure 5.2A). Myotube size shifted with time from small ($<20\ \mu\text{m}$) to mid-range ($20\text{--}40\ \mu\text{m}$) and large ($> 40\ \mu\text{m}$) in all groups except for those treated with INS. The greatest proportion of large myotubes was observed with CR at 2 wk in HG concentrations (40%) concomitant with a decrease in the small size tubes (3%) rather than the mid-range sized tubes (57%; Figure 5.2B).

5.4.3 *Fusion Index*

Nuclei in mature myotubes were observed to be either aligned in a row or clumped in groups within myotubes. The latter was more typical in larger CR-treated myotubes (Figure 3A). The former was more frequently observed in the thinner CR+INS and INS-treated myotubes (Figure 5.3B) with both LG and HG culture concentrations.

To identify if differences in myotube size were associated with the number of myonuclei within myotubes, the fusion index of myotubes treated with CR and CR+INS in HG cultures was compared. The fusion index (nuclei per myotube) was higher but not significantly different for myotubes treated with CR+INS compared to CR (23 ± 2 vs 17 ± 2 nuclei/myotube; $P = 0.06$; Figure 5.3C).

A.



B.

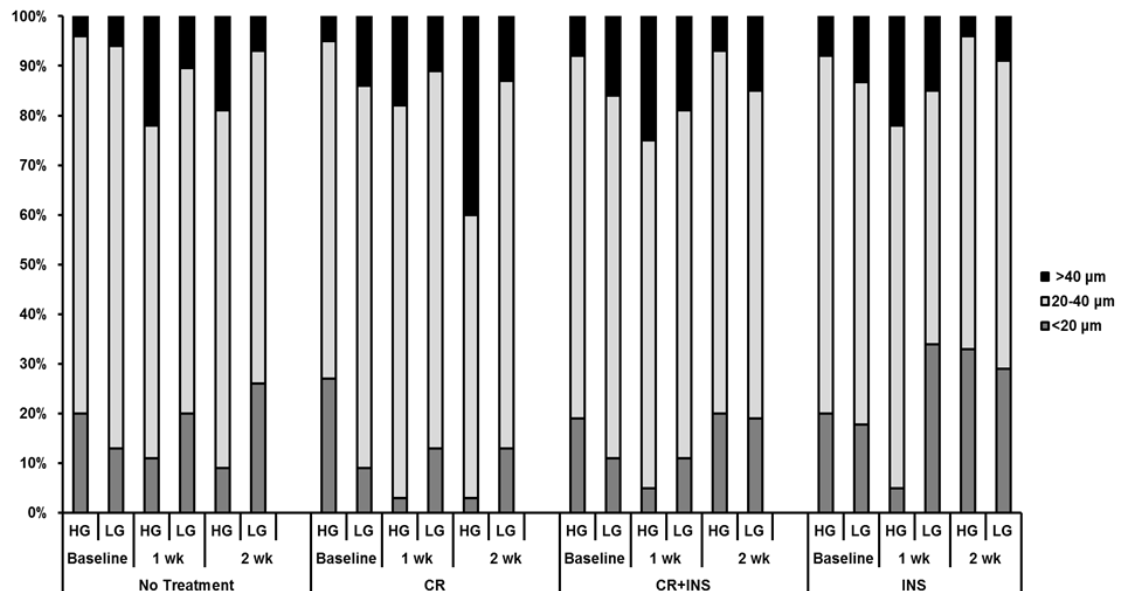


Figure 5.2: Myotube size following treatments with low (LG) or high (HG) glucose medium supplemented with creatine (CR), creatine and insulin (CR+INS) and insulin (INS) for 2 weeks. Myotube size was obtained from 10 randomly selected myotubes from each of 5 microscopic fields per time point (n=50) in 3 separate repeats. **(A)** Fold change in myotube size relative to baseline("1") \pm SEM. **(B)** Relative distribution of myotubes by size; <20 μ m, 20-40 μ m >40 μ m. The proportion of myotubes in each size category is a percentile from the total tubes measured in the field at one time point of each treatment. *, **, *** = Difference (P < 0.05, 0.01 and 0.001, respectively) compared to baseline. %, #, \$ = Difference (P < 0.05, 0.01 and 0.001, respectively) between glucose treatment (LG vs HG) within treatment condition in similar weeks. a, b, c, d = Difference (P < 0.05) between treatment conditions within glucose conditions in similar weeks, where a: no treatment (NT), b: CR, c: CR+INS, d: INS. † = Difference (P < 0.05) between time points (1 wk vs 2 wk) within similar treatment and glucose conditions.

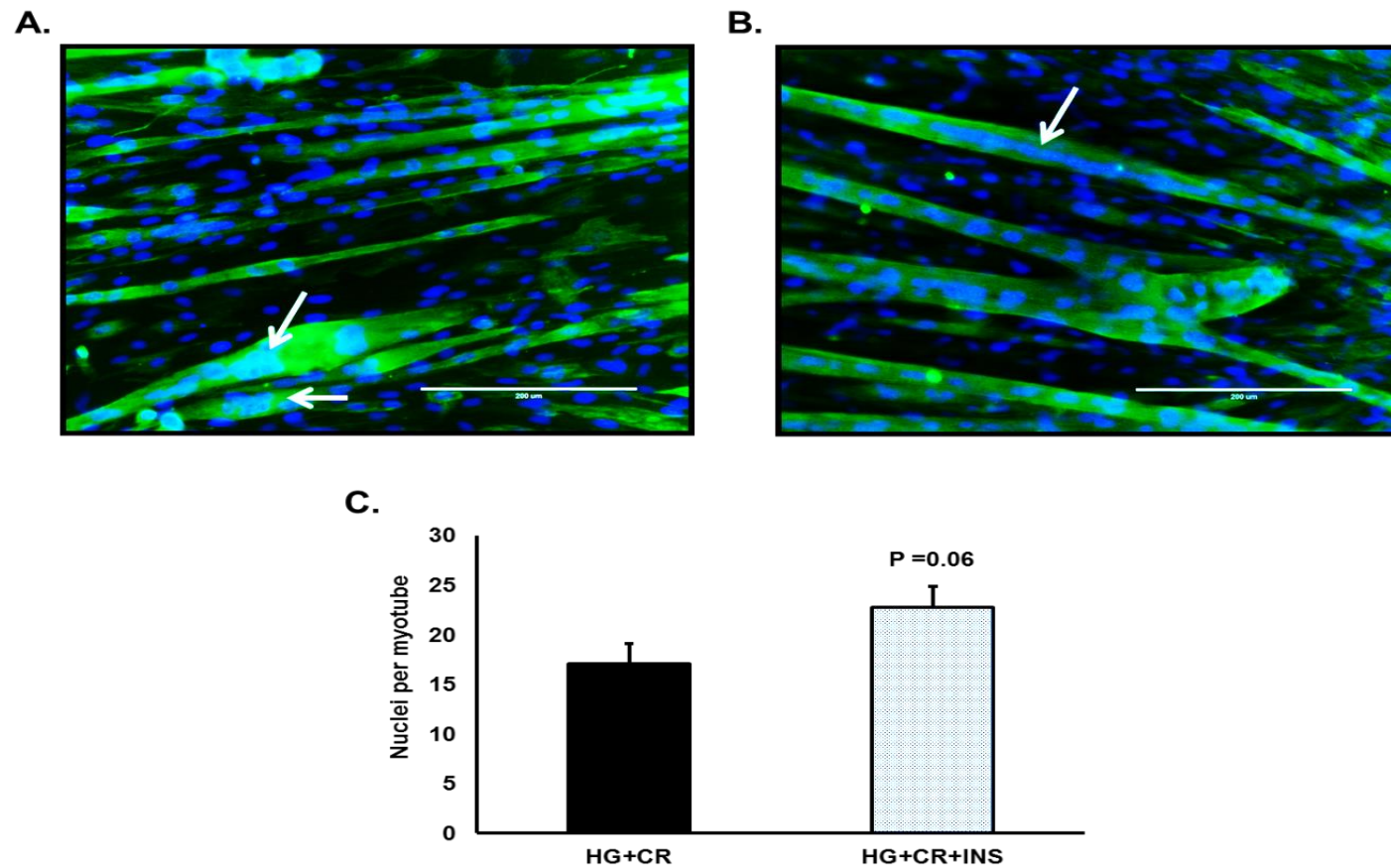


Figure 5.3: Multi-nucleated myotubes cultured with high glucose (4.5 g/L; HG) medium supplemented with (A) creatine (CR) and (B) creatine and insulin (CR+INS) after 2 weeks (x20 magnification; scale bar = 200 μ m). (C) Fusion index calculated as the mean number of nuclei (stained blue with DAPI) within myotube borders (stained green with Desmin) throughout the image \pm SEM (n = 20 images).

5.4.4 Protein Expression

The representative blots of the total and phosphorylated forms of the following hypertrophy associated proteins are presented in Figure 5.4.

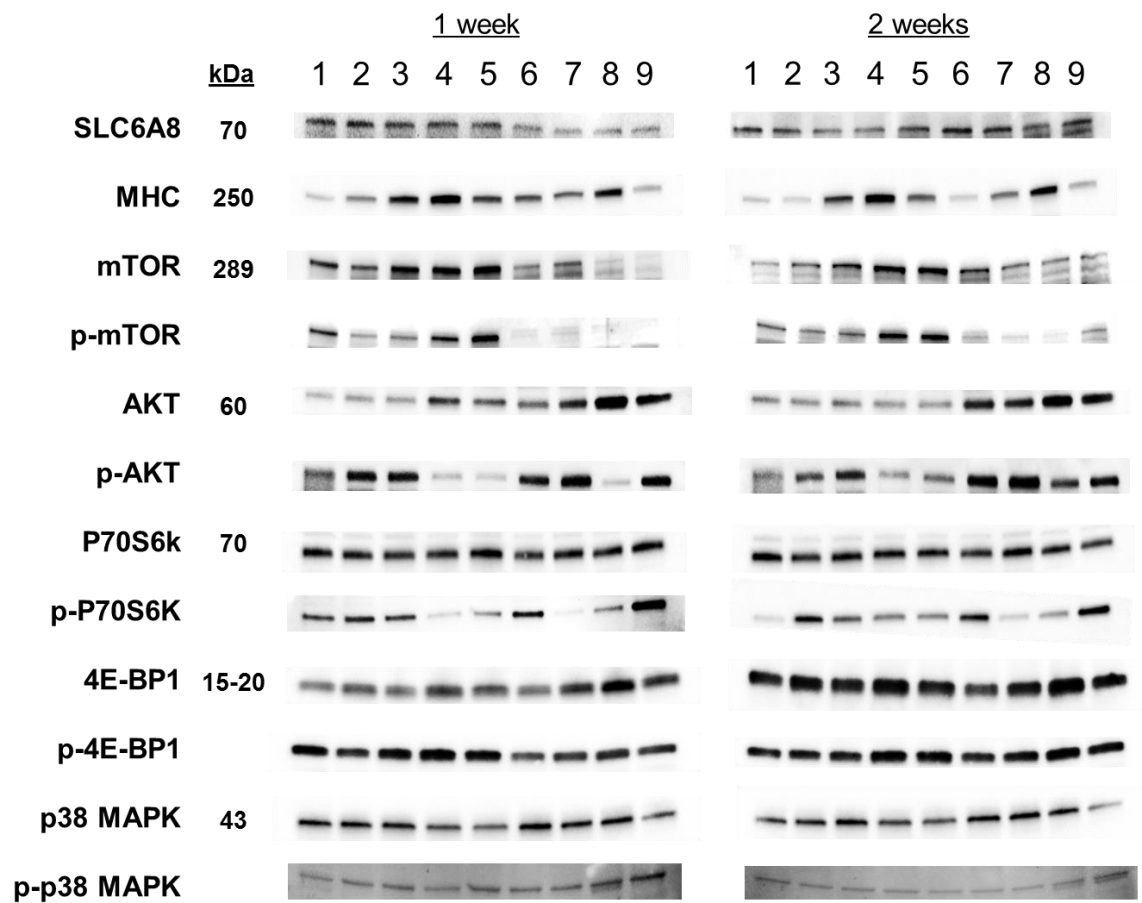


Figure 5.4: Representative Blots for SLC6A8, MHC, and total and phosphorylated signalling protein expression in response to treatments at baseline, 1 and 2 wk. Lane numbers correspond to 1) Baseline; 2) LG; 3) LG+CR; 4) LG+CR+INS; 5) LG+INS; 6) HG; 7) HG+CR; 8) HG+CR+INS; 9) HG+INS. The same baseline was measured for each blot at 1 and 2wk for each respective protein.

SLC6A8

There was no significant effect of treatment or time on the relative expression of SLC6A8 in either LG or HG cultures; although an increase of SLC6A8 protein content was identified in all treatment groups by 1 and 2 wk of culture (Figure 5.5A).

MHC

MHC levels were significantly affected by treatment as well as time with a significant interaction effect observed for both LG and HG concentrations ($P < 0.001$). Treatment with CR+INS in LG cultures resulted in significantly greater levels of MHC compared to NT and all other treatment groups at both 1 and 2 wk (8-10 fold increase; $P < 0.01$ - 0.001 ; Figure 5.5B). Within 2 wk, cultures treated with CR+INS in HG had significantly greater MHC expression compared to CR and INS alone (6 fold increase; $P < 0.05$ - 0.001 ; Figure 5.5B) with a significant increase from 1 to 2 wk observed ($P < 0.05$). Cultures treated with CR, CR+INS and INS in LG had significantly higher levels of MHC than their HG counterpart at 1 and 2 wk ($P < 0.05$ - 0.001 ; Figure 5.5B).

mTOR

There was a strong interaction effect between treatment and time with regard to relative levels of total mTOR ($P < 0.01$). Time alone, however, was not an effector of mTOR levels within either LG or HG conditions. In LG treated cultures, levels of mTOR increased above baseline and NT with all treatment groups at 1 wk and with CR+INS and INS at 2 wk (1-3 fold change; $P < 0.05$ - 0.001 ; Figure 5.5C). There was a significant difference between 1 and 2 wk with CR and CR+INS-treated groups cultured in LG ($P < 0.05$; Figure 5A). LG treated groups increased significantly with CR, CR+INS, and INS at 1 wk and CR+INS and INS at 2 wk above their respective HG counterparts ($P < 0.001$). Increased expression of phosphorylated mTOR was observed with all treatments by 2 wk (Figure 5.5D).

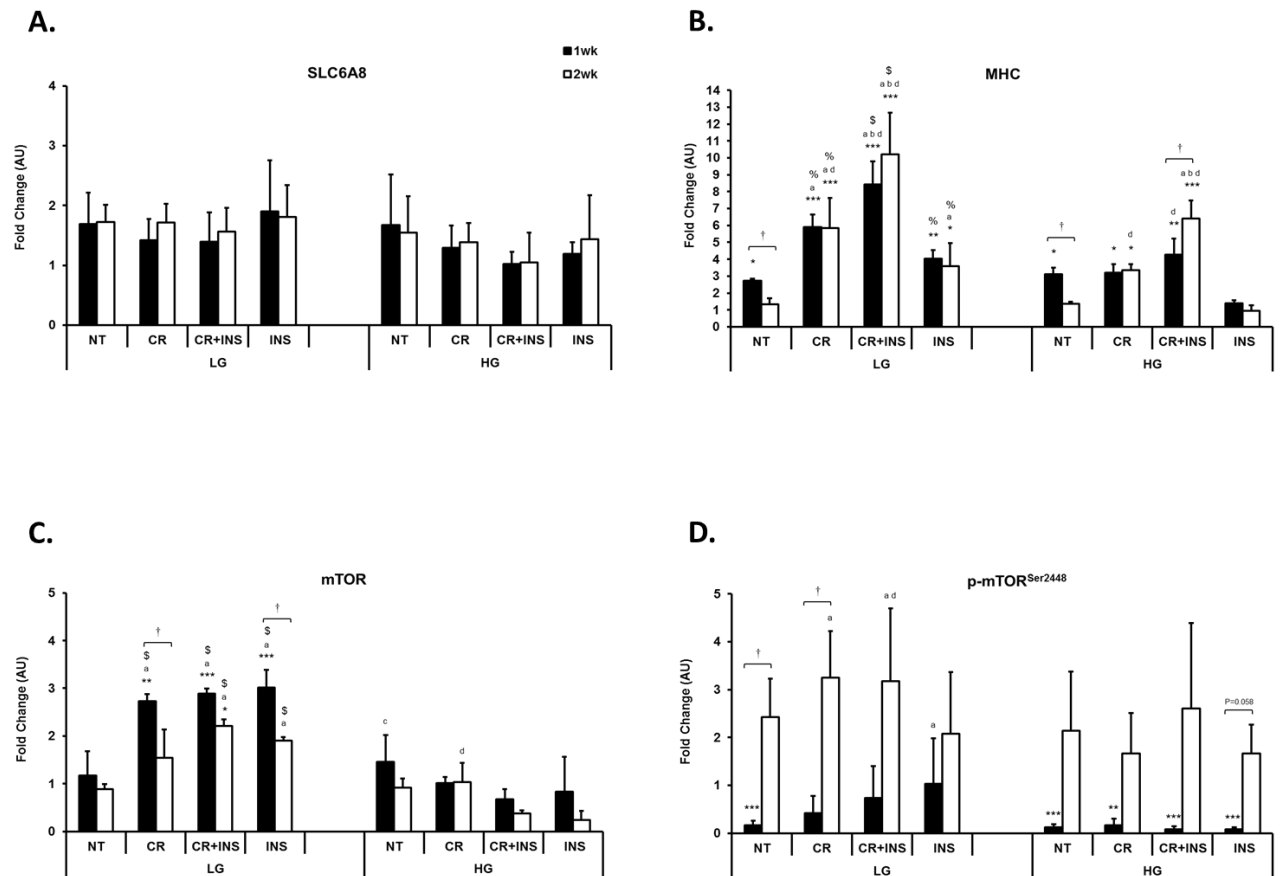


Figure 5.5: (A) SLC6A8 creatine transporter, (B) Myosin Heavy Chain (MHC), (C) total mTOR and (D) phospho-mTOR^{Ser2448} protein expression following 1 and 2 wk treatment with low (LG) or high glucose (HG) only (NT) supplemented with creatine (CR), creatine and insulin (CR+INS), and insulin (INS). Values are expressed as total protein determined by stain-free technology relative to baseline and presented in arbitrary units (AU) as the mean \pm SEM (n=3 biological repeats). *, **, *** = Difference (P < 0.05, 0.01 and 0.001, respectively) compared to baseline. %, #, \$ = Difference (P < 0.05, 0.01 and 0.001, respectively) between glucose treatment (LG vs HG) within treatment condition in similar weeks. a, b, c, d = Difference (P < 0.05) between treatment conditions within glucose conditions in similar weeks, where a: no treatment (NT), b: CR, c: CR+INS, d: INS. † = Difference (P < 0.05) between time points (1 wk vs 2 wk) within similar treatment and glucose conditions.

AKT

There was a significant interaction between treatment and time for AKT expression ($P < 0.05$). No changes between treatments were observed with LG. Within HG treated cultures, the highest AKT expression was observed in those treated with CR+INS at 1 wk (4 fold increase; $P < 0.05$; Figure 5.6A). CR and CR+INS treatments increased AKT levels above baseline at 2 wk (3 fold increase; $P < 0.05$; Figure 5.6A). HG cultures treated with CR and CR+INS had greater AKT expression than their LG counterpart at 1 and 2 wk ($P < 0.05$ -0.001). As with phosphorylated mTOR, increased phosphorylated AKT expression was observed with all treatments by 2 wk (Figure 5.6B).

p70S6K

A significant interaction effect between treatment and time was observed with p70S6K expression ($P < 0.001$). Within LG cultures, CR+INS and INS treatments resulted in greater expression at 2 wk compared to their HG counterpart ($P < 0.05$ - 0.001; Figure 5.6C). At 2 wk, CR treated cultures in HG had significantly higher AKT expression than all other treatment groups (1.7 fold increase; $P < 0.01$ -0.001; Figure 5.6C). By 2 wk, phosphorylated p70S6K expression increased in all treatment groups (Figure 5.6D).

4E-BP1

LG cultures with CR+INS and HG cultures treated with INS showed significantly greater 4E-BP1 expression above baseline 1 wk (1.5-2 fold increase; $P < 0.05$; Figure 5.6E) CR and CR+INS treated groups had a significantly higher expression of phosphorylated 4E-BP1 than baseline at 1 wk (1.2-1.7 fold increase; $P < 0.01$ Figure 5.6F). INS treated groups in LG at 1 wk expressed significantly greater phosphorylated 4E-BP1 compared to its HG counterpart ($P < 0.05$).

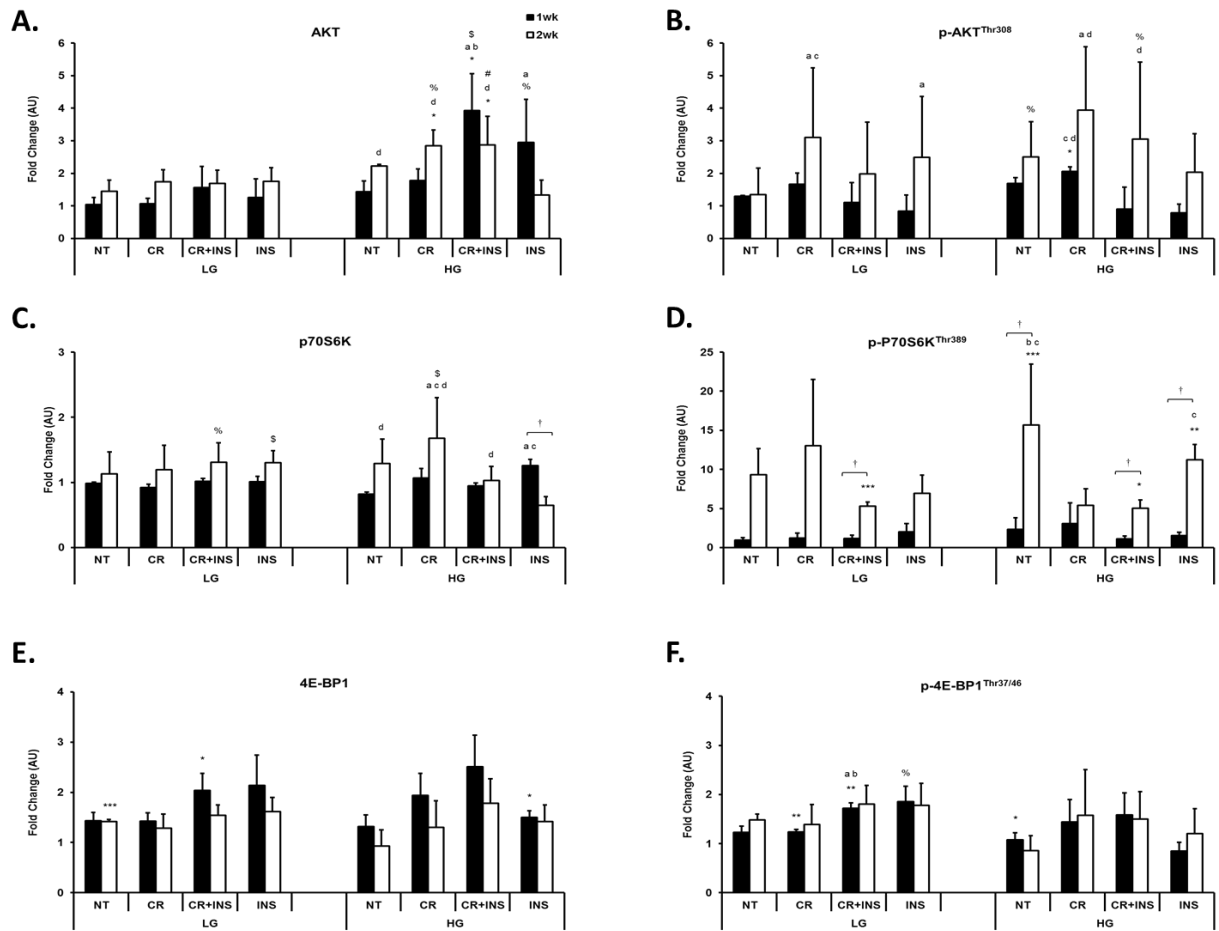


Figure 5.6: (A) Total AKT, (B) phospho-AKT^{Thr308}, (C) Total p70S6K, (D) phospho-p70S6K^{Thr389}, (E) Total 4E-BP1 and (F) phospho-4E-BP1^{Thr37/46} protein expression following 1 and 2 wk treatment with low (LG) or high glucose (HG) only (NT) supplemented with creatine (CR), creatine and insulin (CR+INS), and insulin (INS). Values are expressed as total protein determined by stain-free technology relative to baseline and presented in arbitrary units (AU) as the mean \pm SEM (n=3 biological repeats). *, **, *** = Difference (P < 0.05, 0.01 and 0.001, respectively) compared to baseline. %, #, \$ = Difference (P < 0.05, 0.01 and 0.001, respectively) between glucose treatment (LG vs HG) within treatment condition in similar weeks. a, b, c, d = Difference (P < 0.05) between treatment conditions within glucose conditions in similar weeks, where a: no treatment (NT), b: CR, c: CR+INS, d: INS. † = Difference (P < 0.05) between time points (1 wk vs 2 wk) within similar treatment and glucose conditions.

p38 MAPK

Differences in p38 MAPK between 1 and 2 wk time points were found with LG +INS and HG+CR treated group ($P < 0.05-0.01$) with levels at 2 wk being significantly higher than baseline or 1wk for both groups (1.3 and 2 fold increases with LG +INS and HG+CR, respectively; $P < 0.01-0.001$; Figure 5.7A). In HG cultures, CR treatment had higher levels of p38 MAPK than the INS+CR and INS treated cultures at 2 wk ($P < 0.05-0.001$). HG cultures with CR treatment had significantly higher expression than their LG counterparts at 2 wk ($P < 0.01$; Figure 5.7A). Phosphorylated p38 MAPK levels increased by 1 wk with all treatment groups with HG+CR treatment significantly higher than baseline levels (2.5 fold increase; $P < 0.01$).

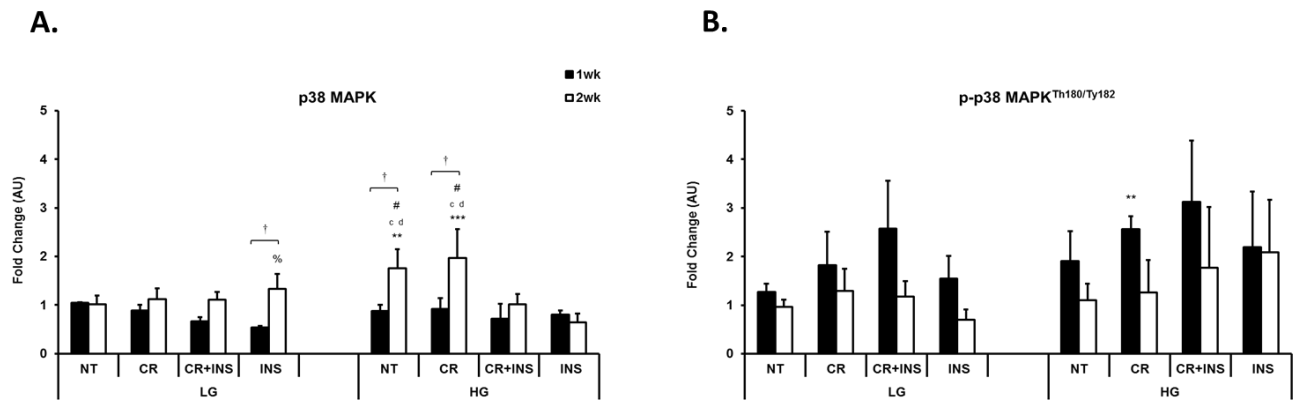


Figure 5.7: (A) Total p38 MAPK and (B) phospho-p38 MAPK^{Th180/Ty182} protein expression following 1 and 2 wk treatment with low (LG) or high glucose (HG) only (NT) supplemented with creatine (CR), creatine and insulin (CR+INS), and insulin (INS). Values are expressed as total protein determined by stain-free technology relative to baseline and presented in arbitrary units (AU) as the mean \pm SEM (n=3 biological repeats). *, **, *** = Difference ($P < 0.05$, 0.01 and 0.001, respectively) compared to baseline. %, #, \$ = Difference ($P < 0.05$, 0.01 and 0.001, respectively) between glucose treatment (LG vs HG) within treatment condition in similar weeks. a, b, c, d = Difference ($P < 0.05$) between treatment conditions within glucose conditions in similar weeks, where a: no treatment (NT), b: CR, c: CR+INS, d: INS. † = Difference ($P < 0.05$) between time points (1 wk vs 2 wk) within similar treatment and glucose conditions.

5.5 Discussion

In vivo, the uptake and transport of creatine into muscle cells is augmented via the co-ingestion of carbohydrates, which is mediated, in part, by the effect of insulin on the SLC6A8 creatine transporter. However, the direct *in vitro* effect(s) of creatine and insulin treatment on established myotubes has not been characterised. The results of the present study demonstrate that morphological differences in size and myofibrillar protein content are indeed observed in C2C12 myotubes cultured with creatine and/or insulin which are also determined by glucose concentration. Larger myotubes developed when cultures are supplemented with creatine in higher glucose concentrations. MHC was upregulated when cultures were supplemented with either creatine or insulin more pronounced in low glucose concentrations. These findings suggest that this increase in MHC protein content in myotubes may not be regulated by the conventional anabolic pathway, which involves mTOR and its downstream substrates, but rather is mediated via p38 MAPK signalling and is largely independent of glucose concentrations.

New skeletal muscle formation is a complex and highly regulated process that involves the proliferation of myoblasts and subsequent differentiation to multinucleated myotubes (Grabiec et al. 2014). The regulation of muscle size is crucial for the maintenance of homeostasis as well as proper skeletal muscle growth and development. Satellite cells are the primary contributors to muscle growth via the provision of myonuclei for the production of new or repair of existing muscle (Wang and Rudnicki 2012). Previous work has demonstrated that an increase in muscle size is not only associated with, but is also dependent on the addition of myonuclei to existing muscle fibres, with particular emphasis on muscle greater than 25% (Petrella et al. 2006).

In vivo, co-supplementation of creatine and carbohydrates synergistically enhances substrate storage capacity as a consequence of greater water retention and subsequently

augmented cell size (Green, Hultman, et al. 1996, van Loon et al. 2004). The addition of creatine to a high glucose medium promoted a further increase in myotube size after 2 wk, commensurate with a greater percentage of large myotubes, which aligns with the results of *in vivo* investigations. High glucose DMEM is known to have “anabolic” effects and thus is commonly recommended for use in C2C12 culture medium for muscle engineering *in vitro* (Khodabukus and Baar 2015). The present study observed that myotubes treated with low glucose concentrations maintained their size after one week.

Previous *in vitro* as well as *in vivo* studies have identified creatine’s ability to augment the number of activated satellite cells suggesting that this may lead to greater number of proliferating and differentiating cells (Vierck et al. 2003, Olsen et al. 2006, Dangott, Schultz, and Mozdziak 2000, Deldicque et al. 2007). The present findings indicate that *in vitro*, creatine’s ability to enhance fusion to form new tubes is augmented by insulin leading to cultures which are highly crowded with thinner myotubes (Figure 5.1A). The current study identified two different modes of myonuclei distribution within the myotubes, rows and groups (Figure 5.3A). Thin myotubes, which were more likely to appear in in INS treated cultures had a greater propensity to have a row-type distribution and also presented a higher fusion index than myotubes formed in cultures treated only with creatine. The latter were more likely to have groups of myonuclei concentrated in one area of the myotube. The distinctive difference in myonuclei distribution in relation to culture conditions and its implications on muscle regeneration warrants further investigation. Furthermore, this investigation also recognised branching myotubes mainly within CR+INS treated cultures in both high and low glucose concentrations. Branching of myofibres can occur via the generation of a new myofibre segment fusing to an existing myofibre or by splitting one myofibre in two (Faber et al. 2014). The activation of satellite cells has been thought to cause

branching in myofibres via the fusion to pre-existing fibres in a non-linear pattern and may explain these observations (Anderson 2000).

Creatine accumulation in skeletal muscle is enhanced via carbohydrate-driven insulin secretion and its direct effect on SLC6A8 creatine transporter (Steenge et al. 1998, Steenge, Simpson, and Greenhaff 2000, Odoom, Kemp, and Radda 1996). Insulin regulates creatine transport into skeletal muscle via the SLC6A8 creatine transporter. Odoom and colleagues (1996) observed a two-fold increase in intracellular creatine content as a result of insulin treatment in cultured myotubes (Odoom, Kemp, and Radda 1996). This observation was confirmed in humans with varying infusion rates of insulin concomitant with creatine consumption (Steenge et al. 1998). In the present study, long-term cultures showed a consistent level of the transporter at both time points regardless of creatine being present suggesting that expression occurs as an outcome of culture conditions. As the primary aim of was to measure prolonged treatment effects on myotube size, a limitation of the present study was the inability to concomitantly measure intracellular creatine content. While such analysis was beyond the scope of the study, the effects of the treatments presented would be of great investigative interest to follow up in future studies, particularly to compare insulin's potential to increase creatine uptake to its effect on muscle size.

Contractile protein MHC and its various isoforms plays a key role in regulating the functionality of skeletal muscle fibres and is subject to a complex system of controls which can be affected by insulin (Sullivan et al. 1995). It has been suggested that creatine's role in increasing muscle protein synthesis occurs by increasing the degree of myosin synthesis (Ingwall, Morales, and Stockdale 1972). A significant increase in MHC protein expression above baseline was observed in the present study with all treatments. The most pronounced effect was observed when CR+INS was added to cultures in low glucose concentrations followed by that of CR only and INS only treatments also in low glucose concentrations.

Work by Khodabukus and Barr (2015) showed that treatment of engineered muscle myotubes in low glucose concentration had significantly lower levels of MHC than myotubes treated with high glucose concentrations (Khodabukus and Baar 2015). Their work also showed that a reduction in glucose results in a shift towards a more slow-oxidative type of MHC, suggesting that glucose plays a prominent role in the regulation of myotube phenotype *in vitro* (Khodabukus and Baar 2015). In the present study, high glucose availability did not affect MHC levels significantly unless combined with CR+INS. Several differences in culture methods may account for the discrepancies observed between the current study and that carried by Khodabukus and Barr (2015). In the present study, myotube differentiation was initiated in monolayers while the results of previous work established myotubes and maintained them in 3-dimensional cultures. Furthermore, myotubes were presently maintained in 2% HS following differentiation for the duration of the treatment period to eliminate other factors within high serum levels that could interfere with CR and/or INS effects, while Khodabukus and Barr (2015) replaced their HS with 7% FBS following 2 days of low serum culture. It was suggested that relative expression levels of MHC are indicative of myotube maturity which, in the present study, was obtained in cultures supplemented with CR+INS predominantly in low glucose concentrations.

In culture, creatine has been shown to upregulate the phosphorylation of a number of key signalling intermediates of the AKT/mTOR protein synthetic pathway (Deldicque et al. 2007). In the present study, the levels of the upstream regulator of mTOR, AKT, were more pronounced in high glucose cultures but were not affected by creatine, or insulin. Additionally, the present study observed no correlation between AKT activity and that of mTOR in the current culture model which is attributed to mTOR's ability to be activated by nutrient availability/stimuli independent of AKT activation (Wang and Proud 2006, Miniaci et al. 2015). mTOR phosphorylates downstream target p70S6K which results in increased

translation (direct or indirect) via the activation of elongation factors including eIF-4E (via 4E-BP1) (Van Wessel et al. 2010). The phosphorylation of p70S6K and inhibition of 4E-BP1 have been closely linked with AKT and mTOR activation. Miniaci and colleagues (2015) demonstrated that an mTOR-mediated increase in p70S6K expression in L6 cells was linked to greater rates of protein synthesis, particularly after a period of glucose starvation (Miniaci et al. 2015). This corresponded with the increased expression of muscle specific proteins, including MHC (Miniaci et al. 2015), which is in agreement with the current observations. It should be noted that due to the transient nature of downstream effects induced by phosphorylation and the chronic time points of the present study, the measurement of total protein were more relevant to the conclusions reached herein.

Neither glucose concentrations nor the different treatments resulted in changes in the expression of total p70S6K or its phosphorylated form. However, the duration of treatment appears to affect the levels of phosphorylated p70S6K as its detection increased significantly in all treatments after 2 wk. The detection of total and phosphorylated 4E-BP1 was similar with increases observed in CR+INS and INS treated groups in LG and CR and CR+INS in HG. Delidicque and colleagues (2007) reported that, when differentiating C2C12 myotubes with creatine, an increase in p70S6K activity does not necessarily have an effect on 4E-BP1 phosphorylation (Deldicque et al. 2007). Furthermore, previous work suggests that 4E-BP1 and p70S6K are regulated by distinct signalling events downstream of mTOR (Wang et al. 2005) and may therefore, be effected via different regulatory pathways.

The MAPK/ERK signalling pathway has also been shown to promote muscle differentiation, growth and repair (Deldicque et al. 2007). p38 MAPK has also been implicated in the activation of a number of transcription factors necessary for differentiation of myoblasts to myotubes (Lechner et al. 1996). In the present study, p38 MAPK levels were affected by treatment and time and to a lesser degree by glucose concentration. The

phosphorylated form of p38 MAPK was more responsive to treatment and time than glucose concentrations with the highest levels found in cultures treated with CR+INS. These findings suggest that the effects of treatments and the varying outcomes may be a result from MAPK signalling pathway. Evidence for “crosstalk” between MAPK and AKT/mTOR pathways is scarce, being limited to observations that one signalling cascade may prevent differentiation of myoblasts by the other (Gardner et al. 2015). Several studies have shown that p38 MAPK activity increased muscle differentiation and forced activation of this pathway could stimulate differentiation and promote myotube formation (Wu et al. 2000, Zetser, Gredinger, and Bengal 1999, Serra et al. 2007). Work by Gardner and colleagues (2015) found that blocking p38 MAPK expression, thereby promoting IGF-1 mediated signalling, resulted in a lack of C2 muscle cell fusion, suggesting p38 MAPK to be essential for the later stages of myotube differentiation which result in multinucleated tubes.

The results of the present study determined that creatine supplementation results in greater myotube size *in vitro*, which is not related to an increase in contractile proteins. The latter is influenced by insulin’s anabolic role, which also supports the fusion of differentiated myoblasts resulting in greater MHC expression. This outcome observed under the present culture conditions is likely mediated, in part, via MAPK signalling cascades.

CHAPTER 6:
Fenugreek Increases Insulin-Stimulated Creatine Content in L6C11
Muscle Myotubes

This chapter is comprised of the following paper which was accepted for publication in the
European Journal of Nutrition:

Fenugreek Increases Insulin-Stimulated Creatine Content in L6C11 Muscle Myotubes

Tomcik KA, Smiles WJ, Camera DM, H Hugel HM, Hawley JA, Watts R.

Preface to Chapter Six

The results of the study described in Chapter Five showed that while glucose concentration can augment the differentiation and growth of muscle, the effects vary over a chronic treatment time course. Insulin, however, appears to play a key role in augmenting size and hypertrophy signaling pathways when in the presence of creatine. In lieu of the *in vitro* observations with combined insulin and creatine treatments in chapter five and in conjunction with the implications of training with low carbohydrate availability, it was speculated whether similar physiological benefits on creatine uptake could be achieved with a compound that mimics the action of insulin to a similar degree of that elicited by insulin alone, thereby eliminating the need for carbohydrate/glucose altogether.

In order to obtain an insulin-like benefit without carbohydrate or insulin present, Study III employed the cell culture model described in the previous study to test the effects of a reported natural insulin mimicking product. Fenugreek is an herb commonly utilized in Indian cooking and has also been studied in alternative Ayurvedic medicine as a treatment for diabetics due to its blood glucose lowering profile which is remarkably similar to that observed with insulin (Figure 6.1).

Fenugreek seeds were obtained from several sources all originating from the same country of origin (India) as well as a purified chemical extract obtained from a commercial vendor. Fenugreek seeds were extracted for their oil components as detailed in the proceeding chapter. In order to ensure quality of the extracts as well as to compare it to the chemical reagent form, a liquid chromatography-mass spectroscopy (LC-MS) method was developed and validated for the analysis of 4-hydroxyisoleucine (4-HIL), the active insulinotropic compound found in fenugreek seed extract.

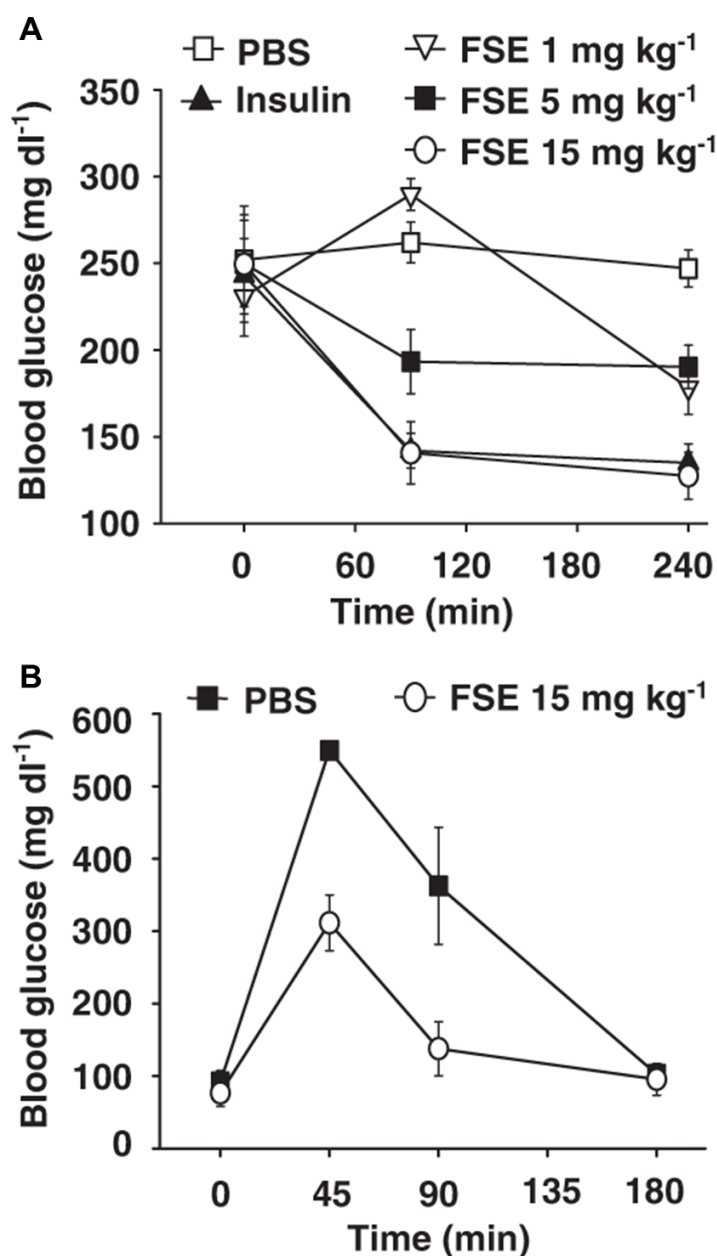


Figure 6.1: The blood glucose lowering effects of fenugreek compared to insulin. **(A)** AXN-induced diabetic Swiss albino mice were injected with vehicle phosphate buffered saline (PBS), insulin or fenugreek seed extract (1, 5 or 15 mg·kg⁻¹). Fenugreek seed extract at a dose of 15 mg·kg⁻¹ produced a significant decrease in blood glucose levels compared to PBS control ($P<0.01$). **(B)** Intraperitoneal glucose tolerance test in glucose-loaded (3 g·kg⁻¹) normal Swiss albino mice. Fenugreek seed extract injected mice had significantly lower levels of blood glucose ($P<0.01$ vs PBS treated) at 45 and 90 min after its administration. (Reproduced from Vijayakumar *et al.* (2005) *British Journal of Pharmacology*)

It was during this process that the present investigation determined the seed extract with the highest concentration of 4-HIL and consequently excluded the chemical reagent from the experiment because it contained the lowest concentration of 4-HIL.

The use of C2C12 cell line was initially employed for the study due to its known application in insulin and creatine studies. Preliminary experiments, however, indicated that the fenugreek extract was toxic to this particular cell line and treatment with a spectrum of fenugreek extract concentrations resulted in 20-60% cell death by 24 h (Figure 6.2). In light of this finding, the subsequent study employed the use of the L6 cell line which, as previously described in the prelude to Chapter Five, have an innate sensitivity to insulin stimulation and remained viable following treatment with fenugreek.

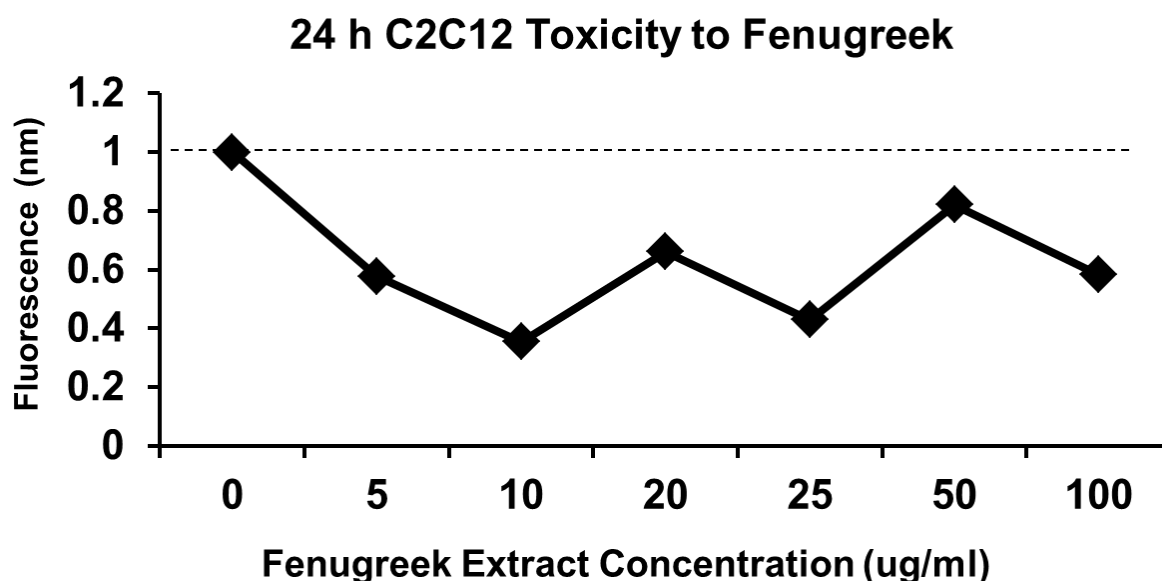


Figure 6.2: Cytotoxic analysis of fenugreek treated C2C12 skeletal muscle cells. Cells were treated with varying concentrations (0-100 $\mu\text{g/ml}$) of fenugreek seed extract for 24 h to establish a suitable non-toxic dose for use in further experiments. A fluorescence-based cell viability assay was used to estimate viable cell number. Per the methodology of the assay, values which fall below the dotted line indicated toxicity and cell death.

6.1 Abstract

Purpose

Creatine uptake by muscle cells is increased in the presence of insulin. Accordingly, compounds with insulin-like actions may also augment creatine uptake. The aim of this study was to investigate whether *Trigonella foenum-graecum* (fenugreek), an insulin-mimetic, increases total intracellular creatine levels *in vitro*.

Methods

Total cellular creatine content was measured fluorometrically in L6C11 muscle myotubes treated for 1, 4, and 24 h with 0.5 mM creatine (CR), CR and 20 μ g/mL fenugreek seed extract (CR+FEN), CR and 100 nM insulin (CR+INS), and CR+INS+FEN (n=6 per treatment group). Alterations in the expression of the sodium-chloride dependent creatine transporter, SLC6A8, and key signalling proteins in the PI3-K/AKT pathway were determined.

Results

Compared to control (CON), CR+INS+FEN increased total creatine content after 4 h ($P<0.05$) whereas all conditions increased SLC6A8 protein expression above CON at this time ($P<0.05$). Changes in insulin signalling were demonstrated via increases in Akt^{Thr308} phosphorylation, with CR+INS > CON and CR at 1 h ($P<0.05$) and with CR+INS+FEN > CON, CR, and CR+INS at 4 h ($P<0.05$). In contrast, no changes in PKC ζ/λ or GLUT4 phosphorylation were detected.

Conclusion

Fenugreek, when combined with insulin, modulates creatine content via a mechanism which is independent of the activity of SLC6A8, suggesting an alternative mechanism is responsible for the regulation and facilitation of insulin-mediated creatine uptake in skeletal muscle cells.

6.2 Introduction

Creatine is an ergogenic supplement commonly used by athletes participating in short-duration, high-intensity exercise (Greenhaff 2001). Creatine becomes phosphorylated to produce phosphocreatine (PCr), which provides skeletal muscle with a rapid store of energy-generating substrates utilized in the replenishment of adenosine triphosphate (ATP) (Wallimann et al. 1992). Basal concentrations of creatine in skeletal muscle can be increased up to 20% using short-term (i.e., 5 days) creatine supplementation strategies (Harris, Soderlund, and Hultman 1992, Greenhaff et al. 1994). Furthermore, the co-ingestion of creatine with carbohydrate (CHO) further augments intramuscular creatine stores by up to 60% above basal levels (Green, Hultman, et al. 1996), presumably via an insulin-dependent mechanism (Odoom, Kemp, and Radda 1996).

While creatine is an important energy source in muscle (Loike et al. 1988, Walker 1979), skeletal muscle cells are incapable of synthesizing their own creatine (Walker 1979). Indeed, approximately 50% of whole body creatine is endogenously synthesised from the amino acids glycine, arginine, and methionine via a series of reactions carried out in the kidney and liver (Kingsley et al. 2009, Schoch, Willoughby, and Greenwood 2006). The remainder is obtained via dietary consumption of meat and fish (Balsom et al. 1993) or nutritional supplementation (Jager et al. 2011). Over 90% of circulating creatine is transported directly into skeletal muscle cells via the membrane-localized transporter protein of the solute carrier family 6 (SLC6), SLC6A8 (Wallimann et al. 1992, Persky, Brazeau, and Hochhaus 2003, Snow and Murphy 2003). The activity of the SLC6A8 creatine transporter and subsequent levels of creatine content in skeletal muscle is affected by a number of variables (Snow and Murphy 2001) including, but not limited to, fluctuations in the sodium/chloride-dependent gradient (Loike, Somes, and Silverstein 1986, Willott et al.

1999), muscle fibre type (Murphy et al. 2001), intra- and extra-cellular creatine concentrations (Loike et al. 1988), and exercise (Harris, Soderlund, and Hultman 1992).

Insulin further augments creatine transport into skeletal muscle, predominantly by increasing muscle Na^+/K^+ pump activity (Steenge et al. 1998, Loike, Somes, and Silverstein 1986, Odoom, Kemp, and Radda 1996, Green, Hultman, et al. 1996, Steenge, Simpson, and Greenhaff 2000, Haugland and Chang 1975, Koszalka and Andrew 1972). Considering the evidence that increased creatine transport into skeletal muscle occurs, in part, via an insulin-stimulated mechanism, it is possible that compounds which mimic insulin's effects may also increase creatine transport. *Trigonella foenum-graecum*, commonly known as fenugreek, is an herbal plant with a demonstrated safety profile in humans (Smith 2003). Its phytochemical constituents include saponins (Kang LP 2013), high-fibre content (Al-Jasass and Al-Jasser 2012), (2*S*,3*R*,4*S*)-4-hydroxyisoleucine (4-HIL) (Sauvaire et al. 1998, Narender et al. 2006), phenolic antioxidants (Kenny et al. 2013) and trigonelline (Nathan et al. 2013) as the major bioactive compounds with reported beneficial effects (Srinivasan 2006). Fenugreek is also used in alternative medicine as a therapeutic agent for the treatment of diabetes due to its inherent insulinotropic effects (Srinivasan 2006, Jaiswal et al. 2012, Vijayakumar et al. 2005, Sauvaire et al. 1998).

Similar to insulin, extracts from fenugreek seeds have been shown to up-regulate key proteins in the PI3-K signalling pathway (Broca et al. 2004). However, discrepancies exist in the activation of key signalling intermediates in this pathway. Unlike insulin, fenugreek treatment does not appear to phosphorylate protein kinase B (herein referred to as AKT), a key intermediate in glucose metabolism (Vijayakumar et al. 2005). Instead, fenugreek is thought to initiate the translocation of protein kinase C (PKC), a similarly sequenced protein kinase. A catalyst for a number of signalling cascades including the insulin signalling pathway, PKC has been shown to translocate to the cell surface of hepatic and adipocyte

cells treated with fenugreek (Vijayakumar et al. 2005) and may similarly do so in muscle cells. Therefore, it was hypothesized that co-incubation of fenugreek with creatine would increase total intracellular creatine content of L6C11 skeletal muscle myotubes to a similar magnitude to that induced by insulin and creatine co-incubation. Furthermore, the present study hypothesized that fenugreek would alter the expression of the principle creatine transporter, SLC6A8 and/or key insulin signalling intermediates as a result of its insulin-mimicking properties.

6.3 Methods

6.3.1 Preparation of Fenugreek Extracts

Raw fenugreek seeds were obtained locally (origin: India) and dried at 105°C for 3 h before being ground into a fine powder. The ground seeds were placed into a sample cell and loaded into an ASE TM 100 System (Dionex, Sunnyvale, CA) and extracted as per manufacturer's instructions. Prepared samples were extracted using 50% ethanol. Upon the completion of the extraction process, ethanol and water were evaporated and any residual solution removed via overnight freeze drying. Fenugreek extract materials were stored in a dry environment until treatment.

6.3.2 Fenugreek Extract Analysis

Known amounts of fenugreek seed extract samples and a commercially available extract (Sigma Aldrich, St Louis, MO USA) were dissolved in 1mL of AR methanol and sonicated for 5min to extract 4-HIL. Standards of 4-HIL (Sigma Aldrich) (Isostd mg/mL) were run on an Electrospray Ionization (ESI) mass spectrometer (Micromass Platform II) to determine peak areas of the standards (Mass 148 was monitored). All samples and standards were made up in acetonitrile: water 50:50 with 0.1% formic acid. A calibration curve was produced using four standard concentrations of 4-HIL. The fenugreek sample areas were

read from the curve to determine the concentrations of 4-HIL (recorded as % weight) in each extract.

6.3.3 *Cell Culture*

L6C11 myoblasts were maintained in high glucose Dulbecco's modified Eagle medium (DMEM) (Life Technologies, CA) supplemented with 10% foetal bovine serum (FBS) (Life Technologies), 0.5% Fungizone (Life Technologies) and penicillin streptomycin (Life Technologies). Cells were cultured in T-75 flasks and maintained at 37°C in a humidified incubator at 5% CO₂. Culture medium was changed every 2-3 d and cells were sub-cultured when >80% confluent. All experiments were performed in passages 6-11.

6.3.4 *Cell Viability*

Cell viability was measured using the CellTiter Blue® Cell Viability Assay Kit (Promega, Madison, WI). Cells were cultured at a density of 2.5×10^3 cells per well in flat-bottomed 96-well plates and equilibrated for 24 h prior to the addition of varying doses (0-25 µg/mL) of fenugreek extract. Following treatment, CellTiter Blue® reagent was added to each well according to manufacturer's instructions. Cell viability was assessed by measuring the fluorescence excited at 550 nm and read at 600 nm using a SpectraMax Paradigm plate reader (Molecular Devices, Sunnyvale, CA). All assays were performed in quadruplicate and independently repeated twice.

6.3.5 *Creatine Content*

Total cellular creatine content was determined using modified versions of fluorometric determination methods previously described (Odoom, Kemp, and Radda 1996, Conn 1960). Briefly, myoblasts were cultured at a density of 12×10^3 cells per well into 24-well plates. When 80-90% confluent, differentiation of L6C11 myoblasts to myotubes was initiated by replacing the mitogen-rich media with DMEM supplemented with 2% horse

serum (Life Technologies), 0.5% Fungizone and penicillin streptomycin for 4 d. Myotubes underwent treatments made with control DMEM (CON), 0.5 mM creatine (CR), CR and 20 µg/mL fenugreek extract (CR+FEN), CR and 100nM insulin (CR+INS), and CR+INS+FEN for up to 24 h (n=6; obtained from 2 independent experiments). Myotubes were rinsed then lysed with 0.1 M NaOH. Lysates were hydrolysed with 1 M HCl and water at 60°C for 40 min. Freshly prepared 1% ninhydrin and 10% KOH (in EtOH) were subsequently added. Following 8 min incubation at room temperature, fluorescence was excited at 410 nm and read at 525 nm using a SpectraMax Paradigm plate reader. Total creatine content was calculated from a creatine standard curve and further normalized to total cellular protein, as determined using the bicinchoninic acid (BCA)-based protein assay kit (Pierce Biotechnology, IL USA) according to manufacturer's instructions. Absorbance was read at 562 nm.

6.3.6 SDS-PAGE and Immunoblotting Analysis

Myotubes were rinsed twice with 1X PBS before lysis with cell lysis buffer (Cell Signaling Technology, Danvers, MA) supplemented with 1 mM PMSF. Extracts were incubated with continuous rotation at 4°C for 1 h then centrifuged at 13,000 rpm at 4°C for 15 min to pellet cell debris. The supernatant was analysed for total protein and sample protein concentration using a BCA protein assay (Pierce, Rockford, IL, USA). Western blotting was carried out as previously described (Camera et al. 2012). Briefly, lysate was re-suspended in Laemmli sample buffer, separated by SDS-PAGE, and transferred to polyvinylidene fluoride membranes blocked with 5% non-fat milk, washed with 10 mM Tris·HCl, 100 mM NaCl, and 0.02% Tween 20, and incubated with primary antibody (1:1,000) overnight at 4°C on a shaker. Membranes were incubated with secondary antibody (1:2,000), and proteins were detected via enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK; Pierce Biotechnology) and quantified by densitometry

(Chemidoc, BioRad, Gladesville, Australia). All samples for each treatment group were run on the same gel. Phospho-AKT^{Thr308} (no. 4056), total AKT (no. 9272), PKC ζ/λ (no. 9378), and GLUT4 (no. 9001) were from Cell Signaling Technology (Danvers, MA, USA). SLC6A8 (no. AV42248) was from Sigma-Aldrich (St. Louis, MO, USA). Data are expressed relative to α -tubulin (no. 3873; Cell Signaling Technology) in arbitrary units.

6.3.7 Statistical Analysis

All data were analysed by one- or two-way ANOVA with Turkey's post hoc analysis and validated by Mauchly's sphericity test. Statistical significance was established when $P < 0.05$. All data are expressed as arbitrary units \pm standard error of the mean (SEM).

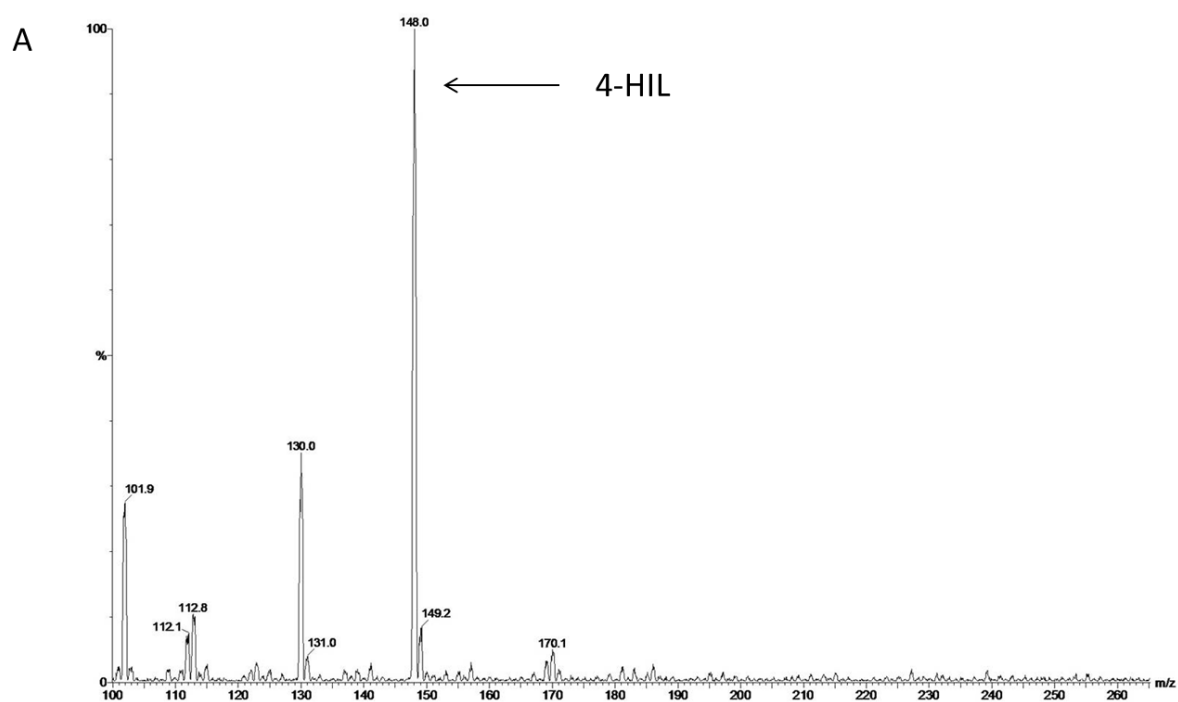
6.4 Results

6.4.1 Extract Sample Quality

To assess the quality of whole fenugreek seed extracts, a comparison of the 4-HIL (a major amino acid with purported insulinotropic properties) concentration between seed extracts and a commercially available fenugreek seed extract was undertaken. Analysis using liquid chromatography-mass spectrometry (LC-MS) determined the 4-HIL component of whole seed fenugreek extract samples to be 50-75 times greater than that of the commercial fenugreek extract. Based on this data, the extract with the greatest 4-HIL concentration (0.303%) was used for all subsequent experiments (Figure 6.3).

6.4.2 Cytotoxicity and Viability in Response to 24 h Fenugreek Treatment

To establish an effective, non-toxic dose of fenugreek extract, cell viability was determined in response to a range of concentrations (0-25 $\mu\text{g/mL}$). L6C11 cells tolerated fenugreek extract at doses up to 20 $\mu\text{g/mL}$ without any reduction in relative fluorescence (indicative of cell number, hence viability). Treatment with 25 $\mu\text{g/mL}$ of fenugreek resulted in a significant decrease in cell number (-37% , $P < 0.05$; Figure 6.4).



B

EXTRACT	Weight % of 4-HIL
Extract 1	0.303
Extract 2	0.204
Extract 3	0.273
Commercial Extract	0.004

Figure 6.3: A liquid chromatography-mass spectroscopy (LC-MS) method developed and validated for the analysis of (2*S*,3*R*,4*S*)- 4-hydroxyisoleucine (4-HIL) in fenugreek seed extract. **(A)** A representative analytical scale of separation and abundance for fenugreek seed extract run on an Electrospray Ionization ESI mass spectrometer. Extract analysis was performed by monitoring the area under the 4-HIL peak at mass/charge of 148 which corresponds to the [4HIL+1] parent ion in the mass spectrum in each extract sample. **(B)** A comparison of the percentage of 4-HIL per extract by weight. A calibration curve was created from 4-HIL standards and fenugreek extract 4-HIL samples. The weight of the 4-HIL content from each fenugreek extract sample was determined using area under the curve and calculated as a percentage.

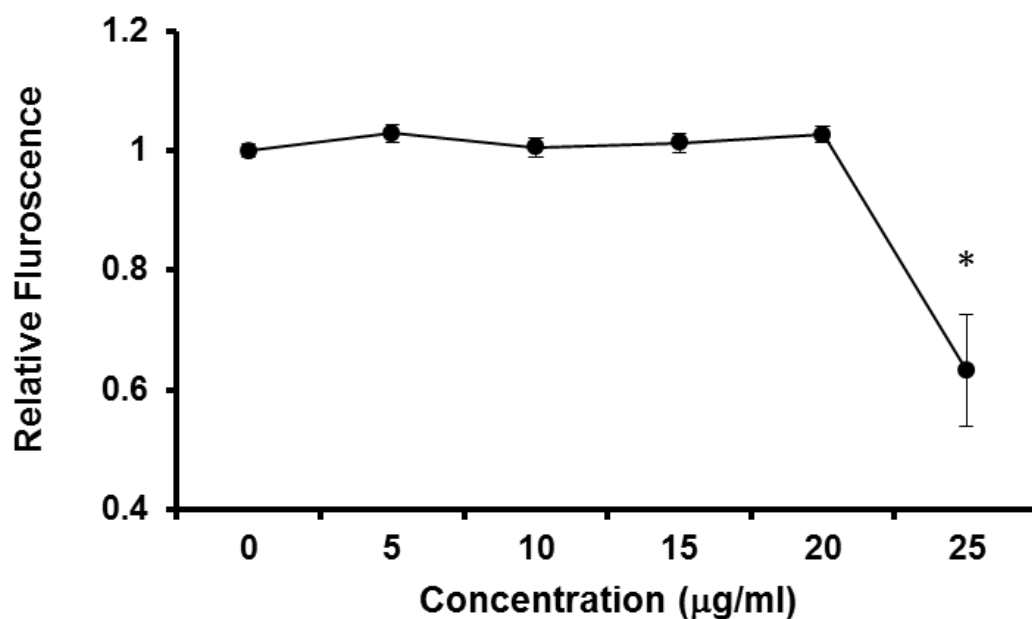


Figure 6.4: Cytotoxic analysis of fenugreek-treated skeletal muscle cells. L6C11 cells were treated with varying concentrations (0-25 µg/ml) of fenugreek seed extract for 24 h to establish a suitable non-toxic dose for use in further experiments. A fluorescence-based cell viability assay was used to estimate viable cell number by measuring fluorescence excited at 550 nm and read at 600 nm. Each point represents the mean \pm SEM of $n=4$ from 2 independent experiments. Statistically significant differences (* $P<0.05$) between control and fenugreek-treated myoblasts were determined by one-way ANOVA.

6.4.3 Fenugreek Increases Total Creatine Content by L6C11 Myotubes

Treatment with CR+INS+FEN induced a significant increase compared to CON in total cellular creatine content at 4 h ($P<0.05$; Figure 6.5). Subsequent pair-wise comparisons identified a significant difference between CR+INS and CR+INS+FEN ($P<0.05$). In contrast, no significant time effects were observed.

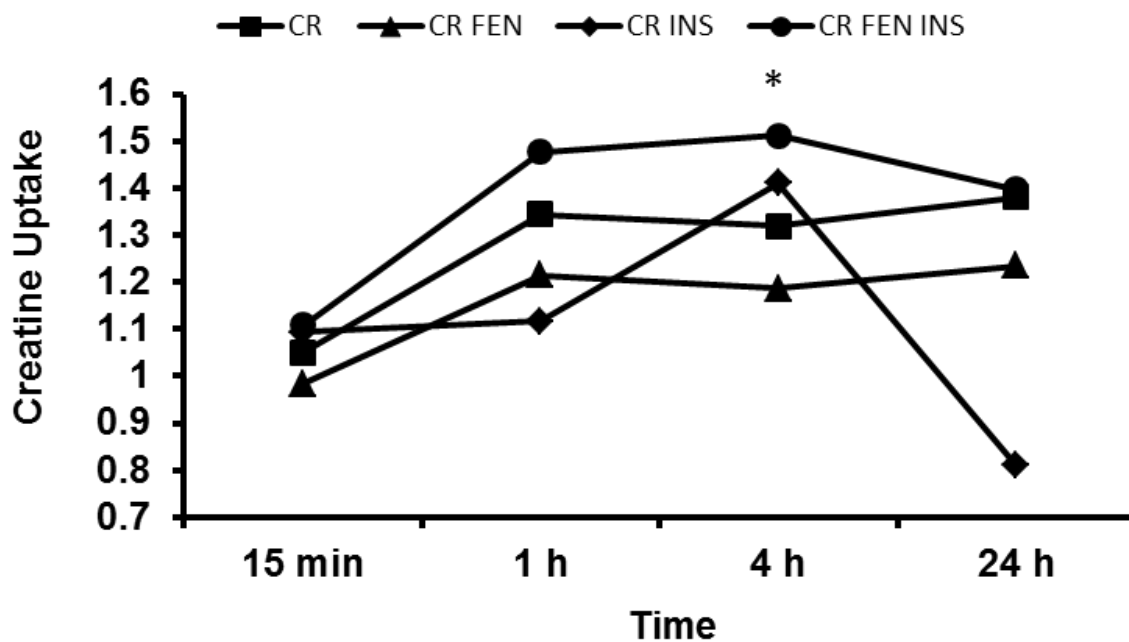


Figure 6.5: Fenugreek increases creatine uptake by skeletal myotubes. Following 4 d of L6C11 cell differentiation, creatine uptake was fluorometrically measured in response to creatine (CR), creatine and fenugreek (CR FEN), creatine and insulin (CR INS) or and creatine, insulin, and fenugreek (CR INS FEN) relative to control. Fluorescence was excited at 410 nm and read at 525 nm. Data points represent the mean of $n=6$ obtained from 2 independent experiments. Statistical significance (* $P<0.05$) was determined by one-way ANOVA with Tukey's post hoc analysis.

6.4.4 Protein Expression

A significant time effect was found between 4 vs. 24 h time points for SLC6A8, PKC ζ/λ , AKT^{Thr308}, and GLUT4 expression ($P<0.05$), 1 vs. 4 h time points for SLC6A8, AKT^{Thr308}, and GLUT4 ($P<0.05$), and 1 vs. 24 h time points for PKC ζ/λ and AKT^{Thr308} ($P<0.05$; Figure 6.6). There was a significant treatment effect observed with insulin for AKT^{Thr308} ($P<0.05$). A significant interaction of treatment and time was also observed for AKT^{Thr308} ($P<0.05$). SLC6A8 protein was increased above CON ($P<0.05$) across all treatments at 4 h (Figure 6.7A). The phosphorylation of AKT^{Thr308} increased with CR+INS above CON and CR at 1 h ($P<0.05$) and with CR+INS+FEN above CON, CR, and CR+INS at 4 h ($P<0.05$; Figure 6.7B). There were no significant changes observed in PKC ζ/λ or GLUT4 protein content (Figure 6.7 C and D).

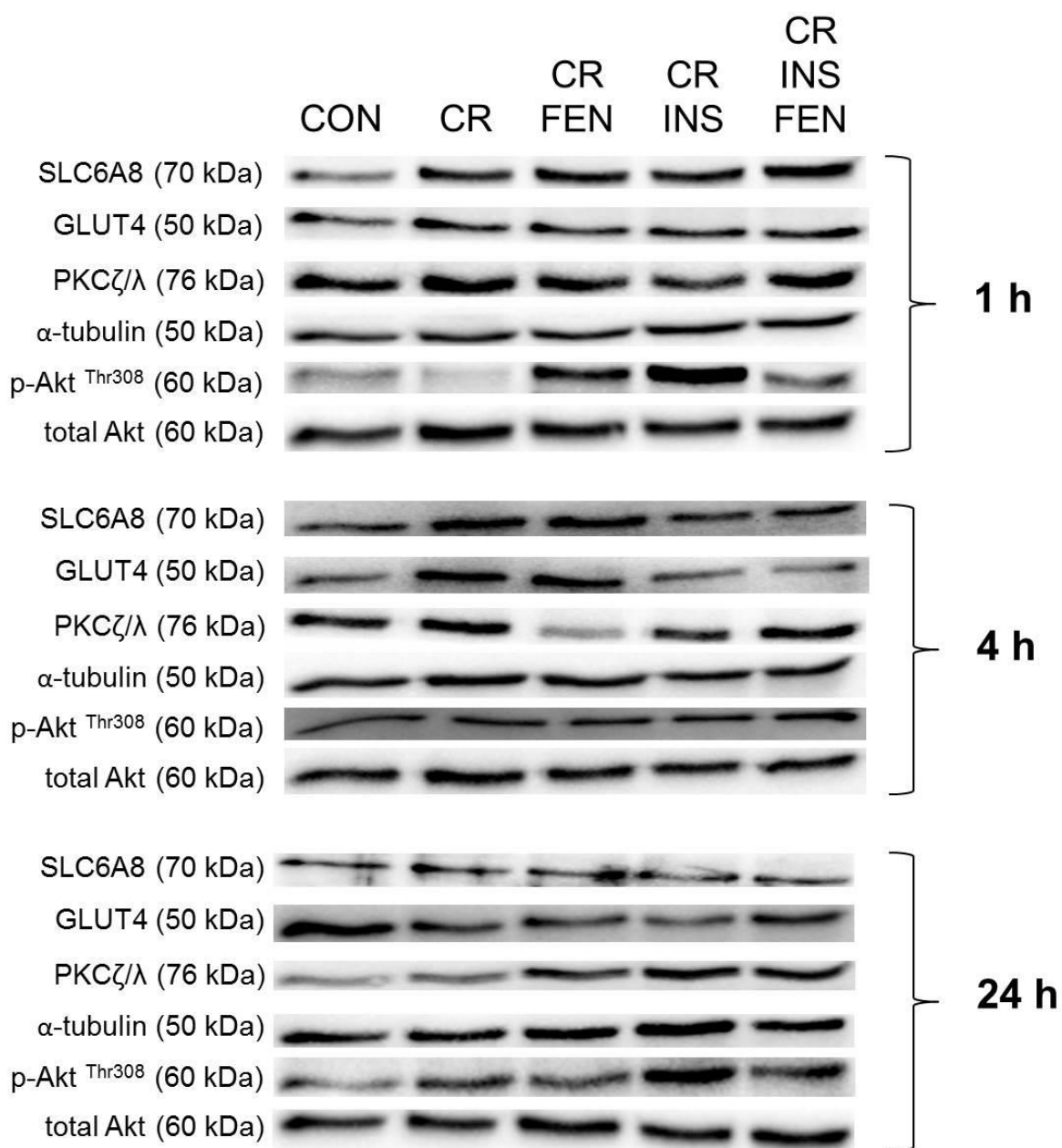


Figure 6.6 Representative Western blots of creatine transporter and insulin signalling proteins. L6C11 myotubes were treated for 1, 4, and 24 h with control (CON), creatine (CR), creatine and fenugreek (CR FEN), creatine and insulin (CR INS) and creatine, insulin, and fenugreek (CR INS FEN) treatments. Membranes were incubated overnight with primary antibody, washed, and incubated with secondary antibody for 1 h before imaging.

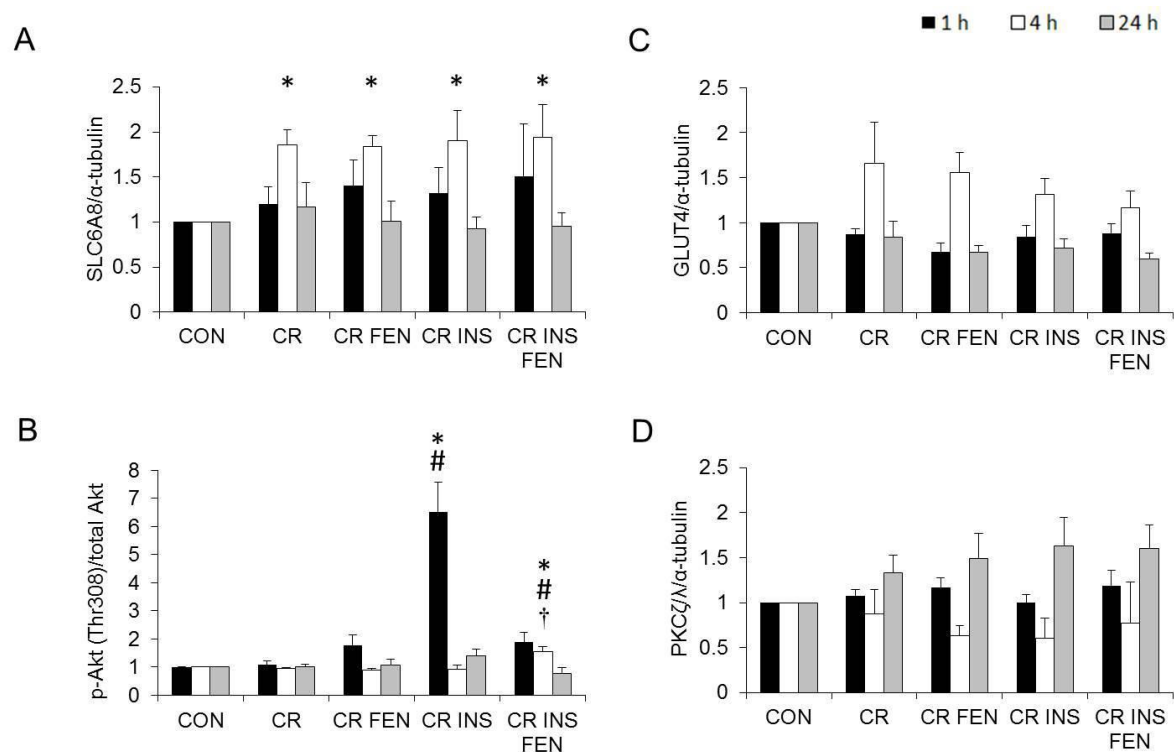


Figure 6.7: The expression of signalling proteins in creatine co-incubated myotubes. The relative protein expression (A) SLC6A8 creatine transporter and key components of the insulin-signalling pathway, (B) AKT^{Thr308} (C) GLUT4, and (D) PKC ζ , were assessed by Western blot in L6C11 myotubes treated for 1, 4, and 24 h. Columns represent the mean \pm SEM of $n=6$ obtained from 2 independent experiments in arbitrary units. Statistically significant ($P < 0.05$) differences between treatments within each time point were determined by one-way ANOVA. * indicates difference vs. CON; # indicates difference vs. CR; and \dagger indicates difference vs. CR+INS

6.5 Discussion

Previous work has shown a variety of creatine supplementation protocols (Green, Hultman, et al. 1996, Greenhaff et al. 1994, Harris, Soderlund, and Hultman 1992) to increase intracellular creatine concentrations toward a maximum ‘threshold’ (Greenhaff 1996). In this study, and in accordance with the first research hypothesis, the current study presents novel data to demonstrate increased total intracellular creatine content in myotubes treated with fenugreek seed extract. This increase in intracellular creatine observed when muscle cells were concomitantly treated with CR+INS+FEN demonstrates an additive effect of fenugreek on increasing creatine content in skeletal muscle. As such, CR+FEN alone was unable to elevate intracellular creatine above those levels observed with CR or CR+INS treated cells.

The creatine dose (0.5 mM) corresponds to the intracellular concentration found in human muscle following a standard 5 g oral dose of creatine (Ceddia and Sweeney 2004). This dose has been demonstrated to elicit maximum creatine uptake in skeletal muscle cells *in vivo* (Sora et al. 1994). The reported insulinotropic effects of fenugreek are largely attributed to the anti-hyperglycaemic 4-HIL, a naturally occurring amino acid that is unique to fenugreek (Ogawa et al. 2011). Sample analysis revealed substantial 4-HIL in the fenugreek seed extracts and is consistent with those obtained in previous studies (Narender et al. 2006). Further, it was demonstrated that a 20 µg/mL dose of fenugreek extract was able to maintain cell viability in the L6C11 cell line in line with previously observed cell viability doses (Shabbeer et al. 2009).

Data pertaining to fenugreek’s interaction with creatine, specifically on creatine storage, however, is limited. Work undertaken by Taylor *et al.* (2011) in humans demonstrated that supplementation with 900 mg of fenugreek combined with 3.5 g of creatine stimulates significant increases in maximum strength measures and increased lean

mass over an 8-week training period (Taylor et al. 2011). However, in that study, no direct measures of creatine content (either via muscle biopsies or magnetic resonance spectroscopy) were undertaken to determine whether their dosing protocol was effective in increasing creatine content in skeletal muscle. It is, therefore, difficult to attribute the changes in strength and body composition to fenugreek's supposed effect on modulating intracellular creatine stores.

The present study hypothesized a mechanism whereby fenugreek-driven increases in creatine concentration would be attributable to increased SLC6A8 activity. Skeletal muscle co-incubation with 0.5 mM creatine and fenugreek seed extract combined with 100 nM insulin elevated total intracellular creatine content. Creatine transporter expression appears to peak at the 4 h time point with all creatine-containing conditions (CR, CR+INS, CR+FEN, and CR+INS+FEN) significantly increasing the expression of SLC6A8 above basal levels. However, at this time point, neither CR+FEN nor CR+INS elicited any further increase in SLC6A8 expression above that of CR alone. Based on these observations, and in accordance with previous work (Willott et al. 1999), the current investigation cannot attribute a role for the involvement of the SLC6A8 transporter in insulin-mediated or insulin-mimicked creatine content augmentation at this time point. Rather, the present data suggest that either 1) fenugreek does not affect creatine uptake via the SLC6A8 transporter in L6C11 skeletal muscle fibres, or 2) the creatine dose used may have maximally up-regulated SLC6A8 expression, thereby masking any potential effects of fenugreek on increased creatine uptake via this creatine transporter (Daly and Seifter 1980). Furthermore, it may be possible that SLC6A8 up-regulation is affected by the activity of other transmembrane proteins. Work by Almilaji *et al.* (2014) determined that the transmembrane protein Klotho, which provides control of insulin sensitivity to an organism, upregulates SLC6A8 expression by stabilizing the transport protein in the cell membrane (Almilaji et al. 2014).

It was also hypothesized that insulin signalling proteins may act as regulatory intermediates by which fenugreek stimulates increased creatine content. Canonical insulin signalling is characterized by the binding and subsequent recruitment of the PI3K subunit to the plasma membrane (Oldham and Hafen 2003). Broca *et al.* (2000) demonstrated that an acute dose of 4-HIL extracted from fenugreek seeds activates PI3-K in the liver and muscle cells of both diabetic and non-diabetic rats, significantly reducing insulin resistance through the activation of early steps in insulin signalling (Broca *et al.* 2004). Jaiswal *et al.* (2012) further examined the effect of 4-HIL on glucose uptake and translocation of GLUT4 to the plasma membrane in L6 skeletal muscle cells (Jaiswal *et al.* 2012). Their findings showed that 4-HIL stimulated glucose uptake by enhancing the translocation of GLUT4 to the cell surface via a PI-3-kinase/AKT-dependent mechanism (Jaiswal *et al.* 2012). Based on this information, the present study determined the effects of fenugreek treatment on selected pathways downstream of PI3-K, specifically on AKT signalling substrates. Previous work has shown that fenugreek does not activate AKT at the Ser⁴⁷³ residue (Vijayakumar *et al.* 2005) and, accordingly, phosphorylation on the Thr³⁰⁸ site was measured due to its known activation by insulin (Kim *et al.* 1999). As expected, AKT^{Thr308} levels were significantly elevated in response to CR+INS at 1 h. Additionally, CR+INS+FEN significantly increased AKT^{Thr308} expression at 4 h. However, in contrast to one of the original hypotheses, no additive effect on AKT^{Thr308} expression was observed with CR+FEN treatment alone across any of the time points tested. It has previously been observed that treatment with fenugreek has little effect on AKT expression in both hepatocytes and adipocytes (Vijayakumar *et al.* 2005). Furthermore, creatine (21 g for 5 d) has been demonstrated to significantly reduce AKT^{Thr308} phosphorylation in resting skeletal muscle samples (Deldicque *et al.* 2008). The presence of creatine, in the absence of insulin co-incubation, may help explain the low levels of phosphorylated AKT at the Thr³⁰⁸ residue with CR and CR+FEN treatments.

Insulin signalling occurs via two divergent pathways: one that is AKT-dependent; and the other that is PKC-dependent. In the absence of altered AKT expression in response to treatment with fenugreek, the current study sought to determine whether phosphorylation of PKC protein was responsible for the observed increase in creatine content. However, no significant change in PKC ζ/λ phosphorylation was seen in response to fenugreek. This finding is in contrast to those of Vijayakumar *et al.* (2005) who found that fenugreek seed extract induces PI3-K dependent activation of the PKC λ isoform in cultured hepatocytes and adipocytes (Vijayakumar et al. 2005). It may be that PKC signalling is dependent on the cell line under investigation, and that the differing metabolic processes and requirements of skeletal muscle cells may account for the observed lack of PKC expression in response to fenugreek treatment.

In conclusion, CR+INS+FEN treatment results in a greater degree of total intracellular creatine content than CR and CR+INS treatments in L6C11 myotubes. However, the increased creatine levels following fenugreek treatment alone occurred independently of changes in the phosphorylation state of both AKT and PKC signalling. Furthermore, fenugreek modulated total intramuscular creatine levels independent of changes in SLC6A8 expression, suggesting the presence of a yet to be identified mechanism of fenugreek regulation in this process. Fenugreek, in combination with creatine and insulin, induces changes in AKT^{Thr308}, offering avenues for future investigation of fenugreek's therapeutic benefits. Based on previous *in vivo* research (Taylor et al. 2011) on the efficacy of creatine and fenugreek co-supplementation on athletic performance, it seems reasonable to hypothesize that a combination of creatine loading, fenugreek supplementation, and a high carbohydrate diet may further increase intracellular creatine storage. The *in vitro* results demonstrate that compounds which mimic insulin's action can further stimulate intramuscular creatine content. These results may provide a platform for future human

studies to determine whether this increased storage translates to further increases in muscle mass, resulting in increased strength for athletes or, on a wider scale, potential attenuation of muscle loss in at risk populations (i.e. sarcopenia).

CHAPTER 7: General Discussion, Limitations and Conclusions

The plasticity of skeletal muscle allows it to alter its phenotype in response to repeated contraction- and nutrition-induced perturbations to cellular homeostasis. To date, the majority of research investigating the ergogenic effects of creatine has been undertaken *in vivo* with a focus on increasing intramuscular substrate stores to enhance exercise capacity. While resistance-based training has been the principal exercise modality studied with respect to creatine supplementation, evidence of an insulin-mediated enhancement of both glycogen and creatine stores in muscle suggests a plausible application of creatine for endurance-based activities. Hence, the primary aims of the studies undertaken for this thesis were to investigate the effects of altered energy substrate availability, both *in vivo* (human) and *in vitro* (mouse and rat cell lines), with specific attention to the effect of creatine availability on skeletal muscle metabolism.

The results of Study I (Chapter Four) showed that creatine and carbohydrate co-supplementation enhanced power output during maximal intensity efforts undertaken during/at the end of a 120 km cycling time trial protocol. This increase in power output was 1) independent of nutrient-induced weight gain that has been previously associated with carbohydrate and creatine loading and 2) independent of increased intramuscular glycogen concentrations. Elevated intramuscular creatine content was, however, associated with increased carbohydrate intake. While changes in selected signalling proteins measured showed no major changes between creatine and placebo groups, a reduction in mTOR was observed following the glycogen depleting time trial and treadmill endurance task. Based on the outcomes of Study I, reservations about using creatine supplementation for endurance events due to a fear of detrimental performance arising from weight gain may be undeserved.

Future applications of creatine in endurance-based activities should focus on athletes participating in events which require intense, short-duration bouts of exercise necessary for “breaking away” from opponents. If timed correctly, these sprints may determine the

outcome of closely contested endurance events (i.e. sprint to the finish line). Creatine supplementation may need to be timed cautiously, however, directly prior to events in which weight classification is critical for competition, such as powerlifting and combat sports, due to the inherent weight gain associated with creatine supplementation.

There were several limitations to Study I. First, the number of biopsies limited the scope of analysis that could be undertaken. A post-time trial biopsy would have allowed for measures of creatine utilisation during the cycling protocol and also provided greater insight into the signalling effects brought about by prolonged endurance exercise under different nutritional conditions. Furthermore, the limited and often inconsistent quantity of muscle sample obtained from biopsies restricted the biochemical analysis that could be undertaken. The absence of intravenous blood samples prevented measurement of metabolites, such as lactate, which may have confirmed the effectiveness of creatine treatment in sparing glycogen breakdown.

To further investigate the combined effect of creatine and carbohydrates, a cell culture model was developed for Studies II and III to provide a controlled *in vitro* environment in which to test the metabolic impact of substrate co-supplementation strategies. In order to simulate *in vivo* conditions as close as possible, Study II (Chapter Five) utilised established murine myotubes as a model for a 2 wk creatine and insulin treatment with low- or high-glucose concentrations. The results showed that culture media supplemented with creatine supported a continual increase in myofibre size. Creatine co-treated with insulin provided further impetus for myotube development as observed in the increased expression of contractile protein myosin heavy chain. However, myotubes treated with insulin were smaller in size but greater in density (i.e. quantity). Both of these outcomes were largely independent of glucose availability. These results suggest the presence of parallel, yet

synergistic, mechanisms in which insulin's anabolic role amplifies creatine's ability to stimulate hypertrophy while also supporting the fusion of differentiated myoblasts.

Both creatine and glucose transport into muscle is mediated by insulin. As the findings from Study II demonstrated, insulin can augment skeletal muscle adaptations arising from creatine supplementation *in vitro*. In humans, however, the major stimulus for the release of insulin from the pancreases is the presence of glucose in the bloodstream. Previous *in vivo* research has identified not only clinical benefits arising from compounds which mimic insulin's regulatory role on blood glucose but also a substantial influence on augmenting creatine's effect on body composition and strength. Utilising similar *in vitro* methodology to Study II, the final study of this thesis (Chapter Six) compared the role of insulin and the natural insulin mimetic fenugreek on creatine uptake. Fenugreek alone did not facilitate greater creatine uptake. However, it did have an additive effect on increasing total intramuscular creatine concentrations in rat myotubes which were also co-incubated with creatine and insulin. While the precise mechanism of action could not be determined, it was identified that fenugreek's action occurs independent of creatine transporter protein expression, suggesting that an alternative mechanism and/or earlier time course of action is responsible for the regulation of insulin-mediated creatine uptake *in vitro*. This finding aligns with the observations of Study II. Inherently, the ability to translate the *in vitro* findings in Study II and III to *in vivo* applications is the primary limitation of tissue culture research. Nevertheless, *in vitro* studies provide a mechanistic framework for future *in vivo* clinical work.

Taken collectively, the work presented for this thesis provides novel insight into creatine's application not only as an ergogenic aid but also as a potential therapeutic compound for the maintenance of muscle mass. Future research should examine the roles of creatine and insulin as agents that could stimulate muscle hypertrophy, particularly in

populations prone to muscle loss (i.e. sarcopenia). Tight regulation of muscle protein turnover (breakdown vs synthesis) is crucial for the prevention of muscle atrophy. In addition to resistance exercise and protein availability, future research on creatine supplementation may further enhance or, at the very least, help maintain total body muscle mass with particular respect to age-related muscle loss, disease states, such as cancer cachexia, or allow for the establishment of muscle regeneration *in vitro* for transplant purposes. Furthermore, a number of studies have implicated fenugreek as a therapeutic agent for the treatment of diabetes. In light of recent evidence on creatine's beneficial effects in type-2 diabetic populations compounded with the findings of Study III, further clinical testing should be employed to further explore creatine and fenugreek's interactive effects and mechanisms which may be beneficial in the treatment of this disease. In this regard, supplementation with fenugreek and/or creatine may serve as a precursor to pharmaceutical-based therapies for the treatment of diabetes or other muscle-effected disorders.

In conclusion, an abundance of research has been performed to evaluate the efficacy of creatine and creatine supplementation strategies on sports performance and contraction-induced increase in hypertrophy. In athletic populations, evidence suggested that creatine's ergogenic properties could be enhanced by co-supplementation with carbohydrate, with particular benefits seen in resistance trained athletes. Such evidence warranted a more thorough investigation into the application of creatine with carbohydrate which was not limited to one modality of exercise, but rather expanded the scope of this synergistic relationship to endurance trained athletes. In applying this strategy to an *in vitro* model investigating the impact of nutritionally-induced cellular perturbations, a better understanding of the role of creatine on muscle growth was identified, which was highlighted by the presence of other anabolic substrates. Thus, in light of emerging multi-disciplinary research, muscle-targeted therapies remain a promising future endeavour for creatine-based

health research. The work completed over the course of this thesis contributes new evidence into the effectiveness of creatine interventions not only in improving athletic performance but also as an aid in promoting muscle growth and development.

CHAPTER 8: References

- Adams, G. R., F. Haddad, and K. M. Baldwin. 1999. "Time course of changes in markers of myogenesis in overloaded rat skeletal muscles." *J Appl Physiol* (1985) 87 (5):1705-12.
- Al-Jasass, Fahad Mohammed, and Mohammed Saud Al-Jasser. 2012. "Chemical composition and fatty acid content of some spices and herbs under Saudi Arabia conditions." *The Scientific World Journal* 2012.
- Alfieri, R. R., M. A. Bonelli, A. Cavazzoni, M. Brigotti, C. Fumarola, P. Sestili, P. Mozzoni, G. De Palma, A. Mutti, D. Carnicelli, F. Vacondio, C. Silva, A. F. Borghetti, K. P. Wheeler, and P. G. Petronini. 2006. "Creatine as a compatible osmolyte in muscle cells exposed to hypertonic stress." *Journal of Physiology-London* 576 (2):391-401. doi: 10.1113/jphysiol.2006.115006.
- Almilaji, Ahmad, Mentor Sopjani, Bernat Elvira, Jose Borrás, Miribane Dermaku-Sopjani, Carlos Munoz, Jamshed Warsi, Undine E Lang, and Florian Lang. 2014. "Upregulation of the creatine transporter Slc6A8 by Klotho." *Kidney and Blood Pressure Research* 39 (6):516-525.
- Anderson, Judy E. 2000. "A role for nitric oxide in muscle repair: nitric oxide-mediated activation of muscle satellite cells." *Molecular biology of the cell* 11 (5):1859-1874.
- Areta, José L, John A Hawley, Ji-Ming Ye, MH Stanley Chan, and Vernon G Coffey. 2014. "Increasing leucine concentration stimulates mechanistic target of rapamycin signaling and cell growth in C2C12 skeletal muscle cells." *Nutrition research* 34 (11):1000-1007.
- Arkinstall, Melissa J, Clinton R Bruce, Sally A Clark, Caroline A Rickards, Louise M Burke, and John A Hawley. 2004. "Regulation of fuel metabolism by preexercise muscle glycogen content and exercise intensity." *Journal of applied physiology* 97 (6):2275-2283.
- Atherton, Philip J, J Babraj, Kenneth Smith, Jaipaul Singh, Michael J Rennie, and Henning Wackerhage. 2005. "Selective activation of AMPK-PGC-1 α or PKB-TSC2-mTOR signaling can explain specific adaptive responses to endurance or resistance training-like electrical muscle stimulation." *The FASEB journal* 19 (7):786-788.
- Baar, Keith, and Karyn Esser. 1999. "Phosphorylation of p70S6k correlates with increased skeletal muscle mass following resistance exercise." *American Journal of Physiology-Cell Physiology* 276 (1):C120-C127.
- Baker, Lindsay B, and Asker E Jeukendrup. 2014. "Optimal Composition of Fluid-Replacement Beverages." *Comprehensive Physiology*.
- Ball, Eric H, and BD Sanwal. 1980. "A synergistic effect of glucocorticoids and insulin on the differentiation of myoblasts." *Journal of cellular physiology* 102 (1):27-36.
- Balsom, Paul D, Karin Söderlund, and Björn Ekblom. 1994. "Creatine in humans with special reference to creatine supplementation." *Sports Medicine* 18 (4):268-280.
- Balsom, PD, SDR Harridge, K Soderlund, B Sjodin, and B Ekblom. 1993. "Creatine supplementation per se does not enhance endurance exercise performance." *Acta Physiologica Scandinavica* 149 (4):521-523.
- Balsom, PD, K Söderlund, B Sjodin, and B Ekblom. 1995. "Skeletal muscle metabolism during short duration high-intensity exercise: influence of creatine supplementation." *Acta Physiologica Scandinavica* 154 (3):303-310.
- Berg, Jeremy M, John L Tymoczko, and Lubert Stryer. 2002. "Fuel Choice During Exercise Is Determined by Intensity and Duration of Activity."
- Bergstrom, J. 1975. "Percutaneous needle biopsy of skeletal muscle in physiological and clinical research." *Scand J Clin Lab Invest* 35 (7):609-16.

- Bergström, Jonas, Lars Hermansen, Eric Hultman, and Bengt Saltin. 1967. "Diet, muscle glycogen and physical performance." *Acta Physiologica Scandinavica* 71 (2-3):140-150.
- Birch, R, D Noble, and PL Greenhaff. 1994. "The influence of dietary creatine supplementation on performance during repeated bouts of maximal isokinetic cycling in man." *European journal of applied physiology and occupational physiology* 69 (3):268-270.
- Bodine, Sue C, Trevor N Stitt, Michael Gonzalez, William O Kline, Gretchen L Stover, Roy Bauerlein, Elizabeth Zlotchenko, Angus Scrimgeour, John C Lawrence, and David J Glass. 2001. "Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo." *Nature cell biology* 3 (11):1014-1019.
- Bosco, C, J Tihanyi, J Pucspk, I Kovacs, A Gabossy, R Colli, G Pulvirenti, C Tranquilli, C Foti, and M Viru. 1997. "Effect of oral creatine supplementation on jumping and running performance." *International Journal of Sports Medicine* 18 (05):369-372.
- Braissant, Olivier, Hugues Henry, Marc Loup, Barbara Eilers, and Claude Bachmann. 2001. "Endogenous synthesis and transport of creatine in the rat brain: an in situ hybridization study." *Molecular brain research* 86 (1):193-201.
- Branch, J David. 2003. "Effect of creatine supplementation on body composition and performance: a meta-analysis." *International journal of sport nutrition and exercise metabolism* 13:198-226.
- Brannon, Tobin A, Gregory R Adams, Cory L Conniff, and Kenneth M Baldwin. 1997. "Effects of creatine loading and training on running performance and biochemical properties of rat skeletal muscle." *Medicine and science in sports and exercise* 29 (4):489-495.
- Broca, Christophe, Vincent Breil, Céline Cruciani-Guglielmacci, Michèle Manteghetti, Christine Rouault, Michel Derouet, Salwa Rizkalla, Bernard Pau, Pierre Petit, and Gérard Ribes. 2004. "Insulinotropic agent ID-1101 (4-hydroxyisoleucine) activates insulin signaling in rat." *American Journal of Physiology-Endocrinology And Metabolism* 287 (3):E463-E471.
- Brooks, George Austin, Thomas Davin Fahey, and Timothy P White. 1996. *Exercise physiology: Human bioenergetics and its applications*: Mayfield publishing company.
- Brosnan, John T, and Margaret E Brosnan. 2007. "Creatine: endogenous metabolite, dietary, and therapeutic supplement." *Annu. Rev. Nutr.* 27:241-261.
- Burattini, S, P Ferri, M Battistelli, R Curci, F Luchetti, and E Falcieri. 2004. "C2C12 murine myoblasts as a model of skeletal muscle development: morpho-functional characterization." *European journal of histochemistry: EJH* 48 (3):223.
- Burattini, S, P Ferri, M Battistelli, R Curci, F Luchetti, and E Falcieri. 2009. "C2C12 murine myoblasts as a model of skeletal muscle development: morpho-functional characterization." *European Journal of Histochemistry* 48 (3):223-234.
- Burke, Louise M, John A Hawley, Elske J Schabort, Alan St Clair Gibson, Iñigo Mujika, and Timothy D Noakes. 2000. "Carbohydrate loading failed to improve 100-km cycling performance in a placebo-controlled trial." *Journal of Applied Physiology* 88 (4):1284-1290.
- Burke, Louise M, Luc JC van Loon, and John A Hawley. 2016. "Post-exercise muscle glycogen resynthesis in humans." *Journal of Applied Physiology:jap*. 00860.2016.
- Camera, Donny M, Daniel WD West, Nicholas A Burd, Stuart M Phillips, Andrew P Garnham, John A Hawley, and Vernon G Coffey. 2012. "Low muscle glycogen

- concentration does not suppress the anabolic response to resistance exercise." *Journal of Applied Physiology* 113 (2):206-214.
- Casey, A, D Constantin-Teodosiu, S Howell, EGPL Hultman, and PL Greenhaff. 1996. "Creatine ingestion favorably affects performance and muscle metabolism during maximal exercise in humans." *American Journal of Physiology-Endocrinology And Metabolism* 34 (1):E31.
- Casey, A, AH Short, E Hultman, and PL Greenhaff. 1995. "Glycogen resynthesis in human muscle fibre types following exercise-induced glycogen depletion." *The Journal of physiology* 483 (1):265-271.
- Ceddia, Rolando B, and Gary Sweeney. 2004. "Creatine supplementation increases glucose oxidation and AMPK phosphorylation and reduces lactate production in L6 rat skeletal muscle cells." *The Journal of physiology* 555 (2):409-421.
- Charge, Sophie BP, and Michael A Rudnicki. 2004. "Cellular and molecular regulation of muscle regeneration." *Physiological reviews* 84 (1):209-238.
- Chen, Nian-Hang, Maarten EA Reith, and Michael W Quick. 2004. "Synaptic uptake and beyond: the sodium-and chloride-dependent neurotransmitter transporter family SLC6." *Pflügers Archiv* 447 (5):519-531.
- Christie, David L. 2007. "Functional insights into the creatine transporter." In *Creatine and creatine kinase in health and disease*, 99-118. Springer.
- Coffey, Vernon G, and John A Hawley. 2007. "The molecular bases of training adaptation." *Sports medicine* 37 (9):737-763.
- Collins, Charlotte A, Viola F Gnocchi, Robert B White, Luisa Boldrin, Ana Perez-Ruiz, Frederic Relaix, Jennifer E Morgan, and Peter S Zammit. 2009. "Integrated functions of Pax3 and Pax7 in the regulation of proliferation, cell size and myogenic differentiation." *PloS one* 4 (2):e4475.
- Collins, Charlotte A, Irwin Olsen, Peter S Zammit, Louise Heslop, Aviva Petrie, Terence A Partridge, and Jennifer E Morgan. 2005. "Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche." *Cell* 122 (2):289-301.
- Conn, Rex B. 1960. "Fluorimetric determination of creatine." *Clinical chemistry* 6 (6):537-548.
- Cooper, Robert, Fernando Naclerio, Judith Allgrove, and Alfonso Jimenez. 2012. "Creatine supplementation with specific view to exercise/sports performance: an update." *Journal of the International Society of Sports Nutrition* 9 (1):33.
- Crameri, Regina M, Henning Langberg, Peter Magnusson, Charlotte H Jensen, Henrik Daa Schrøder, Jens L Olesen, Charlotte Suetta, Børge Teisner, and Michael Kjaer. 2004. "Changes in satellite cells in human skeletal muscle after a single bout of high intensity exercise." *The Journal of physiology* 558 (1):333-340.
- Crim, Marilyn C, DH Calloway, and Sheldon Margen. 1976. "Creatine metabolism in men: creatine pool size and turnover in relation to creatine intake." *J nutr* 106:371-381.
- Cullen, Martin E, Ada HY Yuen, Leanne E Felkin, Ryszard T Smolenski, Jennifer L Hall, Suzanne Grindle, Leslie W Miller, Emma J Birks, Magdi H Yacoub, and Paul JR Barton. 2006. "Myocardial Expression of the Arginine: Glycine Amidinotransferase Gene Is Elevated in Heart Failure and Normalized After Recovery Potential Implications for Local Creatine Synthesis." *Circulation* 114 (1 suppl):I-16-I-20.
- Cunningham, John T, Joseph T Rodgers, Daniel H Arlow, Francisca Vazquez, Vamsi K Mootha, and Pere Puigserver. 2007. "mTOR controls mitochondrial oxidative function through a YY1-PGC-1α transcriptional complex." *nature* 450 (7170):736-740.

- Dai, Wenxuan, Shyamala Vinnakota, Xiaojun Qian, Diana L Kunze, and Hemanta K Sarkar. 1999. "Molecular Characterization of the Human CRT-1 Creatine Transporter Expressed in *Xenopus* Oocytes." *Archives of Biochemistry and Biophysics* 361 (1):75-84.
- Daly, Marie M, and Sam Seifter. 1980. "Uptake of creatine by cultured cells." *Archives of biochemistry and biophysics* 203 (1):317-324.
- Dangott, B, E Schultz, and PE Mozdziak. 2000. "Dietary creatine monohydrate supplementation increases satellite cell mitotic activity during compensatory hypertrophy." *International journal of sports medicine* 21 (01):13-16.
- Delanghe, J, JP De Slypere, M De Buyzere, J Robbrecht, R Wieme, and A Vermeulen. 1989. "Normal reference values for creatine, creatinine, and carnitine are lower in vegetarians." *Clinical chemistry* 35 (8):1802-1803.
- Deldicque, Louise, Philip Atherton, Rekha Patel, Daniel Theisen, Henri Nielens, Michael J Rennie, and Marc Francaux. 2008. "Effects of resistance exercise with and without creatine supplementation on gene expression and cell signaling in human skeletal muscle." *Journal of Applied Physiology* 104 (2):371-378.
- Deldicque, Louise, Daniel Theisen, Luc Bertrand, Peter Hespel, Louis Hue, and Marc Francaux. 2007. "Creatine enhances differentiation of myogenic C2C12 cells by activating both p38 and Akt/PKB pathways." *American Journal of Physiology-Cell Physiology* 293 (4):C1263-C1271.
- Dumont, Nicolas A, Yu Xin Wang, and Michael A Rudnicki. 2015. "Intrinsic and extrinsic mechanisms regulating satellite cell function." *Development* 142 (9):1572-1581.
- Earnest, Conrad P, Anthony L Almada, and Tedd L Mitchell. 1997. "Effects of Creatine Monohydrate Ingestion on Intermediate Duration Anaerobic Treadmill Running to Exhaustion." *The Journal of Strength & Conditioning Research* 11 (4):234-238.
- Ebert, Tammie R, David T Martin, Nicola Bullock, Iñigo Mujika, Marc J Quod, Lesley A Farthing, Louise M Burke, and Robert T Withers. 2007. "Influence of hydration status on thermoregulation and cycling hill climbing." *Medicine and science in sports and exercise* 39 (2):323-329.
- Eichler, Evan E, Fei Lu, Ying Shen, Rachele Antonacci, Vesna Jurecic, Norman A Doggett, Robert K Moyzis, Antonio Baldini, Richard A Gibbs, and David L Nelson. 1996. "Duplication of a gene-rich cluster between 16p11. 1 and Xq28: a novel pericentromeric-directed mechanism for paralogous genome evolution." *Human molecular genetics* 5 (7):899-912.
- Elkalaf, Moustafa, Michal Anděl, and Jan Trnka. 2013. "Low glucose but not galactose enhances oxidative mitochondrial metabolism in C2C12 myoblasts and myotubes." *PloS one* 8 (8):e70772.
- Faber, Rachel M, John K Hall, Jeffrey S Chamberlain, and Glen B Banks. 2014. "Myofiber branching rather than myofiber hyperplasia contributes to muscle hypertrophy in mdx mice." *Skeletal muscle* 4 (1):1.
- Farrell, Peter A, Jazmir M Hernandez, Mark J Fedele, Thomas C Vary, Scot R Kimball, and Leonard S Jefferson. 2000. "Eukaryotic initiation factors and protein synthesis after resistance exercise in rats." *Journal of Applied Physiology* 88 (3):1036-1042.
- Feldman, Elaine B. 1999. "Creatine: a dietary supplement and ergogenic aid." *Nutrition reviews* 57 (2):45-50.
- Fitch, CD. 1977. "Significance of abnormalities of creatine metabolism." In *Pathogenesis of human muscular dystrophies*, 328-336. Excerpta Medica Amsterdam.
- Florini, James R, Daina Z Ewton, Susan L Falen, and Judson J Van Wyk. 1986. "Biphasic concentration dependency of stimulation of myoblast differentiation by

- somatomedins." *American Journal of Physiology-Cell Physiology* 250 (5):C771-C778.
- Francaux, Marc, and JR Poortmans. 1999. "Effects of training and creatine supplement on muscle strength and body mass." *European journal of applied physiology and occupational physiology* 80 (2):165-168.
- Fridén, Jan, Jan Seger, and Björn Eklom. 1985. "Implementation of periodic acid-thiosemicarbazide-silver proteinate staining for ultrastructural assessment of muscle glycogen utilization during exercise." *Cell and tissue research* 242 (1):229-232.
- Fry, Andrew C. 2004. "The role of resistance exercise intensity on muscle fibre adaptations." *Sports medicine* 34 (10):663-679.
- Fry, Christopher S, Erin L Glynn, Micah J Drummond, Kyle L Timmerman, Satoshi Fujita, Takashi Abe, Shaheen Dhanani, Elena Volpi, and Blake B Rasmussen. 2010. "Blood flow restriction exercise stimulates mTORC1 signaling and muscle protein synthesis in older men." *Journal of Applied Physiology* 108 (5):1199-1209.
- Fry, Christopher S, Jonah D Lee, Janna R Jackson, Tyler J Kirby, Shawn A Stasko, Honglu Liu, Esther E Dupont-Versteegden, John J McCarthy, and Charlotte A Peterson. 2014. "Regulation of the muscle fiber microenvironment by activated satellite cells during hypertrophy." *The FASEB Journal* 28 (4):1654-1665.
- Fry, Christopher S, Brian Noehren, Jyothi Mula, Margo F Ubele, Philip M Westgate, Philip A Kern, and Charlotte A Peterson. 2014. "Fibre type-specific satellite cell response to aerobic training in sedentary adults." *The Journal of physiology* 592 (12):2625-2635.
- Garami, Attila, Fried JT Zwartkruis, Takahiro Nobukuni, Manel Joaquin, Marta Roccio, Hugo Stocker, Sara C Kozma, Ernst Hafen, Johannes L Bos, and George Thomas. 2003. "Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2." *Molecular cell* 11 (6):1457-1466.
- Gardner, Samantha, Sean M Gross, Larry L David, John E Klimek, and Peter Rotwein. 2015. "Separating myoblast differentiation from muscle cell fusion using IGF-I and the p38 MAP kinase inhibitor SB202190." *American Journal of Physiology-Cell Physiology* 309 (7):C491-C500.
- Glass, David J. 2003. "Signalling pathways that mediate skeletal muscle hypertrophy and atrophy." *Nature cell biology* 5 (2):87-90.
- Goforth, Harold W, Brad L Bennett, and Patricia G Law. 1997. "Persistence of supercompensated muscle glycogen in trained subjects after carbohydrate loading." *Journal of Applied Physiology* 82 (1):342-347.
- Gollnick, PD, Karin Piehl, and B Saltin. 1974. "Selective glycogen depletion pattern in human muscle fibres after exercise of varying intensity and at varying pedalling rates." *The Journal of physiology* 241 (1):45.
- Grabiec, K, M Gajewska, M Milewska, M Błaszczyk, and K Grzelkowska-Kowalczyk. 2014. "The influence of high glucose and high insulin on mechanisms controlling cell cycle progression and arrest in mouse C2C12 myoblasts: the comparison with IGF-I effect." *Journal of endocrinological investigation* 37 (3):233-245.
- Graham, TE, Z Yuan, AK Hill, and RJ Wilson. 2010. "The regulation of muscle glycogen: the granule and its proteins." *Acta physiologica* 199 (4):489-498.
- Green, A. L., E. Hultman, I. A. Macdonald, D. A. Sewell, and P. L. Greenhaff. 1996. "Carbohydrate ingestion augments skeletal muscle creatine accumulation during creatine supplementation in humans." *Am J Physiol* 271 (5 Pt 1):E821-6.
- Green, AL, EJ Simpson, JJ Littlewood, IA Macdonald, and PL Greenhaff. 1996. "Carbohydrate ingestion augments creatine retention during creatine feeding in humans." *Acta Physiologica Scandinavica* 158 (2):195-202.

- Greenhaff, P. L. 1996. "Creatine supplementation: recent developments." *Br J Sports Med* 30 (4):276-7.
- Greenhaff, P. L. 2001. "The creatine-phosphocreatine system: there's more than one song in its repertoire." *Journal of Physiology-London* 537 (3):657-657.
- Greenhaff, P. L., K. Bodin, K. Soderlund, and E. Hultman. 1994. "Effect of oral creatine supplementation on skeletal muscle phosphocreatine resynthesis." *Am J Physiol* 266 (5 Pt 1):E725-30.
- Greenhaff, Paul L. 1995. "Creatine and its application as an ergogenic aid." *International journal of sport nutrition* 5:S100-10.
- Greenhaff, Paul L. 1997. "The nutritional biochemistry of creatine." *The Journal of Nutritional Biochemistry* 8 (11):610-618.
- Greenhaff, Paul L, Anna Casey, Anthony H Short, Roger Harris, Karin Soderlund, and Eric Hultman. 1993. "Influence of oral creatine supplementation of muscle torque during repeated bouts of maximal voluntary exercise in man." *Clinical Science* 84 (Pt 5):565-571.
- Gualano, B, RB Novaes, GG Artioli, TO Freire, DF Coelho, FB Scagliusi, PS Rogeri, H Roschel, C Ugrinowitsch, and AH Lancha Jr. 2008. "Effects of creatine supplementation on glucose tolerance and insulin sensitivity in sedentary healthy males undergoing aerobic training." *Amino Acids* 34 (2):245-250.
- Guerrero-Ontiveros, Maria Lourdes, and Theo Wallimann. 1998. "Creatine supplementation in health and disease. Effects of chronic creatine ingestion in vivo: down-regulation of the expression of creatine transporter isoforms in skeletal muscle." In *Bioenergetics of the Cell: Quantitative Aspects*, 427-437. Springer.
- Guimbal, Ckile, and MW Kilimann. 1993. "A Na (+)-dependent creatine transporter in rabbit brain, muscle, heart, and kidney. cDNA cloning and functional expression." *Journal of Biological Chemistry* 268 (12):8418-8421.
- Gürtler, Anne, Nancy Kunz, Maria Gomolka, Sabine Hornhardt, Anna A Friedl, Kevin McDonald, Jonathan E Kohn, and Anton Posch. 2013. "Stain-Free technology as a normalization tool in Western blot analysis." *Analytical biochemistry* 433 (2):105-111.
- Haddad, Fadia, and Gregory R Adams. 2002. "Selected contribution: acute cellular and molecular responses to resistance exercise." *Journal of applied physiology* 93 (1):394-403.
- Hahn-Windgassen, Annett, Veronique Nogueira, Chia-Chen Chen, Jennifer E Skeen, Nahum Sonenberg, and Nissim Hay. 2005. "Akt activates the mammalian target of rapamycin by regulating cellular ATP level and AMPK activity." *Journal of Biological Chemistry* 280 (37):32081-32089.
- Harris, R. H. Edwards, E. Hultman, L. O. Nordesjo, B. Ny Lind, and K. Sahlin. 1976. "The time course of phosphorylcreatine resynthesis during recovery of the quadriceps muscle in man." *Pflugers Arch* 367 (2):137-42.
- Harris, R. C., K. Soderlund, and E. Hultman. 1992. "Elevation of creatine in resting and exercised muscle of normal subjects by creatine supplementation." *Clin Sci (Lond)* 83 (3):367-74.
- Harris, RC, E Hultman, and L-O Nordesjö. 1974. "Glycogen, glycolytic intermediates and high-energy phosphates determined in biopsy samples of musculus quadriceps femoris of man at rest. Methods and variance of values." *Scandinavian Journal of Clinical & Laboratory Investigation* 33 (2):109-120.
- Harris, RC, M Viru, PL Greenhaff, and E Hultman. 1993. "The effect of oral creatine supplementation on running performance during maximal short term exercise in

- man." *JOURNAL OF PHYSIOLOGY-LONDON THEN CAMBRIDGE*- 1 (467):74-74.
- Haugland, Rosaria Brivio, and David T Chang. 1975. "Insulin effect on creatine transport in skeletal muscle." *Proceedings of the Society for Experimental Biology and Medicine*. Society for Experimental Biology and Medicine (New York, NY).
- Hawley, John A. 2009. "Molecular responses to strength and endurance training: Are they incompatible? This paper article is one of a selection of papers published in this Special Issue, entitled 14th International Biochemistry of Exercise Conference- Muscles as Molecular and Metabolic Machines, and has undergone the Journal's usual peer review process." *Applied Physiology, Nutrition, and Metabolism* 34 (3):355-361.
- Hawley, John A, Louise M Burke, Stuart M Phillips, Lawrence L Spriet, James A Timmons, L Britton, Ben B Yaspelkis III, Monique Ramaekers, Peter Hespel, and Karen Van Proeyen. 2011. "Skeletal muscle adaptation and performance responses." *J Appl Physiol* 110 (3):834-845.
- Hawley, John A, and Jill J Leckey. 2015. "Carbohydrate dependence during prolonged, intense endurance exercise." *Sports Medicine* 45 (1):5-12.
- Hawley, John A, Elske J Schabort, Timothy D Noakes, and Steven C Dennis. 1997. "Carbohydrate-loading and exercise performance." *Sports Medicine* 24 (2):73-81.
- Hespel, Peter, Bert Op't Eijnde, Marc Van Leemputte, Birgitte Ursø, Paul L Greenhaff, Valery Labarque, Steven Dymarkowski, Paul Van Hecke, and Erik A Richter. 2001. "Oral creatine supplementation facilitates the rehabilitation of disuse atrophy and alters the expression of muscle myogenic factors in humans." *The Journal of physiology* 536 (2):625-633.
- Heszele, Maria F Chacon, and S Russ Price. 2004. "Insulin-like growth factor I: the yin and yang of muscle atrophy." *Endocrinology* 145 (11):4803-4805.
- Hoberman, Henry D, Ethan AH Sims, and John H Peters. 1948. "Creatine and creatinine metabolism in the normal male adult studied with the aid of isotopic nitrogen." *Journal of Biological Chemistry* 172 (1):45-58.
- Holloszy, John O, and Edward F Coyle. 1984. "Adaptations of skeletal muscle to endurance exercise and their metabolic consequences." *Journal of applied physiology: respiratory, environmental and exercise physiology* 56 (4):831-838.
- Hultman, E, K Soderlund, JA Timmons, G Cederblad, and PL Greenhaff. 1996. "Muscle creatine loading in men." *Journal of Applied Physiology* 81 (1):232-237.
- Ingwall, Joanne S, Manuel F Morales, and Frank E Stockdale. 1972. "Creatine and the control of myosin synthesis in differentiating skeletal muscle." *Proceedings of the National Academy of Sciences* 69 (8):2250-2253.
- Iyer, Geeta S, Ralf Krahe, Lynne A Goodwin, Norman A Doggett, Michael J Siciliano, Vicky L Funanage, and Roy Proujansky. 1996. "Identification of a testis-expressed creatine transporter gene at 16p11. 2 and confirmation of the X-linked locus to Xq28." *Genomics* 34 (1):143-146.
- Jager, R., M. Purpura, A. Shao, T. Inoue, and R. B. Kreider. 2011. "Analysis of the efficacy, safety, and regulatory status of novel forms of creatine." *Amino Acids* 40 (5):1369-83. doi: 10.1007/s00726-011-0874-6.
- Jaiswal, Natasha, Chandan K Maurya, K Venkateswarlu, P Sukanya, Arvind K Srivastava, Tadigoppula Narender, and Akhilesh K Tamrakar. 2012. "4-Hydroxyisoleucine stimulates glucose uptake by increasing surface GLUT4 level in skeletal muscle cells via phosphatidylinositol-3-kinase-dependent pathway." *European journal of nutrition* 51 (7):893-898.

- Jeukendrup, Asker E, Neil P Craig, and John A Hawley. 2000. "The bioenergetics of world class cycling." *Journal of Science and Medicine in Sport* 3 (4):414-433.
- Ju, Jeong-Sun, Jill L Smith, Peter J Oppelt, and Jonathan S Fisher. 2005. "Creatine feeding increases GLUT4 expression in rat skeletal muscle." *American Journal of Physiology-Endocrinology and Metabolism* 288 (2):E347-E352.
- Juhn, M. S., and M. Tarnopolsky. 1998a. "Oral creatine supplementation and athletic performance: a critical review." *Clin J Sport Med* 8 (4):286-97.
- Juhn, M. S., and M. Tarnopolsky. 1998b. "Potential side effects of oral creatine supplementation: a critical review." *Clin J Sport Med* 8 (4):298-304.
- Kadi, Fawzi, Nadia Charifi, Christian Denis, Jan Lexell, Jesper L Andersen, Peter Schjerling, Steen Olsen, and Michael Kjaer. 2005. "The behaviour of satellite cells in response to exercise: what have we learned from human studies?" *Pflügers Archiv* 451 (2):319-327.
- Kadi, Fawzi, Fredrik Johansson, Rikard Johansson, Mikael Sjöström, and Jan Henriksson. 2004. "Effects of one bout of endurance exercise on the expression of myogenin in human quadriceps muscle." *Histochemistry and cell biology* 121 (4):329-334.
- Kadi, Fawzi, and Lars-Eric Thornell. 2000. "Concomitant increases in myonuclear and satellite cell content in female trapezius muscle following strength training." *Histochemistry and cell biology* 113 (2):99-103.
- Kang LP, Zhao Y, Pang X, Yu HS, Xiong CQ, Zhang J, Gao Y, Yu K, Ma BP. 2013. "Characterization and identification of steroidal saponins from the seeds of *Trigonalla foem-graecum* by ultra high-performance liquid chromatography and hybrid time-of-flight mass spectrometry." *J Pharm. Biomed. Anal.* 23 (74):11.
- Kataoka, Yoshihisa, Itaru Matsumura, Sachiko Ezoe, Soichi Nakata, Eri Takigawa, Yusuke Sato, Akira Kawasaki, Takashi Yokota, Koichi Nakajima, and Armando Felsani. 2003. "Reciprocal inhibition between MyoD and STAT3 in the regulation of growth and differentiation of myoblasts." *Journal of Biological Chemistry* 278 (45):44178-44187.
- Kenny, O, TJ Smyth, CM Hewage, and NP Brunton. 2013. "Antioxidant properties and quantitative UPLC-MS analysis of phenolic compounds from extracts of fenugreek (*Trigonella foenum-graecum*) seeds and bitter melon (*Momordica charantia*) fruit." *Food chemistry* 141 (4):4295-4302.
- Khodabukus, Alastair, and Keith Baar. 2015. "Glucose concentration and streptomycin alter in vitro muscle function and metabolism." *Journal of cellular physiology* 230 (6):1226-1234.
- Kim, Eunjung, Pankuri Goraksha-Hicks, Li Li, Thomas P Neufeld, and Kun-Liang Guan. 2008. "Regulation of TORC1 by Rag GTPases in nutrient response." *Nature cell biology* 10 (8):935-945.
- Kim, Young-Bum, Svetlana E Nikoulina, Theodore P Ciaraldi, Robert R Henry, and Barbara B Kahn. 1999. "Normal insulin-dependent activation of Akt/protein kinase B, with diminished activation of phosphoinositide 3-kinase, in muscle in type 2 diabetes." *Journal of Clinical Investigation* 104 (6):733-741.
- Kingsley, M., D. Cunningham, L. Mason, L. P. Kilduff, and J. McEneny. 2009. "Role of creatine supplementation on exercise-induced cardiovascular function and oxidative stress." *Oxid Med Cell Longev* 2 (4):247-54. doi: 10.4161/oxim.2.4.9415.
- Kitzmann, Magali, Gilles Carnac, Marie Vandomme, Michael Primig, Ned JC Lamb, and Anne Fernandez. 1998. "The muscle regulatory factors MyoD and myf-5 undergo distinct cell cycle-specific expression in muscle cells." *The Journal of cell biology* 142 (6):1447-1459.

- Kosek, David J, Jeong-su Kim, John K Petrella, James M Cross, and Marcas M Bamman. 2006. "Efficacy of 3 days/wk resistance training on myofiber hypertrophy and myogenic mechanisms in young vs. older adults." *Journal of applied physiology* 101 (2):531-544.
- Koszalka, Thomas R, and Carole L Andrew. 1972. "Effect of insulin on the uptake of creatine-1-14C by skeletal muscle in normal and X-irradiated rats." *Proceedings of the Society for Experimental Biology and Medicine*. Society for Experimental Biology and Medicine (New York, NY).
- Kraemer, William J, and Jeff S Volek. 1999. "Creatine supplementation: its role in human performance." *Clinics in sports medicine* 18 (3):651-666.
- Kreider, Richard B. 2003. "Effects of creatine supplementation on performance and training adaptations." *Molecular and cellular biochemistry* 244 (1-2):89-94.
- Kreider, Richard B, Maria Ferreira, Michael Wilson, Pamela Grindstaff, Steven Plisk, Jeff Reinardy, Edward Cantler, and AL Almada. 1998. "Effects of creatine supplementation on body composition, strength, and sprint performance." *Medicine and science in sports and exercise* 30:73-82.
- Kuang, Shihuan, Sophie B Chargé, Patrick Seale, Michael Huh, and Michael A Rudnicki. 2006. "Distinct roles for Pax7 and Pax3 in adult regenerative myogenesis." *The Journal of cell biology* 172 (1):103-113.
- Kubica, Neil, Douglas R Bolster, Peter A Farrell, Scot R Kimball, and Leonard S Jefferson. 2005. "Resistance exercise increases muscle protein synthesis and translation of eukaryotic initiation factor 2B ϵ mRNA in a mammalian target of rapamycin-dependent manner." *Journal of Biological Chemistry* 280 (9):7570-7580.
- Kubo, YOSHIHIRO. 1991. "Comparison of initial stages of muscle differentiation in rat and mouse myoblastic and mouse mesodermal stem cell lines." *The Journal of physiology* 442 (1):743-759.
- Kumegawa, Masayoshi, Eiko Ikeda, Satoko Hosoda, and Taishin Takuma. 1980. "In vitro effects of thyroxine and insulin on myoblasts from chick embryo skeletal muscle." *Developmental biology* 79 (2):493-499.
- Lechner, Cornelia, Muayad A Zahalka, Jean-Francois Giot, NP Møller, and Axel Ullrich. 1996. "ERK6, a mitogen-activated protein kinase involved in C2C12 myoblast differentiation." *Proceedings of the National Academy of Sciences* 93 (9):4355-4359.
- Leto, D., and A. R. Saltiel. 2012. "Regulation of glucose transport by insulin: traffic control of GLUT4." *Nat Rev Mol Cell Biol* 13 (6):383-96. doi: 10.1038/nrm3351.
- Levesque, Daniel G, Robert W Kenefick, and Timothy J Quinn. 2007. "Creatine Supplementation: Impact on Cycling Sprint Performance." *Journal of Exercise Physiology Online* 10 (4).
- Lluís, Frederic, Eusebio Perdiguero, Angel R Nebreda, and Pura Muñoz-Cánoves. 2006. "Regulation of skeletal muscle gene expression by p38 MAP kinases." *Trends in cell biology* 16 (1):36-44.
- Loenneke, JP, CA Fahs, LM Rossow, T Abe, and MG Bembien. 2012. "The anabolic benefits of venous blood flow restriction training may be induced by muscle cell swelling." *Medical hypotheses* 78 (1):151-154.
- Loike, J. D., D. L. Zalutsky, E. Kaback, A. F. Miranda, and S. C. Silverstein. 1988. "Extracellular creatine regulates creatine transport in rat and human muscle cells." *Proc Natl Acad Sci U S A* 85 (3):807-11.
- Loike, JOHN D, MICHELE Somes, and SAMUEL C Silverstein. 1986. "Creatine uptake, metabolism, and efflux in human monocytes and macrophages." *American Journal of Physiology-Cell Physiology* 251 (1):C128-C135.

- Louis, Magali, Jacques R Poortmans, Marc Francaux, Jacques Berré, Nathalie Boisseau, Eric Brassine, Daniel JR Cuthbertson, Kenneth Smith, John A Babraj, and Tom Waddell. 2003. "No effect of creatine supplementation on human myofibrillar and sarcoplasmic protein synthesis after resistance exercise." *American Journal of Physiology-Endocrinology and Metabolism* 285 (5):E1089-E1094.
- Louis, Magali, Jacques R Poortmans, Marc Francaux, Eric Hultman, Jacques Berré, Nathalie Boisseau, Vernon R Young, Kenneth Smith, Wolfram Meier-Augenstein, and John A Babraj. 2003. "Creatine supplementation has no effect on human muscle protein turnover at rest in the postabsorptive or fed states." *American Journal of Physiology-Endocrinology and Metabolism* 284 (4):E764-E770.
- Louis, Magali, Ronald Van Beneden, Mischaël Dehoux, Jean Paul Thissen, and Marc Francaux. 2004. "Creatine increases IGF-I and myogenic regulatory factor mRNA in C₂ C₁₂ cells." *FEBS letters* 557 (1):243-247.
- Low, Sylvia Y, Michael J Rennie, and Peter M Taylor. 1996. "Modulation of glycogen synthesis in rat skeletal muscle by changes in cell volume." *The Journal of physiology* 495 (Pt 2):299-303.
- Lowe, Dawn A, Troy Lund, and Stephen E Alway. 1998. "Hypertrophy-stimulated myogenic regulatory factor mRNA increases are attenuated in fast muscle of aged quails." *American Journal of Physiology-Cell Physiology* 275 (1):C155-C162.
- Macpherson, PC, Robert G Dennis, and John A Faulkner. 1997. "Sarcomere dynamics and contraction-induced injury to maximally activated single muscle fibres from soleus muscles of rats." *The Journal of Physiology* 500 (Pt 2):523.
- Marchand, I, M Tarnopolsky, KB Adamo, JM Bourgeois, K Chorneyko, and TE Graham. 2007. "Quantitative assessment of human muscle glycogen granules size and number in subcellular locations during recovery from prolonged exercise." *The Journal of physiology* 580 (2):617-628.
- Maughan, Ronald J. 2013. *The Encyclopaedia of Sports Medicine: An IOC Medical Commission Publication, Sports Nutrition*. Vol. 19: John Wiley & Sons.
- Mauro, Alexander. 1961. "Satellite cell of skeletal muscle fibers." *The Journal of biophysical and biochemical cytology* 9 (2):493-495.
- McBride, J. J., M. M. Guest, and E. L. Scott. 1941. "The storage of the major liver components; emphasizing the relationship of glycogen to water in the liver and the hydration of glycogen." *J Biol Chem* 139 (2):943-952.
- McCarthy, John J, Jyothi Mula, Mitsunori Miyazaki, Rod Erfani, Kelcye Garrison, Amreen B Farooqui, Ratchakrit Srikuea, Benjamin A Lawson, Barry Grimes, and Charles Keller. 2011. "Effective fiber hypertrophy in satellite cell-depleted skeletal muscle." *Development* 138 (17):3657-3666.
- McCully, Kevin K, and John A Faulkner. 1985. "Injury to skeletal muscle fibers of mice following lengthening contractions." *Journal of Applied Physiology* 59 (1):119-126.
- McInerney, Patrick, Sarah J Lessard, Louise M Burke, Vernon G Coffey, Sonia L Lo Giudice, Robert J Southgate, and John A Hawley. 2005. "Failure to repeatedly supercompensate muscle glycogen stores in highly trained men." *Med Sci Sports Exerc* 37 (3):404-411.
- McManus, Edward J, Barry J Collins, Peter R Ashby, Alan R Prescott, Victoria Murray-Tait, Laura J Armit, J Simon C Arthur, and Dario R Alessi. 2004. "The in vivo role of PtdIns (3, 4, 5) P3 binding to PDK1 PH domain defined by knockin mutation." *The EMBO journal* 23 (10):2071-2082.
- McNaughton, Lars R, Brad Dalton, and Janine Tarr. 1998. "The effects of creatine supplementation on high-intensity exercise performance in elite performers." *European journal of applied physiology and occupational physiology* 78 (3):236-240.

- Mesa, José LM, Jonatan R Ruiz, M Marcela González-Gross, Ángel Gutiérrez Sáinz, and Manuel J Castillo Garzón. 2002. "Oral creatine supplementation and skeletal muscle metabolism in physical exercise." *Sports Medicine* 32 (14):903-944.
- Meyer, RA, H Lee Sweeney, and MJ Kushmerick. 1984. "A simple analysis of the phosphocreatine shuttle." *American Journal of Physiology-Cell Physiology* 246 (5):C365-C377.
- Meyer, Ronald A, Truman R Brown, and Martin J Kushmerick. 1985. "Phosphorus nuclear magnetic resonance of fast-and slow-twitch muscle." *American Journal of Physiology-Cell Physiology* 248 (3):C279-C287.
- Miniaci, Maria Concetta, Maria Gabriella Dattolo, Carlo Irace, Antonella Capuozzo, Rita Santamaria, and Pietro Scotto. 2015. "Glucose deprivation promotes activation of mTOR signaling pathway and protein synthesis in rat skeletal muscle cells." *Pflügers Archiv-European Journal of Physiology* 467 (6):1357-1366.
- Moore, NP. 2000. "The distribution, metabolism and function of creatine in the male mammalian reproductive tract: A review." *international journal of andrology* 23 (1):4-12.
- Mora, A., D. Komander, D. M. van Aalten, and D. R. Alessi. 2004. "PDK1, the master regulator of AGC kinase signal transduction." *Semin Cell Dev Biol* 15 (2):161-70.
- Mountjoy, Margo, Jorunn Sundgot-Borgen, Louise Burke, Susan Carter, Naama Constantini, Constance Lebrun, Nanna Meyer, Roberta Sherman, Kathrin Steffen, and Richard Budgett. 2014. "The IOC consensus statement: Beyond the female athlete triad—Relative Energy Deficiency in Sport (RED-S)." *British journal of sports medicine* 48 (7):491-497.
- Mujika, I, and S Padilla. 1997. "Creatine supplementation as an ergogenic aid for sports performance in highly trained athletes: a critical review." *International journal of sports medicine* 18 (7):491-496.
- Murphy, R, G McConell, David Cameron-Smith, K Watt, Leigh Ackland, B Walzel, T Wallimann, and Rodney Snow. 2001. "Creatine transporter protein content, localization, and gene expression in rat skeletal muscle." *American Journal of Physiology-Cell Physiology* 280 (3):C415-C422.
- Murphy, Robyn M, Rebecca J Tunstall, Kate A Mehan, David Cameron-Smith, Michael J McKenna, Lawrence L Spriet, Mark Hargreaves, and Rodney J Snow. 2003. "Human skeletal muscle creatine transporter mRNA and protein expression in healthy, young males and females." In *Guanidino Compounds in Biology and Medicine*, 151-157. Springer.
- Nader, Gustavo A. 2005. "Molecular determinants of skeletal muscle mass: getting the "AKT" together." *The international journal of biochemistry & cell biology* 37 (10):1985-1996.
- Nader, Gustavo A, and Karyn A Esser. 2001. "Intracellular signaling specificity in skeletal muscle in response to different modes of exercise." *Journal of Applied Physiology* 90 (5):1936-1942.
- Narender, Tadigoppula, Anju Puri, Tanvir Khaliq, Rashmi Saxena, Geetika Bhatia, and Ramesh Chandra. 2006. "4-Hydroxyisoleucine an unusual amino acid as antidiabetic and antihyperglycemic agent." *Bioorganic & medicinal chemistry letters* 16 (2):293-296.
- Nash, SR, B Giros, SF Kingsmore, JM Rochelle, ST Suter, P Gregor, MF Seldin, and MG Caron. 1993. "Cloning, pharmacological characterization, and genomic localization of the human creatine transporter." *Receptors & channels* 2 (2):165-174.
- Nathan, J, Siddika Panjwani, V Mohan, Veena Joshi, and Prasad Arvind Thakurdesai. 2013. "Efficacy and Safety of Standardized Extract of *Trigonella foenum-graecum* L Seeds

- as an Adjuvant to L-Dopa in the Management of Patients with Parkinson's Disease." *Phytotherapy Research*.
- Nedachi, Taku, Hideaki Fujita, and Makoto Kanzaki. 2008. "Contractile C2C12 myotube model for studying exercise-inducible responses in skeletal muscle." *American Journal of Physiology-Endocrinology and Metabolism* 295 (5):E1191-E1204.
- Nedachi, Taku, Akito Kadotani, Miyako Ariga, Hideki Katagiri, and Makoto Kanzaki. 2008. "Ambient glucose levels qualify the potency of insulin myogenic actions by regulating SIRT1 and FoxO3a in C2C12 myocytes." *American Journal of Physiology-Endocrinology and Metabolism* 294 (4):E668-E678.
- Nelson, Arnold G, DAVID A Arnall, Joke Kokkonen, Randy Day, and Jared Evans. 2001. "Muscle glycogen supercompensation is enhanced by prior creatine supplementation." *Medicine and science in sports and exercise* 33 (7):1096-1100.
- Nielsen, Joachim, Hans-Christer Holmberg, Henrik D Schrøder, Bengt Saltin, and Niels Ørtenblad. 2011. "Human skeletal muscle glycogen utilization in exhaustive exercise: role of subcellular localization and fibre type." *The Journal of physiology* 589 (11):2871-2885.
- Nygren, AT, M Karlsson, B Norman, and L Kaijser. 2001. "Effect of glycogen loading on skeletal muscle cross-sectional area and T2 relaxation time." *Acta Physiologica Scandinavica* 173 (4):385-390.
- Odoom, J. E., G. J. Kemp, and G. K. Radda. 1996. "The regulation of total creatine content in a myoblast cell line." *Mol Cell Biochem* 158 (2):179-88.
- Ogawa, Jun, Tomohiro Kodera, Sergey V Smirnov, Makoto Hibi, Natalia N Samsonova, Ryoukichi Koyama, Hiroyuki Yamanaka, Junichi Mano, Takashi Kawashima, and Kenzo Yokozeki. 2011. "A novel L-isoleucine metabolism in *Bacillus thuringiensis* generating (2S, 3R, 4S)-4-hydroxyisoleucine, a potential insulinotropic and anti-obesity amino acid." *Applied microbiology and biotechnology* 89 (6):1929-1938.
- Ohanna, Mickaël, Andrew K Sobering, Thomas Lapointe, Lazaro Lorenzo, Christophe Praud, Emmanuel Petroulakis, Nahum Sonenberg, Paul A Kelly, Athanassia Sotiropoulos, and Mario Pende. 2005. "Atrophy of S6K1^{-/-} skeletal muscle cells reveals distinct mTOR effectors for cell cycle and size control." *Nature cell biology* 7 (3):286-294.
- Oldham, S., and E. Hafen. 2003. "Insulin/IGF and target of rapamycin signaling: a TOR de force in growth control." *Trends Cell Biol* 13 (2):79-85.
- Oliver, Jonathan M, Dustin P Joubert, Steven E Martin, and Stephen F Crouse. 2013. "Oral creatine supplementation's decrease of blood lactate during exhaustive, incremental cycling." *International Journal of Sport Nutrition & Exercise Metabolism* 23 (3).
- Olsen, Steen, Per Aagaard, Fawzi Kadi, Goran Tufekovic, Julien Verney, Jens L Olesen, Charlotte Suetta, and Michael Kjær. 2006. "Creatine supplementation augments the increase in satellite cell and myonuclei number in human skeletal muscle induced by strength training." *The Journal of physiology* 573 (2):525-534.
- Olsson, K. E., and B. Saltin. 1970a. "Variation in total body water with muscle glycogen changes in man." *Acta Physiol Scand* 80 (1):11-8. doi: 10.1111/j.1748-1716.1970.tb04764.x.
- Olsson, Karl-Erik, and Bengt Saltin. 1970b. "Variation in total body water with muscle glycogen changes in man." *Acta Physiologica Scandinavica* 80 (1):11-18.
- Op't Eijnde, B, Birgitte Ursø, Erik A Richter, Paul L Greenhaff, and Peter Hespel. 2001. "Effect of oral creatine supplementation on human muscle GLUT4 protein content after immobilization." *Diabetes* 50 (1):18-23.
- Ørtenblad, Niels, Håkan Westerblad, and Joachim Nielsen. 2013. "Muscle glycogen stores and fatigue." *The Journal of physiology* 591 (18):4405-4413.

- Orvig, C, KH Thompson, M Battell, and JH McNeill. 1995. "Vanadium compounds as insulin mimics." *Metal ions in biological systems* 31:575.
- Palmer, Robert M, Michael G Thompson, Rachel M Knott, Gillian P Campbell, Amanda Thom, and Kenneth S Morrison. 1997. "Insulin and insulin-like growth factor-I responsiveness and signalling mechanisms in C 2 C 12 satellite cells: effect of differentiation and fusion." *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* 1355 (2):167-176.
- Park, In-Hyun, Ebru Erbay, Paul Nuzzi, and Jie Chen. 2005. "Skeletal myocyte hypertrophy requires mTOR kinase activity and S6K1." *Experimental cell research* 309 (1):211-219.
- Patel, DK, SK Prasad, R Kumar, and S Hemalatha. 2012. "An overview on antidiabetic medicinal plants having insulin mimetic property." *Asian Pacific Journal of Tropical Biomedicine* 2 (4):320-330.
- Peral, MJ, M García-Delgado, ML Calonge, JM Duran, MC Horra, T Wallimann, O Speer, and AA Ilundáin. 2002. "Human, rat and chicken small intestinal Na⁺-Cl⁻-creatine transporter: functional, molecular characterization and localization." *The Journal of physiology* 545 (1):133-144.
- Persky, Adam M, and Gayle A Brazeau. 2001. "Clinical pharmacology of the dietary supplement creatine monohydrate." *Pharmacological Reviews* 53 (2):161-176.
- Persky, Adam M, Gayle A Brazeau, and Günther Hochhaus. 2003. "Pharmacokinetics of the dietary supplement creatine." *Clinical pharmacokinetics* 42 (6):557-574.
- Petrella, John K, Jeong-su Kim, James M Cross, David J Kosek, and Marcos M Bamman. 2006. "Efficacy of myonuclear addition may explain differential myofiber growth among resistance-trained young and older men and women." *American Journal of Physiology-Endocrinology and Metabolism* 291 (5):E937-E946.
- Philp, Andrew, Mark Hargreaves, and Keith Baar. 2012. "More than a store: regulatory roles for glycogen in skeletal muscle adaptation to exercise." *American Journal of Physiology-Endocrinology And Metabolism* 302 (11):E1343-E1351.
- Pilegaard, Henriette, Takuya Osada, Lisbeth T Andersen, Jørn W Helge, Bengt Saltin, and P Darrell Neufer. 2005. "Substrate availability and transcriptional regulation of metabolic genes in human skeletal muscle during recovery from exercise." *Metabolism* 54 (8):1048-1055.
- Pittner, Richard A, Deborah Wolfe-Lopez, Andrew A Young, and Kevin Beaumont. 1996. "Different pharmacological characteristics in L6 and C2C12 muscle cells and intact rat skeletal muscle for amylin, CGRP and calcitonin." *British journal of pharmacology* 117 (5):847-852.
- Ponticos, Markella, Qi Long Lu, Jennifer E Morgan, D Grahame Hardie, Terence A Partridge, and David Carling. 1998. "Dual regulation of the AMP-activated protein kinase provides a novel mechanism for the control of creatine kinase in skeletal muscle." *The EMBO Journal* 17 (6):1688-1699.
- Powers, Michael E, Brent L Arnold, Arthur L Weltman, David H Perrin, Dilawaar Mistry, David M Kahler, William Kraemer, and Jeff Volek. 2003. "Creatine supplementation increases total body water without altering fluid distribution." *Journal of athletic training* 38 (1):44.
- Reeds, PJ, and RM Palmer. 1983. "The possible involvement of prostaglandin F 2 α in the stimulation of muscle protein synthesis by insulin." *Biochemical and biophysical research communications* 116 (3):1084-1090.
- Ren, JIAN-MING, CF Semenkovich, and JO Holloszy. 1993. "Adaptation of muscle to creatine depletion: effect on GLUT-4 glucose transporter expression." *American Journal of Physiology-Cell Physiology* 264 (1):C146-C150.

- Richter, E. A., and M. Hargreaves. 2013. "Exercise, GLUT4, and Skeletal Muscle Glucose Uptake." *Physiol Rev* 93 (3):993-1017. doi: 10.1152/physrev.00038.2012.
- Richter, EA, C Macdonald, B Kiens, G Hardie, and JFP Wojtaszewski. 2001. "Dissociation of 5'AMP-activated protein kinase activity and glucose clearance in human skeletal muscle during exercise." *Diabetes*.
- Richter, Joel D, and Nahum Sonenberg. 2005. "Regulation of cap-dependent translation by eIF4E inhibitory proteins." *Nature* 433 (7025):477-480.
- Robergs, Robert A, and Scott Roberts. 2000. *Fundamental principles of exercise physiology: for fitness, performance, and health*: McGraw-Hill College.
- Roberts, Paul A, John Fox, Nicholas Peirce, Simon W Jones, Anna Casey, and Paul L Greenhaff. 2016. "Creatine ingestion augments dietary carbohydrate mediated muscle glycogen supercompensation during the initial 24 h of recovery following prolonged exhaustive exercise in humans." *Amino acids*:1-12.
- Robinson, Tristan M, Dean A Sewell, Eric Hultman, and Paul L Greenhaff. 1999. "Role of submaximal exercise in promoting creatine and glycogen accumulation in human skeletal muscle." *Journal of Applied Physiology* 87 (2):598-604.
- Rommel, Christian, Sue C Bodine, Brian A Clarke, Roni Rossman, Lorna Nunez, Trevor N Stitt, George D Yancopoulos, and David J Glass. 2001. "Mediation of IGF-1-induced skeletal myotube hypertrophy by PI (3) K/Akt/mTOR and PI (3) K/Akt/GSK3 pathways." *Nature cell biology* 3 (11):1009-1013.
- Roschel, Hamilton, Bruno Gualano, Marcelo Marquezi, André Costa, and Antonio H Lancha Jr. 2010. "Creatine supplementation spares muscle glycogen during high intensity intermittent exercise in rats." *Journal of the International Society of Sports Nutrition* 7 (1):6.
- Roth, SM, GF Martel, FM Ivey, JT Lemmer, BL Tracy, EJ Metter, BF Hurley, and MA Rogers. 2001. "Skeletal muscle satellite cell characteristics in young and older men and women after heavy resistance strength training." *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* 56 (6):B240-B247.
- Roux, Philippe P, and John Blenis. 2004. "ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions." *Microbiology and molecular biology reviews* 68 (2):320-344.
- Russell, Aaron P, Lobna Ghobrial, Craig R Wright, Séverine Lamon, Erin L Brown, Michihiro Kon, Matthew R Skelton, and Rodney J Snow. 2014. "Creatine transporter (SLC6A8) knockout mice display an increased capacity for in vitro creatine biosynthesis in skeletal muscle." *Frontiers in physiology* 5.
- Ruvinsky, Igor, and Oded Meyuhas. 2006. "Ribosomal protein S6 phosphorylation: from protein synthesis to cell size." *Trends in biochemical sciences* 31 (6):342-348.
- Safdar, Adeel, Nicholas J Yardley, Rodney Snow, Simon Melov, and Mark A Tarnopolsky. 2008. "Global and targeted gene expression and protein content in skeletal muscle of young men following short-term creatine monohydrate supplementation." *Physiological genomics* 32 (2):219-228.
- Saiti, Deshira, and Orly Lacham-Kaplan. 2007. "Mouse Germ Cell Development in-vivo and in-vitro." *Biomarker insights* 2:241.
- Sakamoto, Kei, and Laurie J Goodyear. 2002. "Invited review: intracellular signaling in contracting skeletal muscle." *Journal of Applied Physiology* 93 (1):369-383.
- Sakurai, Hiromu, Hiromi Watanabe, Hideyuki Tamura, Hiroyuki Yasui, Rokuji Matsushita, and Jitsuya Takada. 1998. "Insulin-mimetic vanadyl—dithiocarbamate complexes." *Inorganica chimica acta* 283 (1):175-183.
- Salamon, Michela, Caterina Millino, Anna Raffaello, Marco Mongillo, Claudia Sandri, Camilla Bean, Enrico Negrisola, Alberto Pallavicini, Giorgio Valle, and Manuela

- Zaccolo. 2003. "Human MYO18B, a novel unconventional myosin heavy chain expressed in striated muscles moves into the myonuclei upon differentiation." *Journal of molecular biology* 326 (1):137-149.
- Salomons, Gajja S, Silvy JM van Dooren, Nanda M Verhoeven, Kim M Cecil, William S Ball, Ton J Degrauw, and Cornelis Jakobs. 2001. "X-linked creatine-transporter gene (SLC6A8) defect: a new creatine-deficiency syndrome." *The American Journal of Human Genetics* 68 (6):1497-1500.
- Saltarelli, MD, AL Bauman, KR Moore, CC Bradley, and RD Blakely. 1996. "Expression of the rat brain creatine transporter in situ and in transfected HeLa cells." *Developmental neuroscience* 18 (5-6):524-534.
- Sarabia, V, L Lam, E Burdett, LA Leiter, and A Klip. 1992. "Glucose transport in human skeletal muscle cells in culture. Stimulation by insulin and metformin." *Journal of Clinical Investigation* 90 (4):1386.
- Sarbassov, Dos D, Siraj M Ali, Do-Hyung Kim, David A Guertin, Robert R Latek, Hediye Erdjument-Bromage, Paul Tempst, and David M Sabatini. 2004. "Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton." *Current biology* 14 (14):1296-1302.
- Sarbassov, Dos D, David A Guertin, Siraj M Ali, and David M Sabatini. 2005. "Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex." *Science* 307 (5712):1098-1101.
- Sartorelli, Vittorio, and Marcella Fulco. 2004. "Molecular and cellular determinants of skeletal muscle atrophy and hypertrophy." *Sci. STKE* 2004 (244):re11-re11.
- Sauvaire, Yves, Pierre Petit, Christophe Broca, Michèle Manteghetti, Yves Baissac, Josepha Fernandez-Alvarez, Renè Gross, Michèle Roye, Agnès Leconte, and Ramon Gomis. 1998. "4-Hydroxyisoleucine: a novel amino acid potentiator of insulin secretion." *Diabetes* 47 (2):206-210.
- Schoch, R. D., D. Willoughby, and M. Greenwood. 2006. "The regulation and expression of the creatine transporter: a brief review of creatine supplementation in humans and animals." *J Int Soc Sports Nutr* 3 (1):60-6. doi: 10.1186/1550-2783-3-1-60.
- Seale, Patrick, Luc A Sabourin, Adele Girgis-Gabardo, Ahmed Mansouri, Peter Gruss, and Michael A Rudnicki. 2000. "Pax7 is required for the specification of myogenic satellite cells." *Cell* 102 (6):777-786.
- Serra, Carlo, Daniela Palacios, Chiara Mozzetta, Sonia V Forcales, Ianessa Morantte, Meri Ripani, David R Jones, Keyong Du, Ulupi S Jhala, and Cristiano Simone. 2007. "Functional interdependence at the chromatin level between the MKK6/p38 and IGF1/PI3K/AKT pathways during muscle differentiation." *Molecular cell* 28 (2):200-213.
- Sestili, Piero, Elena Barbieri, Chiara Martinelli, Michela Battistelli, Michele Guescini, Luciana Vallorani, Lucia Casadei, Alessandra D'Emilio, Elisabetta Falcieri, and Giovanni Piccoli. 2009. "Creatine supplementation prevents the inhibition of myogenic differentiation in oxidatively injured C2C12 murine myoblasts." *Molecular nutrition & food research* 53 (9):1187-1204.
- Sewell, Dean A, Tristan M Robinson, and Paul L Greenhaff. 2008. "Creatine supplementation does not affect human skeletal muscle glycogen content in the absence of prior exercise." *Journal of Applied Physiology* 104 (2):508-512.
- Shabbeer, Shabana, Michelle Sobolewski, Ravi Kumar Anchoori, Sushant Kachhap, Manuel Hidalgo, Antonio Jimeno, Nancy E Davidson, Michael Carducci, and Saeed R Khan. 2009. "Fenugreek: a naturally occurring edible spice as an anticancer agent." *Cancer biology & therapy* 8 (3):272-278.

- Shainberg, Asher, Gad Yagil, and David Yaffe. 1971. "Alterations of enzymatic activities during muscle differentiation in vitro." *Developmental biology* 25 (1):1-29.
- Shearer, Jane, and Terry E Graham. 2002. "New perspectives on the storage and organization of muscle glycogen." *Canadian journal of applied physiology* 27 (2):179-203.
- Sherman, WM, MJ Plyley, RL Sharp, PJ Van Handel, RM McAllister, WJ Fink, and DL Costill. 1982. "Muscle glycogen storage and its relationship with water." *International journal of sports medicine* 3 (01):22-24.
- Shima, Hiroshi, Mario Pende, Yi Chen, Stefano Fumagalli, George Thomas, and Sara C Kozma. 1998. "Disruption of the p70s6k/p85s6k gene reveals a small mouse phenotype and a new functional S6 kinase." *The EMBO journal* 17 (22):6649-6659.
- Shojaiefard, Manzar, David L Christie, and Florian Lang. 2006. "Stimulation of the creatine transporter SLC6A8 by the protein kinase mTOR." *Biochemical and biophysical research communications* 341 (4):945-949.
- Siu, Parco M, David A Donley, Randall W Bryner, and Stephen E Alway. 2004. "Myogenin and oxidative enzyme gene expression levels are elevated in rat soleus muscles after endurance training." *Journal of Applied Physiology* 97 (1):277-285.
- Smith, Michael. 2003. "Therapeutic applications of fenugreek." *Alternative Medicine Review* 8 (1):20-27.
- Snow, Rodney J, and Robyn M Murphy. 2001. "Creatine and the creatine transporter: a review." *Molecular and cellular biochemistry* 224 (1-2):169-181.
- Snow, Rodney J, and Robyn M Murphy. 2003. "Factors influencing creatine loading into human skeletal muscle." *Exercise and sport sciences reviews* 31 (3):154-158.
- Sobolewski, Eric J, Brennan J Thompson, Abbie E Smith, and Eric D Ryan. 2011. "The physiological effects of creatine supplementation on hydration: A review." *American Journal of Lifestyle Medicine* 5 (4):320-327.
- Sora, Ichiro, Jeremy Richman, Giovanna Santoro, HB Wei, Yu Wang, Todd Vanderah, Robert Horvath, Mike Nguyen, Sue Waite, and William R Roeske. 1994. "The cloning and expression of a human creatine transporter." *Biochemical and biophysical research communications* 204 (1):419-427.
- Spurway, Neil, and Henning Wackerhage. 2006. *Genetics and molecular biology of muscle adaptation*: Elsevier Health Sciences.
- Srinivasan, K. 2006. "Fenugreek (*Trigonella foenum-graecum*): A review of health beneficial physiological effects." *Food reviews international* 22 (2):203-224.
- Stapleton, SR. 2000. "Selenium: an insulin mimetic." *Cellular and Molecular Life Sciences CMLS* 57 (13-14):1874-1879.
- Steenge, GR, J Lambourne, A Casey, IA Macdonald, and PL Greenhaff. 1998. "Stimulatory effect of insulin on creatine accumulation in human skeletal muscle." *American Journal of Physiology-Endocrinology And Metabolism* 275 (6):E974-E979.
- Steenge, GR, EJ Simpson, and PL Greenhaff. 2000. "Protein-and carbohydrate-induced augmentation of whole body creatine retention in humans." *Journal of Applied Physiology* 89 (3):1165-1171.
- Stephens, Francis B, and Paul L Greenhaff. 2014. "Creatine." *The Encyclopaedia of Sports Medicine: An IOC Medical Commission Publication, Volume 19*:301-312.
- Sullivan, Vicki K, Scott K Powers, DAVID S Criswell, NIHAL Tumer, JEFFREY S Larochelle, and DAVID Lowenthal. 1995. "Myosin heavy chain composition in young and old rat skeletal muscle: effects of endurance exercise." *Journal of Applied Physiology* 78 (6):2115-2120.
- Sumitani, Satoru, Kayoko Goya, Joseph R Testa, Haruhiko Kouhara, and Soji Kasayama. 2002. "Akt1 and Akt2 differently regulate muscle creatine kinase and myogenin gene

- transcription in insulin-induced differentiation of C2C12 myoblasts." *Endocrinology* 143 (3):820-828.
- Takahashi, Hideyuki, Mitsuharu Inaki, Koichi Fujimoto, Shigeru Katsuta, Izumi Anno, Mamoru Nütsu, and Yuji Itai. 1995. "Control of the rate of phosphocreatine resynthesis after exercise in trained and untrained human quadriceps muscles." *European journal of applied physiology and occupational physiology* 71 (5):396-404.
- Tang, Fu-Chun, Chun-Chen Chan, and Po-Ling Kuo. 2013. "Contribution of creatine to protein homeostasis in athletes after endurance and sprint running." *European journal of nutrition*:1-11.
- Taniguchi, Cullen M, Brice Emanuelli, and C Ronald Kahn. 2006. "Critical nodes in signalling pathways: insights into insulin action." *Nature reviews Molecular cell biology* 7 (2):85-96.
- Tarnopolsky, Mark, Gianni Parise, Min-Hua Fu, Andrea Brose, Andrew Parshad, Oliver Speer, and Theo Wallimann. 2003. "Acute and moderate-term creatine monohydrate supplementation does not affect creatine transporter mRNA or protein content in either young or elderly humans." In *Guanidino Compounds in Biology and Medicine*, 159-166. Springer.
- Taylor, Lem, Chris Poole, Earnest Pena, Morgan Lewing, Richard Kreider, Cliffa Foster, and Colin Wilborn. 2011. "Effects of Combined Creatine Plus Fenugreek Extract Vs. Creatine Plus Carbohydrate Supplementation on Resistance Training Adaptations." *Journal of Sports Science and Medicine* 10 (2):254-260.
- Thein, Lori A, Jill M Thein, and Gregory L Landry. 1995. "Ergogenic aids." *Physical Therapy* 75 (5):426-439.
- Thomson, David M, and Scott E Gordon. 2006. "Impaired overload-induced muscle growth is associated with diminished translational signalling in aged rat fast-twitch skeletal muscle." *The Journal of physiology* 574 (1):291-305.
- Tomcik, Kristylen A, William J Smiles, Donny M Camera, Helmut M Hügel, John A Hawley, and Rani Watts. 2016. "Fenugreek increases insulin-stimulated creatine content in L6C11 muscle myotubes." *European journal of nutrition*:1-7.
- Treebak, Jonas T, Stephan Glund, Atul Deshmukh, Ditte K Klein, Yun Chau Long, Thomas E Jensen, Sebastian B Jørgensen, Benoit Viollet, Leif Andersson, and Dietbert Neumann. 2006. "AMPK-mediated AS160 phosphorylation in skeletal muscle is dependent on AMPK catalytic and regulatory subunits." *Diabetes* 55 (7):2051-2058.
- Tsuka, Shintaro, Fumiko Aonuma, Sen Higashi, Tomoko Ohsumi, Koki Nagano, Akiko Mizokami, Tomoyo Kawakubo-Yasukochi, Chihiro Masaki, Ryuji Hosokawa, and Masato Hirata. 2015. "Promotion of insulin-induced glucose uptake in C2C12 myotubes by osteocalcin." *Biochemical and biophysical research communications* 459 (3):437-442.
- van Loon, L. J. C., A. M. Oosterlaar, F. Hartgens, M. K. C. Hesselink, R. J. Snow, and A. J. M. Wagenmakers. 2003. "Effects of creatine loading and prolonged creatine supplementation on body composition, fuel selection, sprint and endurance performance in humans." *Clinical Science* 104 (2):153-162. doi: Doi 10.1042/Cs20020159.
- van Loon, Luc JC, Robyn Murphy, Audrey M Oosterlaar, David Cameron-Smith, Mark Hargreaves, Anton JM Wagenmakers, and Rodney Snow. 2004. "Creatine supplementation increases glycogen storage but not GLUT-4 expression in human skeletal muscle." *Clinical science* 106 (1):99-106.
- Van Pilsum, John F, Beatta Olsen, Dorris Taylor, Thomas Rozycki, and James C Pierce. 1963. "Transamidinase activities, in vitro, of tissues from various mammals and from

- rats fed protein-free, creatine-supplemented and normal diets." *Archives of biochemistry and biophysics* 100 (3):520-524.
- Van Wessel, T, A De Haan, WJ Van der Laarse, and RT Jaspers. 2010. "The muscle fiber type–fiber size paradox: hypertrophy or oxidative metabolism?" *European journal of applied physiology* 110 (4):665-694.
- Vandebuerie, Francis, Eynde B Vanden, K Vandenberghe, and Peter Hespel. 1998. "Effect of creatine loading on endurance capacity and sprint power in cyclists." *International journal of sports medicine* 19 (7):490-495.
- Vandenberghe, K, Marina Goris, Paul Van Hecke, Marc Van Leemputte, L Vangerven, and Peter Hespel. 1997. "Long-term creatine intake is beneficial to muscle performance during resistance training." *Journal of Applied Physiology* 83 (6):2055-2063.
- Vierck, JANET L, DERI L Icenogge, LUKE Bucci, and MICHAEL V Dodson. 2003. "The effects of ergogenic compounds on myogenic satellite cells." *Medicine and science in sports and exercise* 35 (5):769-776.
- Vijayakumar, Malepillil Vavachan, Sandeep Singh, Rishi Raj Chhipa, and Manoj Kumar Bhat. 2005. "The hypoglycaemic activity of fenugreek seed extract is mediated through the stimulation of an insulin signalling pathway." *British journal of pharmacology* 146 (1):41-48.
- Vincent, EE, DJE Elder, EC Thomas, L Phillips, C Morgan, J Pawade, M Sohail, MT May, MR Hetzel, and JM Tavaré. 2011. "Akt phosphorylation on Thr308 but not on Ser473 correlates with Akt protein kinase activity in human non-small cell lung cancer." *British journal of cancer* 104 (11):1755-1761.
- Vissing, Kristian, Jesper L Andersen, Stephen DR Harridge, Claudia Sandri, Andreas Hartkopp, Michael Kjaer, and Peter Schjerling. 2005. "Gene expression of myogenic factors and phenotype-specific markers in electrically stimulated muscle of paraplegics." *Journal of Applied Physiology* 99 (1):164-172.
- Volek, Jeff S, Noel D Duncan, Scott A Mazzetti, Robert S Staron, Margot Putukian, Al Gomez, David R Pearson, William J Fink, and William J Kraemer. 1999. "Performance and muscle fiber adaptations to creatine supplementation and heavy resistance training." *Medicine and science in sports and exercise* 31:1147-1156.
- Volek, Jeff S, William J Kraemer, Jill A Bush, Mark Boetes, Thomas Incledon, Kristine L Clark, and James M Lynch. 1997. "Creatine supplementation enhances muscular performance during high-intensity resistance exercise." *Journal of the American Dietetic Association* 97 (7):765-770.
- Vorobiev, DV, EG Vetrova, IM Larina, IA Popova, and AI Grigoriev. 1996. "Energy substrates, hormone responses and glucocorticoid binding in lymphocytes during intense physical exercise in humans following phosphocreatine administration." *European journal of applied physiology and occupational physiology* 74 (6):534-540.
- Vyas, Dharmesh R, Espen E Spangenburg, Tsghe W Abraha, Thomas E Childs, and Frank W Booth. 2002. "GSK-3 β negatively regulates skeletal myotube hypertrophy." *American Journal of Physiology-Cell Physiology* 283 (2):C545-C551.
- Walker, J. B. 1979. "Creatine: biosynthesis, regulation, and function." *Adv Enzymol Relat Areas Mol Biol* 50:177-242.
- Wallimann, T., M. Wyss, D. Brdiczka, K. Nicolay, and H. M. Eppenberger. 1992. "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 (Pt 1) (Pt 1):21-40.
- Wang, Xuemin, Anne Beugnet, Mirei Murakami, Shinya Yamanaka, and Christopher G Proud. 2005. "Distinct signaling events downstream of mTOR cooperate to mediate

- the effects of amino acids and insulin on initiation factor 4E-binding proteins." *Molecular and cellular biology* 25 (7):2558-2572.
- Wang, Xuemin, and Christopher G Proud. 2006. "The mTOR pathway in the control of protein synthesis." *Physiology* 21 (5):362-369.
- Wang, Yu Xin, and Michael A Rudnicki. 2012. "Satellite cells, the engines of muscle repair." *Nature reviews Molecular cell biology* 13 (2):127-133.
- Watt, Kenneth KO, Andrew P Garnham, and Rodney J Snow. 2004. "Skeletal muscle total creatine content and creatine transporter gene expression in vegetarians prior to and following creatine supplementation." *International journal of sport nutrition & exercise metabolism* 14 (5).
- Willott, CA, ME Young, B Leighton, GJ Kemp, EA Boehm, GK Radda, and K Clarke. 1999. "Creatine uptake in isolated soleus muscle: kinetics and dependence on sodium, but not on insulin." *Acta physiologica scandinavica* 166 (2):99-104.
- Willoughby, Darryn S, and John M Rosene. 2003. "Effects of oral creatine and resistance training on myogenic regulatory factor expression." *Medicine and science in sports and exercise* 35 (6):923-929.
- Winder, WW. 2001. "Energy-sensing and signaling by AMP-activated protein kinase in skeletal muscle." *Journal of Applied Physiology* 91 (3):1017-1028.
- Wojtaszewski, Jørgen FP, Marina Mourtzakis, Thore Hillig, Bengt Saltin, and Henriette Pilegaard. 2002. "Dissociation of AMPK activity and ACC β phosphorylation in human muscle during prolonged exercise." *Biochemical and biophysical research communications* 298 (3):309-316.
- Wu, Zhenguo, Pamela J Woodring, Kunjan S Bhakta, Kumiko Tamura, Fang Wen, James R Feramisco, Michael Karin, Jean YJ Wang, and Pier Lorenzo Puri. 2000. "p38 and extracellular signal-regulated kinases regulate the myogenic program at multiple steps." *Molecular and cellular biology* 20 (11):3951-3964.
- Xiong, Cheng-Juan, Ping-Fa Li, Yang-Liu Song, Li-Xiang Xue, Zhu-Qing Jia, Chun-Xia Yao, Qing-Xia Wei, Shu-Feng Zhang, Shan-Feng Zhang, and Yan-Yan Zhang. 2013. "Insulin induces C2C12 cell proliferation and apoptosis through regulation of cyclin D1 and BAD expression." *Journal of cellular biochemistry* 114 (12):2708-2717.
- Yaffe, David. 1968. "Retention of differentiation potentialities during prolonged cultivation of myogenic cells." *Proceedings of the National Academy of Sciences* 61 (2):477-483.
- Yang, Yifan, Andrew Creer, Bozena Jemiolo, and Scott Trappe. 2005. "Time course of myogenic and metabolic gene expression in response to acute exercise in human skeletal muscle." *Journal of applied physiology* 98 (5):1745-1752.
- Zaid, Hilal. 2013. "In vitro evaluations of cytotoxicity of eight antidiabetic medicinal plants and their effect on GLUT4 translocation." *Evidence-Based Complementary and Alternative Medicine* 2013.
- Zammit, Peter S, Jon P Golding, Yosuke Nagata, Valérie Hudon, Terence A Partridge, and Jonathan R Beauchamp. 2004. "Muscle satellite cells adopt divergent fates a mechanism for self-renewal?" *The Journal of cell biology* 166 (3):347-357.
- Zammit, Peter S, Frederic Relaix, Yosuke Nagata, Ana Pérez Ruiz, Charlotte A Collins, Terence A Partridge, and Jonathan R Beauchamp. 2006. "Pax7 and myogenic progression in skeletal muscle satellite cells." *Journal of cell science* 119 (9):1824-1832.
- Zetser, Anna, Eran Gredinger, and Eyal Bengal. 1999. "p38 mitogen-activated protein kinase pathway promotes skeletal muscle differentiation participation of the MEF2C transcription factor." *Journal of Biological Chemistry* 274 (8):5193-5200.

- Ziegenfuss, Tim N, Lonnie M Lowery, and Peter WR Lemon. 1998. "Acute fluid volume changes in men during three days of creatine supplementation." *Journal of Exercise Physiology Online*.
- Zierath, Juleen R. 2002. "Invited review: exercise training-induced changes in insulin signaling in skeletal muscle." *Journal of Applied Physiology* 93 (2):773-781.
- Zorzano, Antonio, César FANDOS, and Manuel PALACÍN. 2000. "Role of plasma membrane transporters in muscle metabolism." *Biochemical Journal* 349 (3):667-688.

CHAPTER 9: Research Portfolio Appendix

APPENDIX I: Publication Contributions

Chapter 2: Literature Review

Tomcik KA, Hawley JA, Burke LM. (2016) The Interactive Roles of Muscel Creatine and Glycogen: New Insights (Commentary). *Sports Medicine*. Under review.

Contribution statement:

KAT was primarily responsible for writing and submitting the commentary manuscript. JAH and LMB assisted in writing and reviewing the manuscript.

Approximate percentage contributions:

Tomcik KA 60%; Hawley JA 20%; Burke LM 20%.

I acknowledge, as primary author, that my contribution to the aforementioned study is as stated:



9 December 2016

Kristylen A Tomcik

Date

I certify, as primary supervisor, on behalf of all co-authors that the above is true and correct:



9 December 2016

Prof. John A Hawley

Date

Chapter 4: STUDY 1

Tomcik KA, Camera DM, Bone JL, Ross ML, Jeacocke NA, Tachtsis B, Senden J, van Loon LJC, Hawley JA, Burke LM. (2016) Effects of Combined Creatine and Carbohydrate Loading on Cycling Time Trial Performance. *Medicine & Science in Sports and Exercise*. Under second review (Manuscript # MSSE-D-16-01178).

Contribution statement:

KAT was primarily responsible for gaining ethical approval from Australian Catholic University, experimental design, participant recruitment, data collection, data analysis, statistical analysis, writing and submitting the manuscript and addressing reviewer's comments. MLR, NAJ, JAH, and LMB were involved in experimental design. DMC, BT, JS, and LJCvL assisted in data analysis. JLB and MLR assisted in data collection and subject recruitment. DMC, JAH and LMB assisted in writing and reviewing the manuscript. LMB, MLR and JLB were involved in gaining ethical approval from the Australian Institute of Sport.

Approximate percentage contributions:

Tomcik KA 60%; Camera DM 5%; Bone JL: 2.5%; Ross ML 2.5%; Jeacocke NA 2.5 %; Tachtsis B 2.5 %; Senden J 2.5%; van Loon LJC 2.5%; Hawley JA 10%; Burke LM 10%.

I acknowledge, as primary author, that my contribution to the aforementioned study is as stated:



9 December 2016

Kristy A Tomcik

Date

I certify, as primary supervisor, on behalf of all co-authors that the above is true and correct:



9 December 2016

Prof. John A Hawley

Date

Chapter 5: STUDY 2

Tomcik KA, Hawley JA, Lacham-Kaplan O. (2016) The *in vitro* Effects of Creatine and Insulin on Skeletal Myotubes Cultured in Different Glucose Concentrations. *Journal of Nutritional Biochemistry*. Under Review (manuscript # JNB_2016_593).

Contribution statement:

KAT was primarily responsible for experimental design, data collection, data analysis, statistical analysis, and wrote and submitted the manuscript. OLK was involved in experimental design, data collection and data analysis. OLK and JAH assisted in writing and reviewing the manuscript.

Approximate percentage contributions:

Tomcik KA 70%; Hawley JA 5%; Lacham-Kaplan O 25%.

I acknowledge, as primary author, that my contribution to the aforementioned study is as stated:



9 December 2016

Kristylen A Tomcik

Date

I certify, as primary supervisor, on behalf of all co-authors that the above is true and correct:



9 December 2016

Prof. John A Hawley

Date

Chapter 6: STUDY 3

Tomcik KA, Smiles WJ, Camera DM, H Hgel HM, Hawley JA, Watts R. (2016). Fenugreek Increases Insulin-Stimulated Creatine Content in L6C11 Muscle Myotubes. *European Journal of Nutrition*. pp 1-7. DOI: 10.1007/s00394-015-1145-1

Due to copyright restrictions, the published version of this article is not available here. Please view the published version online at: <http://dx.doi.org/10.1007/s00394-015-1145-1>

Contribution statement:

KAT was primarily responsible for experimental design, data collection, data analysis, statistical analysis, and writing and submitted the manuscript, addressing reviewer's comments, and approving final proofs. RW was involved in experimental design. WJS, DMC, and HMH assisted in data analysis. RW and JAH assisted in writing and reviewing the manuscript.

Approximate percentage contributions:

Tomcik KA 70%; Smiles WJ 2.5%; Camera DM 5%; Hgel HM 2.5%; Hawley JA 10%; Watts R10%.

I acknowledge, as primary author, that my contribution to the aforementioned study is as stated:



9 December 2016

Kristy A Tomcik

Date

I certify, as primary supervisor, on behalf of all co-authors that the above is true and correct:



9 December 2016

Prof. John A Hawley

Date

APPENDIX II: Ethics Approval, Letters to Participants and Consent Forms



Human Ethics Application


Application ID :	2014 2544
Application Title :	Interactive effect of manipulating muscle creatine and glycogen stores on endurance performance and DXA measurements of lean mass
Date of Submission :	21/08/2014
Primary Investigator :	Dr Louise Burke
Other Investigators :	Julia Bone Grishen Tomdik Prof John Hawley


Introduction

Introduction

Introduction

You will be asked a series of questions related to your external ethics approval.

The help icon () provides help / examples to relevant questions / page.

You may save your progress at any point by clicking on the save button () near the top-right corner of the page.

Please click on the green arrow () below-right to continue

Contact

If you have any questions regarding your Ethics application, please contact the ETHICS TEAM on:

Phone: (02) 9739 2646

Email: res.ethics@acu.edu.au

References

- [National Statement on Ethical Conduct in Human Research](#)
- [Australian Code for the Responsible Conduct of Research](#)
- [ACU Code of Conduct for Research](#)
- [ACU Ethics website including Guidelines for Applicants](#)
- [NHMRC ethics page](#)

Section A: Application

A.1: General Information

[Registration](#) of external ethics approval:

A.1.1 Application ID

2014 254N

A.1.2 Application Date (to be filled by Orion)

21/08/2014

A.1.3 School*

Nat Sch Exercise Science

A.1.4 Ethics category*

Human External Approval
Registration

A.1.5 What is the formal title of the research project? *

Interactive effect of manipulating muscle creatine and glycogen stores on endurance performance and DXA measurements of lean mass

A.2: Application Details

A.2.1 Please provide details of other HRECs and attach the approval documentation (at Section A.6).

The original HREC application must be attached together with the approvals and any other supporting documentation.

Australian Institute of Sport (AIS) - 23/05/2014
AIS - 30/06/2014

A.2.2 Description of the project in plain language (National Statement, 2007, s.1.2).

Give a concise and simple description (not more than 400 words), in plain language, of the:

- Aims of the project
- The proposal research design
- The methods to be used to achieve those aims

In addition, please attach a 2-4 page research proposal outlining of the research design, objectives and methodology.*

Individual protocols to increase the muscle content of glycogen (carbohydrate) and creatine (Cr) are commonly undertaken by athletes to increase their capacity for endurance and repeated high-intensity exercise respectively. Two novel areas regarding creatine and glycogen loading

will be examined within a single study. The first involves the interaction of creatine and glycogen loading on endurance performance. An early study found impaired performance of a 6 km cross country run following Cr loading; this was attributed to the weight gain associated with Cr loading and the lack of a limiting role for muscle Cr in such an event. Newer studies, however, have shown that glycogen storage is increased in a previously Cr-loaded muscle due to intracellular swelling. The functional outcome of these findings (performance of a glycogen-limited exercise task) is unknown. The second will study the effect of systematically altering the creatine and glycogen content of the skeletal muscle on DXA-derived estimates of lean mass in athletes. This continues our work to establish Best Practice Protocols for the reliable monitoring of body composition of athletes using whole body DXA scans. Our current protocol achieves reliable estimation of lean mass and fat mass with a test-retest error of 0.5 kg and 0.3 kg respectively, yet longitudinal monitoring of athletes over a season of training reveals unexplained between-assessment fluctuations of ~ 1 kg in lean mass which could be significant for athletic performance, but appear to be artefacts despite our standardisation protocols. Systematically investigating the DXA errors in lean mass estimates that can be attributed to acute changes in muscle creatine and glycogen (and their effect of cellular hydration), will help us to further refine the DXA protocol and/or better interpret the results.

This study also provides an opportunity to validate the use of a patented and commercialised methodology and software technology (MuscleSound®) which claims it provides a non-invasive measurement of muscle glycogen content, against our gold standard muscle biopsy technique.

A.2.3 Start Date (project)*

11/08/2014

A.2.4 Anticipated Finish Date (project)*

30/05/2015

A.2.5 Start Date (data collection)

09/09/2014

A.2.6 Anticipated Finish Date (data collection)

30/05/2015

[Note: Multi-year approval may be given by the Committee. All projects, however, are subject to annual review. The Annual Renewal of projects is covered by the Progress/Final Report Form. Extensions beyond the approved durations are also covered by this form]

A.3: Researcher Details

A.3.1 Is this a student application?*

☐ Yes ☒ No

Please ensure:

- Exactly 1 **Chief (Primary) Investigator** is present
- Chief Investigator is an **ACU staff member**
- The Chief Investigator is selected as **"Primary"**
- **Required details of the research / investigator are filled in** (click on their name to bring up their details):
 - Position of investigator
 - Qualifications
 - Expertise relevant to this project

A.3.2 Researchers / Investigators:

Can't find a student? Click here to add from Banner*

1	Title	Dr
	Given Name	Louise
	Surname	Burke
	Full Name	Dr Louise Burke
	Gender	Female
	Work Number	
	Mobile Number	
	Home Number	
	Email Address	Louise.Burke@acu.edu.au
	School	621N
	Faculty	FHS
	Organisation	Australian Institute of Sport
	Organisation Name (Enter the organisation name here if you cannot find it above)	Australian Institute of Sport
	Primary?	Yes
	Position of investigator	Chief Investigator
	Qualifications	PhD & Deakin University BSc (Nutr) Deakin University Grad Dip Diet Deakin University Accredited Practising Dietitian

	Expertise relevant to this project	Co-investigator in carbohydrate loading, muscle glycogen and creatine research >150 peer reviewed papers and book chapters across various sport nutrition topics
2	Title	Not Specified
	Given Name	Julia
	Surname	Bone
	Full Name	Julia Bone
	Gender	Female
	Work Number	0433472566
	Mobile Number	0487780843
	Home Number	
	Email Address	jlbone001@myacu.edu.au
	School	621N
	Faculty	RHS
	Organisation	
	Organisation Name (Enter the organisation name here if you cannot find it above)	
	Primary?	No
	Position of investigator	HDR Student
	Qualifications	BAppSc (Sports & Exercise Nutrition) (Hons) University of Otago MSc (Human Nutrition) University of Otago MDietStud The University of Queensland ANZBMS Certified DXA technician
	Expertise relevant to this project	Previous research experience in study design, data collection, analytical lab work and data analysis and interpretation. AIS DXA technician
3	Title	Not Specified
	Given Name	Kristyen
	Surname	Tomcik
	Full Name	Kristyen Tomcik
	Gender	Male
	Work Number	
	Mobile Number	0405903080
	Home Number	
	Email Address	kktomc001@myacu.edu.au
	School	621N
	Faculty	RHS
	Organisation	
	Organisation Name (Enter the organisation name here if you cannot find it above)	
	Primary?	No
	Position of investigator	HDR Student
	Qualifications	PhD - Candidate ACU
	Expertise relevant to this project	Laboratory assay work
4	Title	Prof
	Given Name	John
	Surname	Hawley
	Full Name	Prof John Hawley
	Gender	Male
	Work Number	+6139953 3039
	Mobile Number	
	Home Number	
	Email Address	John.Hawley@acu.edu.au
	School	621N
	Faculty	RHS
	Organisation	Royal Melbourne Institute of Technology

Organisation name (Enter the organisation name here if you cannot find it above)	Royal Melbourne Institute of Technology
Primary?	No
Position of Investigator	Co-Investigator
Qualifications	PhD
Expertise relevant to this project	Head of the Exercise & Nutrition Research Group Professor of Exercise Metabolism in the Department of Exercise Sciences.

A.3.3 Primary Contact:

Dr Louise Mary Burke

 If you cannot continue to the next page, please check to ensure you have selected a primary investigator.

A.4: Level of Risk

A.4.1 Please indicate the level of risk to the participant in this research: ¹

- ☐ More than Low Risk
☒ Low Risk
☐ Negligible Risk

A.5: Participants

A.5.1 Does your project specifically target Aboriginal and Torres Strait Islander participants? ¹

- ☐ Yes ☒ No

A.5.2 Does your project specifically target minors? ¹

- ☐ Yes ☒ No

A.6: Attachments

A.6.1 Please attach the relevant documents listed below (please use ZIP file if there are more than 1 document per item): (Please refer to details in help for items with an asterisk next to them)

[Investigator's Signatures template](#)

Description	Reference	Soft copy	Hard copy
Research Proposal	Creatine_Glycozen_Study_Research_Protocol_AQU.docm	✓	
Participant Information Letter	Information to Participants Updated.doc	✓	
External Ethics Application	creatine glycozen ethics application (2).pdf	✓	
External HRE Approval letters	Ethics Approval Cr-Gly Study.pdf	✓	
Consent Form	JKFOR MED CONSENT.doc	✓	
Advertisement text / script	AIS Cr-Gly Study_Plan.doc	✓	
Questionnaires / sample interview questions	MUSCLE BIOPSY QUESTIONNAIRE.doc	✓	
Investigator's Signatures**	AQU-Investigators Signatures.pdf	✓	
Minor Variation application of ultrasound	Minor variation Creatine-Gly Study Bone_20140612.pdf	✓	
Minor Variation Approval letter	Approval Minor variation Cr-Gly study 20140612.pdf	✓	

** Investigator's Signatures: All investigators in section C.1.3 **MUST** sign the [signature document](#) to acknowledge their participation in the specified research project, unless:

- There is only 1 investigator, who creates the application - the univue login to Orion serves as an electronic signature.
- Applications created by students, where there are only 1 supervisor and 1 student listed - the supervisor will be required to sign off on the application after the student has submitted the application

Section B: Office Use Only

B.1: Ethics Officer Review

B.1.1 Risk Assessment Outcome¹

29/11/2016

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Multi Site

Continue

Next

Please click the "Submit Application" action in the "Action" tab to continue (2nd tab on the left).

Date of Submission: August 25 2014

Resubmission / Version #: **R2** (Required for minor variations and resubmissions, please include original approval number followed by version number .R1 for first revision / .R2 for second revision)

Project Title: Interactive effect of manipulating muscle creatine and glycogen stores on endurance performance and DXA measurements of lean mass.

Principal Researcher	Organisation	Contact details
Louise Burke	Australian Institute of Sport Postal address: PO Box 176, Belconnen, ACT 2617	Tel: 02 6214 1351 Email: louise.burke@ausport.gov.au

Co-Researchers	Organisation(s)
1.Kristyen Tomick	Australian Catholic University
2.John Hawley	Australian Catholic University
3.Julia Bone	Sports Nutrition, Australian Institute of Sport

Brief description of the project:

Individual protocols to increase the muscle content of glycogen (carbohydrate) and creatine (Cr) are commonly undertaken by athletes to increase their capacity for endurance and repeated high-intensity exercise respectively. Two novel areas regarding creatine and glycogen loading will be examined within a single study. **The first involves the interaction of creatine and glycogen loading on endurance performance.** An early study found impaired performance of a 6 km cross country run following Cr loading [1]; this was attributed to the weight gain associated with Cr loading and the lack of a limiting role for muscle Cr in such an event. Newer studies, however, have shown that glycogen storage is increased in a previously Cr-loaded muscle due to intracellular swelling [10,12,13]. The functional outcome of these findings (performance of a glycogen-limited exercise task) is unknown. **The second will study the effect of systematically altering the creatine and glycogen content of the skeletal muscle on DXA-derived estimates of lean mass in athletes.** This continues our work to establish Best Practice Protocols for the reliable monitoring of body composition of athletes using whole body DXA scans [8,9]. Our current protocol achieves reliable estimation of lean mass and fat mass with a test-retest error of 0.5 kg and 0.3 kg respectively, yet longitudinal monitoring of athletes over a season of training reveals unexplained between-assessment fluctuations of ~ 1 kg in lean mass which could be significant for athletic performance, but appear to be artefacts despite our standardisation protocols. Systematically investigating the DXA errors in lean mass estimates that can be attributed to acute changes in muscle creatine and glycogen (and their effect of cellular hydration), will help us to further refine the DXA protocol and/or better interpret the results.

Project Aim(s):

1. The **specific aim** of the first part of this project is to investigate whether prior creatine loading (5 d) enhances muscle glycogen loading, **measured** via muscle biopsy; and to determine its impact on subsequent performance of a weight-sensitive cycling protocol. This outcome is **highly novel and relevant** and will potentially provide a new application for creatine supplements in sports nutrition.
2. A **second specific aim**, is to investigate the alterations in estimates of lean mass, **measured** using DXA, due to creatine loading and the depletion and supercompensation of muscle glycogen stores. This application is **highly relevant** as it will enhance the practice and interpretation of a commonly available tool for body composition assessment.

Background: (This box is limited in size, what you see is all the area you get, please include references at the bottom of the page)

Please include the statement of the problem, relevant literature and justification for the study.

Concentrations of skeletal muscle substrates can be manipulated by diet and exercise. For example, the muscle content of Cr is elevated by ~10-40% above resting levels after five days of supplementation with Cr monohydrate [3,4]. The resting content of the muscle glycogen is increased by training but can be increased by a further ~50% by carbohydrate (CHO) loading in which an athlete rests or reduces training and consumes a CHO-rich diet for 24-48 hours [5]. Conversely, a bout of prolonged exercise, particularly involving intermittent efforts of high-intensity work, can deplete muscle glycogen stores to very low levels. Nutritional strategies that increase muscle substrate concentrations are commonly used by athletes to enhance performance in workouts or events which would otherwise be limited by the depletion of these substrates. For example, Cr loading enhances performance repeated bouts of high-intensity work interspersed by short rest intervals in which there is inadequate recovery of and a gradual decline in, muscle phosphocreatine stores [3]. It is therefore most associated as a training aid for athletes who undertake resistance training or interval training (e.g. swimmers, rowers, body builders) or who compete in sports of a “stop and go” nature (e.g. team sports) [7]. By comparison, CHO loading is associated with endurance events where it promotes performance by delaying the point at which the athlete “hits the wall” due to the depletion of muscle glycogen stores [5].

More recent discoveries have identified the potential for intracellular substrates to facilitate the adaptations to exercise. Paradoxically, undertaking aerobic exercise with *low* muscle glycogen stores has been shown to enhance the cascade of signalling pathways which underpin the adaptive responses associated with endurance training; this had led to “train low” strategies which purposefully manipulate low glycogen stores prior to, and even after, some training sessions [6]. Furthermore, the storage of additional creatine and water within the muscle cell, causing cellular swelling, has been associated with an upregulation of the activation of a large number of genes involved in protein and glycogen synthesis, satellite cell proliferation and differentiation, and DNA replication and repair [12]. Therefore creatine loading could play other roles in athletic preparation apart from simply providing a substrate for high-intensity exercise.

Although the principal reason for manipulating muscle substrate content is to enhance performance, training capacity or training adaptations, a side-effect of these strategies is a small but potentially detectable change in muscle mass or volume due to changes in muscle solute content and the binding/storing of water to counter the alteration in intracellular osmotic load. Indeed, carbohydrate loading and creatine loading are both known to cause a small (~1-2 kg) but acute increase in body mass principally via additional storage of intracellular water [11,12,14]. These acute changes have been detected in terms of an increase in muscle cross-sectional area via Magnetic Resonance Imaging (MRI) in the case of glycogen loading [11] and an increase in lean mass via DXA measurements of body composition in the case of creatine loading [12].

The project will address two separate areas of knowledge regarding creatine and glycogen loading. The first theme focuses on the potential application of creatine supplementation to the preparation of endurance athletes. The second theme addresses the possibility that alterations in these muscle substrates introduce an artefact into DXA estimates of body composition in athletes. The novelty of this research includes the potential for a new application of creatine supplementation and a new population of athletes who might benefit from it. The study design is innovative in tackling this theme for the first time, in constructing a laboratory-based performance test that is weight-sensitive and glycogen limited, and in allowing a secondary data set from the study to investigate the separate issue of the reliability of DXA estimates of physique. This research will potentially produce two separate and practical outcomes: 1) a new application for creatine supplementation involving a novel nutritional strategy for endurance/ultra-endurance athletes and 2) an opportunity to enhance the reliability and/or interpretation of DXA estimates of body composition. Both will have widespread use in sports nutrition.

1. Balsom PD, Hamidge SD, Söderlund K, Sjodin B, Ekblom B. Creatine supplementation per se does not enhance endurance exercise performance. *Acta Physiol Scand*. 149(4):521-3, 1993.
2. Ebert TR, Martin DT, Bullock N, Mujika I, Quod MJ, Farthing LA, Burke LM, Withers RT. Influence of hydration status on thermoregulation and cycling hill climbing. *Med Sci Sports Exerc*. 39(2):323-9, 2007.
3. Greenhaff PL, Casey A, Short AH, Harris R, Soderlund K, Hultman E. Influence of oral creatine supplementation of muscle torque during repeated bouts of maximal voluntary exercise in man. *Clin Sci (Lond)* 84: 565-571, 1993.
4. Harris RC, Soderlund K, Hultman E. Elevation of creatine in resting and exercised muscle of normal subjects by creatine supplementation. *Clin Sci (Lond)* 83: 367-374, 1992.
5. Hawley JA, Schabert EJ, Noakes TD, Dennis SC. Carbohydrate-loading and exercise performance: An update. *Sports Med*. 24(2):73-91, 1997.
6. Hawley JA, Burke LM. Carbohydrate availability and training adaptation: effects on cell metabolism. *Exerc Sport Sci Rev*. 38(4):152-60, 2010.
7. Juhn MS, Tamopolsky M. Oral creatine supplementation and athletic performance: a critical review. *Clin J Sport Med* 8: 286-297, 1998.
8. Nana A, Slater GJ, Hopkins WG, Burke LM. Effects of daily activities on dual-energy X-ray absorptiometry measurements of body composition in active people. *Med Sci Sports Exerc*. 44(1):180-9, 2012.
9. Nana A, Slater GJ, Hopkins WG, Burke LM. Effects of exercise sessions on DXA measurements of body composition in active people. *Med Sci Sports Exerc*. 45(1):178-85, 2013.
10. Nelson AG, Arnall DA, Kokkonen J, Day R, Evans J. Muscle glycogen supercompensation is enhanced by prior creatine supplementation. *Med Sci Sports Exerc*. 33(7):1096-100, 2001.
11. Nygren AT, Karlsson M, Norman B, Kaijser L. Effect of glycogen loading on skeletal muscle cross-sectional area and T2 relaxation time. *Acta Physiol Scand*. 173(4):385-90, 2001.
12. Safdar A, Yardley NJ, Snow R, Melov S, Tamopolsky MA. Global and targeted gene expression and protein content in skeletal muscle of young men following short-term creatine monohydrate supplementation. *Physiol Genomics* 32(2):219-28, 2008.
13. van Loon LJ, Murphy R, Oosterlaar AM, Cameron-Smith D, Hargreaves M, Wagenmakers AJ, Snow R. Creatine supplementation increases glycogen storage but not GLUT-4 expression in human skeletal muscle. *Clin Sci (Lond)*. 106(1):99-106, 2004.

Subject Information and Recruitment

Does the project involve subjects who are: Yes No N/A

Mentally Disabled (NS 5)? ☐ ☒ ☐

Physically Disabled (NS 6)? ☐ ☒ ☐

Minors (<18yrs) (NS 4.1, NS 4.2)? ☐ ☒ ☐

How are you recruiting subjects:

E-mail? ☒ ☐ ☐

Word of mouth? ☒ ☐ ☐

Referral? ☐ ☐ ☐

Direct correspondence with a team or coach? ☐ ☐ ☐

Other? Please elaborate

Will subjects receive any monetary or other benefits ☒ ☐ ☐

for their participation (NS 1.10)?

If **Yes** please provide further detail

1. Subjects will receive an honorarium of \$600AUD to cover travel and food expenses, as well as the time involved in fulfilling the requirements of the study as well as.
2. To provide an incentive for performance during the time trials, a prize money scheme will be devised to reward performances outcomes relative to standardised parameters that give each cyclist an equal chance of being rewarded. Although the prize pool will be relatively minor (\$10,000 shared between 24 subjects), past experiences have shown this to be a valuable way to promote within-subject competitiveness.

Description of Subjects:

Projected Number of participants:24

Number of male: 24

Number of Female: 0

Sport/s: Cycling / Triathlon

Age Range: 18-40 y

Institutions involved: Bike Clubs, Triathlon clubs and chat rooms

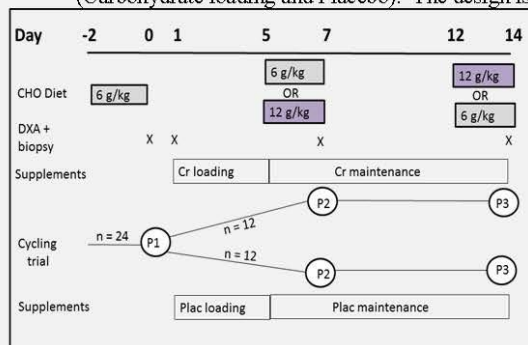
Athletic Status (Elite, sub-elite, novice, recreational, sedentary): Sub-elite

Criteria for participation:

- **Inclusion:** Participants should be sub-elite cyclists or triathletes, aged 18-40 y who are currently completing a weekly cycling training load of > 250 km/week and have a > 2 y history of competing in road races or triathlons.
- **Exclusion:** 1. History of abnormal bleeding/clotting or needle phobia. 2. Exclusion criteria for having 4 whole body DXA scan (e.g. exposure to recent radiation load from medical imaging procedures) and > 6 wk free of Creatine supplementation

Methodology: (Please use the grey text boxes)

- **Experimental Design:** Parallel group design with two separate cohorts (Creatine loading and Placebo) who each undertake a cross-over application of the second intervention (Carbohydrate loading and Placebo). The design is summarised in the following table



Detailed Methodology:

Test Protocols: Subjects will undergo preliminary testing to characterise VO_2max and peak power output (PPO) and to undertake a familiarisation of two of the key performance measurements intended for the cycling trial:

1. A 60 km version (representing half) of the main exercise protocol involving cycling at a set speed on a Velotron ergometer interspersed with 1 km and 4 km sprints (as fast as possible) alternating every 10 km (10-11 km, 20-24 km, 30-31 km, 40-44 km, 50-51 km, 56-60 km). These sprints represent a weight-supported test of high-intensity performance
2. time-to-exhaustion cycling @ a speed equivalent to 88% VO_2max on the subjects' own bicycle on an inclined treadmill (8%) to simulate a weight-dependent cycling hill-climb [2]

To ensure that subjects are comfortable with the pacing strategies and technical elements of these

protocols, they will be offered a second occasion to undertake a familiarisation trial within the next week. Prior to the first Performance Trial (P1), all subjects will be provided with a pre-packaged diet providing a controlled carbohydrate intake (6 g/kg/d) for 48 hours. Standardised training and hydration strategies will be followed over the last 24 hours.

Subjects will report to the laboratory on the morning of P1 after an overnight fast and will undertake a whole body DXA scan following the AIS Best Practice Protocol [9] followed by measurement of total body water via Bioelectrical Impedance Spectroscopy (BIS). A muscle sample will then be collected using the subcutaneous biopsy technique for measurement of muscle glycogen and creatine content. Subjects will then consume a standardised pre-event meal (2 g/kg carbohydrate) and rest for 2 hours prior to undertaking the Performance trial

The Performance trial will consist of 120 km of cycling on the Velotron cycle ergometer at a set pace during which subjects will be instructed to undertake ten intermittent bouts of high-intensity exercise (alternating 1 km and 4 km segments at 10-14, 20-21, 30-34, 40-41, 50-54, 60-61, 70-74, 80-81, 90-94, 100-101, 110-114, 119-120 km) "as fast as possible". This protocol is expected to lead to significant depletion of muscle glycogen stores. During this trial, they will be required to follow a standardised hydration plan and carbohydrate intake (60 g/h). Once completed, subjects will quickly mount their own cycles and complete the time to exhaustion "hill climb" on the inclined treadmill, as previously discussed.

Following the completion of P1, subjects will be provided with a pre-packaged carbohydrate-restricted (< 1 g/kg) diet to prevent significant repletion of muscle glycogen stores over the next 15-18 hours. A repeat DXA and muscle biopsy will be undertaken the following morning, again using the AIS Best Practice Protocol which requires rested, fasted conditions.

Subjects will be pair-matched into two groups based on PPO (W/kg), results of P1 and DXA lean mass estimates. Group 1 will be allocated a validated creatine loading and maintenance protocol for the next 2 weeks (5 d @ 20 g/d + 9 days @ 3 g/d) while Group 2 will receive a placebo treatment. Treatments will be allocated in a double-blinded protocol by placing creatine monohydrate powder or polyjoule in capsules for consumption. The creatine monohydrate will be sourced from a company with sound manufacturing processes to ensure that it is pure and at low risk of being contaminated.

Over the next 2 weeks, subjects will undertake 2 further Performance Trials (P2 and P3), one week apart. These trials will be undertaken in a double-blinded, placebo-controlled design in which subjects will receive pre-packaged diets (normal carbohydrate = 6 g/kg BM/d or Glycogen loading = 12 g/kg/d) for the 48 hours prior to the Performance trial. The diets will be blinded in terms of carbohydrate content by providing the carbohydrate in the form of an artificially sweetened drink and jelly which may or may not contain a glucose polymer (Polycose). This will serve a second goal of matching the fibre content of the diets in terms of gastrointestinal contents.

Data Analysis:

- DXA and BIS measurements on Days 0, 1, 7 and 14 will allow the following estimates of BM and lean mass to be made under conditions of
 - resting glycogen (Day 0);
 - glycogen depletion (Day 1);
 - glycogen supercompensation (Day 7 or 14 – Group 2);
 - creatine loaded/resting glycogen (Day 7 or 14 – Group 1)
 - creatine loaded/glycogen supercompensation (Day 7 or 14 - Group 1)
- Blood lactate concentrations post sprint (within 120 km TT) and 5 min post hill climb TTE
- Analysis of muscle samples taken on Days 0, 1, 7 and 14 will allow the investigation of the

effects of creatine loading on normal glycogen storage and ability to supercompensate glycogen stores using a recognised glycogen loading protocol in endurance-trained muscle

- Performance trials on Days 0, 7 and 14 will allow the investigation of the effect of individual and interactive effects of creatine loading and glycogen loading on the performance of a glycogen limited cycling protocol, with the following individual parameters
 - Performance of 12 higher intensity segments (6 x 4 km and 6 x 1 km) interspersed throughout the 120 km (weight supported)
 - Time to exhaustion of hill climbing at a set speed (weight sensitive)

This protocol has some ecological validity to the demands of a road race in cycling (individual road race or stage within a cycling tour) in which there are changing work intensities due to riding tactics within the peloton and changes in terrain, and in which the stage finishes with a prolonged hill climb. During hill climbing, the rider must attempt to stay with the pre-determined speed of a group bunch or peloton to retain the advantage of drafting. An inability to maintain this speed will result in being “dropped” and having to ride individually. This justifies the use of the open ended measurement of endurance in this circumstance to maintain this speed will result in being “dropped” and having to ride individually.

Ethical Considerations:

Biomedical Procedures:

Yes No N/A

Does this proposal involve Biomedical Procedures, drugs or chemical agents (NS 14, NS 15)?

☒ ☐ ☐

If **YES** explain the procedures:

1. Muscle biopsies will be undertaken by an experienced physician according to standardised procedures that have been routinely undertaken by the current group of researchers and within the AIS Physiology lab. A Best Practice Protocol has been previously prepared for this procedure. Each subject will have 4 biopsies
2. Capillary blood samples will be taken post sprint (12 samples) and post hill climb (1 sample) for measurement of blood lactate concentration via Lactate-Pro

Has a qualified medical practitioner approved the procedures: ☒ ☐

If **NO** explain why:

*Please ensure medical officer signs the bottom of this application.

Radiation Exposure:

Will this study involve ionising radiation, non-ionising radiation or high intensity sound (Including DEXA) (NS 10)? ☒ ☐

If <u>YES</u> , have you sought advice from the external Radiation Safety Committee?	<input checked="" type="checkbox"/>	<input type="checkbox"/>
<u>Blinding:</u>		
Does this proposal involve procedures specifically designed to directly modify the knowledge, thinking, attitudes, feelings or other aspects of the behaviour of subjects (NS 17.1 NS 17.2)?	<input checked="" type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>
If <u>YES</u> , does this study involve giving false/misleading information to subjects or withholding information such that their "informed" consent is in question (NS 17.1, NS 17.2)?	<input type="checkbox"/>	<input checked="" type="checkbox"/>
 <u>Procedural:</u>		
Are the procedures new or innovative (not established) (NS 13)?	<input type="checkbox"/>	<input checked="" type="checkbox"/> <input type="checkbox"/>
Explain:		
Will the procedures cause and degree of discomfort, harassment, invasion of privacy, risk of physical injury, threat to dignity of subjects or be otherwise potentially harmful to subjects (NS 1.3)?	<input checked="" type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>
Please provide further detail: DXA does expose subjects to radiation (0.5µSv per one whole body scan), it is at very low levels, less than that during a 7h aeroplane (~50µSv) and much less than the typical radiation exposure with conventional x-rays (25-60 µSv). The collection of biopsies is an invasive process and some participants may have a phobia of needles. However all testing will be performed by an appropriately qualified professional to minimise any discomfort and will not be expected to carry any negative health consequences.		
<u>Security and Anonymity:</u>		
Describe how you will maintain the anonymity of participants (NS 1.19, NS 15.9, NS 18)?		
Confidential information will be kept by the principal investigator in a lockable filing cabinet and in a personal computer protected by password. In addition, the participants will be identified by number only. All address lists will be kept securely in the primary investigator's care and only be available to the primary investigator and appropriate co-researchers. The presentation of results will not make reference to individual participants when the data are published. Personal results will be provided to each participant. Raw data will be retained by the principal researcher for five years in a secure		

cabinet at Australian Institute of Sport. At the conclusion of this five year period all material containing confidential information will be destroyed.

What will be gained by undertaking the research?

Explain how the benefits outweigh the risks (NS 1.14)?

- The project will address four separate areas of knowledge regarding creatine and glycogen loading.
- The novelty of this research includes the potential for a new application of creatine supplementation and a new population of athletes who might benefit from it. The study design is innovative in tackling this theme for the first time, in constructing a laboratory-based performance test that is glycogen limited and includes weight independent and weight-sensitive elements. Furthermore, the design allows a secondary data set from the study to investigate the separate issue of the reliability of DXA estimates of physique

Project Details: (Note: Time frame has been amended to acknowledge delay in signing contract: see general research agreement)

<u>Proposed time-frame</u> (NS 1.1.6):	Month / Year
Design:	02 / 2014
Ethics:	03 / 2014
Recruitment:	04-05 / 2014
Commencement:	09 / 2014
Data Analysis:	06-09 / 2015
Report:	12 / 2015
Absolute completion:	12 / 2015

Give estimates for:

Total average time required for subject's participation (in hours)- Total average time required for subject's participation (in hours)- maximum of 40 hours spread over 3 testing days and 1-2 familiarisation/preliminary testing days

The total number of items if questionnaires/tests are involved- N/A

(Please note that Ethics Approval is only valid for 3 months post proposed written completion date, notification to the Committee via the Secretary will be required for an extension to the time frame)

Budget required: 80,000

Approved level of funding: \$ 80,000

Source(s) of funding: ACU Project Grant provided to Louise Burke

Note: budget has been amended to allow additional data measurements. Funding is available from ACU project grant

How will the results of the study be implemented or used? (eg what impact will the results have on the daily training environment)

- This research will potentially produce two separate and practical outcomes: 1) a new application for creatine supplementation involving a novel nutritional strategy for endurance/ultra-endurance athletes and 2) an opportunity to enhance the reliability and/or interpretation of DXA estimates of body composition. Both will have widespread use in sports nutrition. Both aspects could change practice of working with elite athletes. The new creatine/glycogen loading strategy will have application to several highly ranked individuals within the AWE pathway (e.g. Jared Tallent - 50 km race walker and Jess Trengrove - marathon runner) as well as other HP athletes (e.g. road cyclists). Meanwhile a more reliable or better interpreted DXA method of body composition assessment will have application across a wide number of athlete servicing and research activities.

How will the results of the study be presented? (eg written report, published papers, thesis, conference, seminars)

- A written report will be made available to appropriate sports science professionals and coaches within the AWE environment to publicise the results and recommended changes in practice. Other venues for communicating results (e.g. HP conferences, coaches meetings, PIT meetings in Rio preparations) will be identified and utilised.
- The results of the proposed study will be further published in appropriate peer-reviewed scientific literature and presented at relevant national/international conferences. The timing of such wider communication will be considered in view of the opportunity to retain important IP for Rio Preparation.

Appendix 2: Project budget

Item	Based on n= 24	
	Quantity	Price
EQUIPMENT		
Gas Calibration and consumables: subject testing		
\$50 per subject	1200	\$1200
Muscle biopsy		
Muscle biopsy kit @ \$15 per kit	24 x4x15	\$1440
BIS/DXA/muscle sound consumables		
Electrodes and wipes: \$10 per subject per trial	10x3x24	\$720
Diets and supplements		
Standardised diets (\$20 per subject per diet)	24x4x20	\$1920
Creatine supplements (\$80 per loading regimen)	12X 80	\$960
Placebo capsules and filler (\$20 per subject)	12x20	\$240
Subject payments and incentives		
Incentive scheme for performance	\$10,000	\$10,000
Travel/food expenses (600 per subject)	\$14,400	\$14,400
Payment for medical doctor		
\$750 per testing day (7 days x 4 cohorts)	(750x4x7)	\$21,000
Muscle analyses		
Creatine and glycogen	1000	\$1000
Muscle proteins and signalling proteins	10000	\$10,000
Other metabolites (lactate)	1000	\$1000
Blood analysis		
Lactate analyses: 2.75 per sample	2.75 x 3 x 13 x 24	\$2574
Travel and accommodation		
Muscle sound technicians (2 international airfares + 3 nights hotel)	7000	\$7000
ACU PhD travel (2 x Melbourne-canberra return)	1500	\$1500
ACU PhD accommodation (10 w @ \$400)	4000	\$4000
Shipping costs for muscle samples	600	\$600
Total		\$79,554



MINUTE

TO: Dr Louise Burke CC:
FROM: Ms Helene Rushby
SUBJECT: Approval from AIS Ethics Committee DATE: 23rd May 2014

On the 24th April 2014, the AIS Ethics Committee gave consideration out of session to your submission titled **"Interactive effect of manipulating muscle creatine and glycogen stores on endurance performance and DXA measurements of lean mass"**. The Committee saw no ethical reason why your project should not proceed.

The approval number for this project: 20140612

It is a requirement of the AIS Ethics Committee that the Principal Researcher (you) advise all researchers involved in the study of Ethics Committee approval and any conditions of that approval. You are also required to advise the Ethics Committee immediately (via the Secretary) of:

Any proposed changes to the research design,
Any adverse events that may occur,

Researchers are required to submit **annual status reports** and **final reports** to the secretary of the AIS Ethics Committee. Details of status report requirements are contained in the "Guidelines" for ethics submissions.

Please note the approval for this submission expires on the 30th June 2016 after which time an extension will need to be sought.

If you have any questions regarding this matter, please don't hesitate to contact me on (02) 6214 1577

A handwritten signature in black ink, appearing to be 'Helene Rushby', written over a faint, stylized graphic that resembles a flame or a ribbon.

Sincerely
Helène Rushby
Secretary, AIS EC



MINUTE

TO: Ms Helene Rushby **CC:** Louise Burke
FROM: Dr Meg Ross, ACU Postdoctoral Fellow, AIS Sports Nutrition
SUBJECT: Ethics – Minor Variation (R2)
DATE: 20 Jun 2014

Minor Variation (R2) to project # 20140612

Interactive effect of manipulating muscle creatine and glycogen stores on endurance performance and DXA measurements of lean mass (Creatine-Glycogen study)

We are seeking approval from the AIS Ethics Committee to include two minor variations to the current research study design, which would further improve the rigour and outcomes of the study. Please view the amended version (changes amended by highlighted text) of our submission to AIS Ethics, that we would like the committee to consider. To serve as a reminder:

The original protocol has been approved for the following minor variation:

- the Addition of MuscleSound ® (ultrasound) measurement of muscle glycogen content at time points at which a biopsy has taken place to allow correlation study of muscle glycogen estimates via direct and indirect techniques


This version (R2) adds further variation:

- The extension of the TT protocol from 100 km to 120 km to ensure that it is a glycogen limiting exercise protocol
- The addition of capillary blood samples (finger-prick) to measure blood lactate concentrations on completion of each sprint (n=12) and the hill climb (n=1; total n=13)

It is anticipated these two variations will add only minor discomfort to the research subject, in having a finger-pick by a lancet, for the purpose of sampling capillary blood— a regularly-performed routine procedure in our laboratory, and cycling an additional 20 km—an increase in total distance that is well within the capability of subjects that will be recruited for this study. The estimated time commitment for each subject will remain the same.

Sincere thanks to the members of the committee for considering these changes.

Sincerely,

Signed: 

Principle Researcher and Head of Department



MINUTE

TO: Ms Julia Bone CC: Louise Burke
FROM: Ms Helene Rushby
SUBJECT: Approval from AIS Ethics Committee DATE: 30th June 2014

On the 10th June 2014, the AIS Ethics Committee gave consideration to the minor variation in your submission titled **"Interactive effect of manipulating muscle creatine and glycogen stores on endurance performance and DXA measurements of lean mass"**. The Committee saw no ethical reason why your project should not proceed.

The approval number for this project: 20140612

It is a requirement of the AIS Ethics Committee that the Principal Researcher (you) advise all researchers involved in the study of Ethics Committee approval and any conditions of that approval. You are also required to advise the Ethics Committee immediately (via the Secretary) of:

Any proposed changes to the research design,
Any adverse events that may occur,

Researchers are required to submit **annual status reports** and **final reports** to the secretary of the AIS Ethics Committee. Details of status report requirements are contained in the "Guidelines" for ethics submissions.

Please note the approval for this submission expires on the 30th June 2016 after which time an extension will need to be sought.

If you have any questions regarding this matter, please don't hesitate to contact me on (02) 6214 1577

A handwritten signature in black ink, appearing to be 'H. Rushby', written over a horizontal line.

Sincerely
Helene Rushby
Secretary, AIS EC



INFORMATION TO PARTICIPANTS

Research Title Interactive effect of manipulating muscle creatine and glycogen stores on endurance performance and DXA measurements of lean mass

Principal Researcher:

Prof. Louise Burke (Australian Institute of Sport)

- Tel: 02 6214 1351 0422 635 869
- Email: louise.burke@ausport.gov.au

Co-Researchers:

Mr. Kristylen Tomcik (Australian Catholic University)

- Tel: 0405 903 080
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- Tel: 03 995335523 0408 089 959
- Email: john.hawley@acu.edu.au

Julia Bone (Australian Institute of Sport)

- Tel 02 6214 1641 0487 780 843
- Email: Julia.bone@ausport.gov.au

Dr Meg Ross (Australian Institute of Sport)

- Tel 02 6214 7980
- Email: meg.ross@ausport.gov.au

We would like to invite you to participate in this original research project, a collaboration between the AIS and Australian Catholic University (ACU). You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to understand why the research is being done and what your participation will involve. We realise this information sheet is lengthy, but please take time to read it carefully and discuss it with others if you wish. Ask us if you would like more information.

Background

The performance of endurance sports is reliant on muscle stores of glycogen (carbohydrate). Indeed, strategies that increase this muscle fuel (carbohydrate loading) are able to enhance race outcomes by extending the time that optimal pacing strategies can be maintained. Some studies have shown that glycogen stores can be further increased if the muscle has been previously loaded with creatine. Creatine supplementation protocols, which increase this muscle fuel associated with brief high-intensity exercise, are familiar to athletes who undertake repeated sprint events and resistance training. However, if they can also enhance glycogen stores, they may provide an ergogenic aid for endurance athletes such as road cyclists and marathon runners. To date the performance benefits of increased levels of carbohydrate loading in endurance events haven't been investigated. One particular issue that might need to be explored is the effect of the extra body mass associated with both creatine and carbohydrate loading that occurs due to the extra fuel stored in the muscle and the water bound to it.

While the primary aim of manipulating muscle glycogen and creatine stores is to enhance performance, a secondary outcome of changing muscle fuel and water content is to change the results of a DXA scan of lean mass of the region. This might explain why we observe that DXA measurements of what we interpret as "muscle" can suddenly fluctuate by 0.5-1 kg without reflecting a real change. Since muscle size and strength are important in sports

Please note NS refers to the NHMRC 'National Statement' for the ethical conduct of research. For further information please go to <http://www.nhmrc.gov.au> and follow the links

performance, we are always searching for ways to either improve our ability to measure them reliably or to better interpret the results of DXA scans.

The current study has been designed to achieve three separate outcomes.

Aim:

1. The **primary aim** is to investigate whether creatine loading increases the glycogen storage achieved by a carbohydrate loading program, and whether this translates into better performance of a prolonged cycling protocol designed to mimic elements of real-life racing
2. A secondary **specific aim** is to investigate the size of the alterations in estimates of lean mass, **measured** using DXA, due to creatine loading and the depletion and supercompensation of muscle glycogen stores.
3. The study will also be used to validate a non-invasive technique of measuring muscle glycogen content using ultrasound technology

Benefits:

We are aiming for two separate benefits from undertaking this study of glycogen and creatine loading.

- The first theme focuses on the potential application of creatine supplementation to the performance of endurance athletes using a protocol that simulates some important aspects of real-life sport. One particular element of interest is the effect of any increase in body mass on performance of a weight-sensitive sporting protocol
- The second theme addresses the possibility that alterations in these muscle fuels introduce an artefact into DXA estimates of body composition in athletes. Knowing this can help us to either create a more reliable set of standardised conditions for measuring lean mass, or to better interpret the results of measurements under certain conditions.

Both aspects of this study are novel and will help with the preparation of athletes.

What is involved?

- Your first task will be to undertake testing of your aerobic capacity (VO₂max) and a chance to practice part of the performance protocol we have chosen for this study. The performance trial is described in more detail more below, but on this testing day, you will only do half of the Velotron ride (60 km including sprints) followed by riding your own bike on a treadmill which is at an 8% incline to simulate riding up a hill.
- You will then undertake a second practice day within the next week to make sure you are familiar with the best pacing strategies and technical skills needed to perform well in the real trials
- Performance trials will be undertaken three times, each a week apart from the other. In each case, you will be provided with a packaged diet for 48 hours beforehand, which you must follow faithfully. During this time you will also undertake a standardised training program but you are not required to attend the AIS for those sessions.
 - All participants will receive the same dietary program before Performance Trial 1 – a menu providing a carbohydrate intake of 6 g per kg of your body mass, which should allow you to recover normal stores of muscle glycogen.
 - At the end of Performance Trial 1, the group will be divided into two – with one half receiving a creatine loading and maintenance supplementation protocol for the following 2 weeks, while the other half will receive placebo (no active ingredients) supplements. This supplementation program will require you to undertake 5 days in which you will consume a number of capsules of a creatine or placebo powder at 4 separate times of the day (breakfast, lunch, after training and dinner). For the remainder of the two week period you will only need to consume a single dose of capsules with your breakfast meal. This activity will mean that Trials 2 and 3 will allow a comparison between a Creatine Group and a Placebo Group
 - Before Performance Trials 2 and 3, you will receive each of two different diets in an order that we will keep secret from you until the end of the study. One menu will provide the same amount of carbohydrate as in Trial 1, while the other will contain twice as much carbohydrate to allow you to carbohydrate

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load. Half of each of the two groups (Creatine or Placebo) will receive the carbohydrate loading one first while the other half will do the opposite pattern. This part of the study will investigate the effects of carbohydrate loading with or without prior creatine loading

- Each Performance Trial day will follow the same procedures
 - You will arrive at the Lab fasted (drinking only water since ~ 9 pm the night before the trial) on the morning of the trial). The first activity will be to undertake a whole body DXA scan which requires you to lie still on the DXA scanning bed for ~ 5 minutes. A separate booklet explaining the DXA procedure will be provided to you.
 - A measurement of total body water using a BIS machine will be undertaken while you remain lying down. This scan will require us to place 4 electrodes on your wrists and ankles. No radiation is involved.
 - Following the scans, we will take an ultrasound image and perform a biopsy to take a small amount of muscle from your quadriceps. The biopsy will be performed by an experienced sports physician, under local anaesthetic and following the AIS Best Practice Protocol that is described in a separate booklet.
 - You will then consume a standard breakfast simulating a pre-race meal and wait for 2 hours before commencing your Performance Trial.
 - The Performance trial will start with a 120 km cycling protocol undertaken on the Velotron ergometer which has been set up to mimic your bike geometry. You will undertake most of this at a set workload, determined by the results of your initial testing. However at prescribed segments, you will be asked to undertake a 1 km or 4 km interval as fast as possible where we will measure your performance time (10-14, 20-21, 30-34, 40-41, 50-54, 60-61, 70-74, 80-81, 90-94 and 99-100 km). This represents some of the challenges of road cycling on a flat course where your weight is supported. You will be aware of the elapsed distance during the 120 km protocol, but not the time or your power outputs during the sprint phases.
 - At the finish of this segment, you will switch to riding your own bike on a treadmill set to an incline of 8% to mimic an uphill finish to a road race. You will be asked to ride for as long as you can at a speed that is equivalent to 88% of your Peak Power Output. Again, your elapsed and final finishing time will be kept hidden from you until the end of the study.
 - During each Performance Trial you will consume carbohydrate and fluid according to a standardised race nutrition plan, and we will also take a drop of blood from your finger-tip or earlobe at various distances throughout the ride, in order to measure blood lactate.
 - To encourage the best performance possible, we will organise a prize bank of \$10,000 based on a formula in which the mean performance over the three rides (120km, sprint times and hill ride to exhaustion) is calculated and normalised to baseline physiological test results so that each rider has an equal chance of performing well
- At the conclusion of Performance trial 1, you will undergo another ultrasound of your quadriceps. No biopsy will be taken at this time. You will then be provided with a diet that is low in carbohydrate that you must consume for the rest of the day. The aim of this diet is to keep your "race" depleted glycogen stores low until the next morning, when we will repeat a DXA scan, BIS, ultrasound and muscle biopsy under our AIS Best Practice Protocols. This "depletion" measure will only occur once during the study. At the completion of Performance Trials 2 and 3 you will be allowed to consume a menu of your choice for the next couple of days while you recover and complete your training program until the next pre-Trial preparation period. Overall, we will work with you to negotiate your training program over the ~ 21 days of your participation in the study so that it remains constant/standardised

Summary of your commitment

- We anticipate that your participation in this study will span ~ 35 hours, over the course of 6 visits to the laboratory
 - Preliminary testing
 - Practice day,

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- Performance Trial 1 (DXA, BIS scan, ultrasound, muscle biopsy and Riding protocol),
 - Depletion testing day (DXA, BIS scan, ultrasound and muscle biopsy
 - Performance Trial 2(DXA, BIS scan, ultrasound, muscle biopsy and Riding protocol),
 - Performance Trial 3 (DXA, BIS scan, ultrasound, muscle biopsy and Riding protocol)
 - We will provide you with an honorarium of \$600 to compensate you for travel costs, food costs outside the packaged diets, and lost work time during your participation in the trial
 - You will undertake 4 periods of dietary control: 3 x 48 hours prior to each Performance Trial, and 1 x 12-18 hours post Performance Trial 1. Diets will be prepared to meet individual preferences and intolerances.
- A total of 4 biopsies, 8 ultrasounds and 4 DXA/BIS scans will be undertaken

Who we are recruiting?

- Participants who are endurance trained, sub-elite cyclists or triathletes, aged 18-40 y who are currently completing a weekly cycling training load of > 250 km/week and have a > 2 y history of competing in road races or triathlons.
- Exclusion criteria:
- History of abnormal bleeding/clotting or needle phobia that would prevent you from having a muscle biopsy
- Taken a creatine supplement within 6 weeks of first performance trial
- Recent exposure to radiation (e.g. medical imaging techniques) that would prevent you from having 4 DXA scans)

Adverse Effects and Withdrawal:

- As explained in more detail in our booklet on the procedures in our trial, it is common for subjects to experience some mild muscle soreness during the initial 48 hrs of recovery after a muscle biopsy. However this should not restrict movement or function. In some rare cases mild bruising has occurred but these symptoms disappear within a week. The principal researcher will monitor the status of subjects in the days after an experimental trial. In addition, although the possibility of infection and significant bruising is small, subjects are informed to contact the principal researcher if by chance this should eventuate. The principal researcher will then refer any individuals with these symptoms to the project doctor.
- Participation in this clinical trial is voluntary and participants are free to withdraw from the study at any point if they deem they can no longer adhere or complete the requirements of the trials.

Confidentiality:

- You will be provided with a full copy of your own results on the completion of the study, as well as the general findings of the study. No data from any other individual participant will be provided to you by the researchers.
- Confidential information will be kept by the principal investigator in a lockable filing cabinet and in a personal computer protected by password. In addition, the participants will be identified by number only. All address lists will be kept securely in the primary investigator's care and only be available to the primary investigator and appropriate co-researchers. The presentation of results will not make reference to individual participants when the data are published. The raw data will be retained by the principal investigator for five years in a secure cabinet at the Australian Institute of Sport. At the conclusion of this five year period all material containing confidential information will be destroyed.

Ethics Approval:

- The project set out in the attached application, including the adequacy of its research design and compliance with recognised ethical standards, has the approval of the Australian Institute of Sport (AIS). If you have any concerns, please contact the secretary of the AIS Ethics Committee Helene Rushby on 02 6214 1577.

Further information:

Please note NS refers to the NHMRC 'National Statement' for the ethical conduct of research. For further information please go to <http://www.nhmrc.gov.au> and follow the links

- Please contact Julia Bone on Julia.bone@ausport.gov.au or 02 6214 1641 if you have any questions, concerns, or require further information in regards to any aspect of participating in this study.

Please note NS refers to the NHMRC 'National Statement' for the ethical conduct of research. For further information please go to <http://www.nhmrc.gov.au> and follow the links

'INFORMED CONSENT' FORM (Adult)

Project Title: Interactive effect of manipulating muscle creatine and glycogen stores on endurance performance and DXA measurements of lean mass

Principal Researchers: Prof. Louise Burke

This is to certify that I, [REDACTED] hereby agree to participate as a volunteer in a scientific investigation as an authorised part of the research program of the Australian Sports Commission under the supervision of Prof. Louise Burke, Mr. Kristylen Tomcik, Miss Julia Bone and Prof. John Hawley..

The investigation and my part in the investigation have been defined and fully explained to me by Prof. Louise Burke/Miss Julia Bone and I understand the explanation. A copy of the procedures of this investigation and a description of any risks and discomforts has been provided to me and has been discussed in detail with me.

- I have been given an opportunity to ask whatever questions I may have had and all such questions and inquiries have been answered to my satisfaction.
- I understand that I am free to deny any answers to specific items or questions in interviews or questionnaires.
- I understand that I am free to withdraw consent and to discontinue participation in the project or activity at any time, without disadvantage to myself.
- I understand that I am free to withdraw my data from analysis without disadvantage to myself.
- I understand that any data or answers to questions will remain confidential with regard to my identity.
- I certify to the best of my knowledge and belief, I have no physical or mental illness or weakness that would increase the risk to me of participating in this investigation.
- I am participating in this project of my (his/her) own free will and I have not been coerced in any way to participate.

Privacy Statement: The information submitted will be managed in accordance with the ASC Privacy Policy.

☐ *I consent to the ASC keeping my personal information.*

Signature of Subject: _____ Date: ____/____/____

I, the undersigned, was present when the study was explained to the subject/s in detail and to the best of my knowledge and belief it was understood.

Signature of Researcher: _____ Date: ____/____/____

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