METABOLIC ADAPTATION TO HIGH-INTENSITY EXERCISE

METABOLIC ADAPTATION TO HIGH-INTENSITY EXERCISE:

MANIPULATION OF TRAINING STIMULUS AND NUTRITIONAL SUPPORT

By

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the degree

Doctor of Philosophy

McMaster University

DOCTOR OF PHILOSOPHY (2013) McMaster University

(Kinesiology) Hamilton, Ontario

TITLE: Metabolic adaptation to high-intensity exercise: manipulation of training stimulus and nutritional support

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NUMBER OF PAGES: xvii, 168

Abstract

This thesis investigated the acute and chronic responses of human skeletal muscle to high-intensity exercise, with a particular focus on markers of mitochondrial content, and the potential for nutrition to manipulate the adaptive response in recreationally active individuals. The acute response was primarily assessed via measurement of signalling proteins and mRNA species linked to exercise-induced mitochondrial biogenesis. The chronic response was determined via changes in the protein content or maximal activities of mitochondrial enzymes after training. Study 1 examined whether the manner in which a given amount of high-intensity cycling work was performed (i.e., in an intermittent or continuous fashion) altered the acute metabolic response to exercise, and whether the acute response was indicative of longer-term adaptations. Despite the similar acute activation of signalling proteins after the intermittent and continuous matched-work exercise protocols, 6 wk of training with the continuous protocol did not increase mitochondrial content, contrary to what we have previously shown after 6 wk training with the intermittent protocol. This suggests that the intermittent application of a low-volume, high-intensity stimulus is important to elicit training-induced increases in mitochondrial content. Furthermore, Study 1 showed that acute changes in specific signalling proteins did not necessarily predict chronic adaptations. Studies 2 and 3 examined whether specific nutritional interventions, previously shown to modulate acute exercise capacity or metabolic response, altered the mitochondrial adaptive response to several weeks of HIT. Neither manipulating carbohydrate availability between twice daily training sessions, or chronic ingestion of β-alanine, augmented skeletal muscle

adaptations in response to 2-6 wk of HIT. It is possible that small influences of nutrition were overwhelmed by the potency of HIT, which stimulated marked increases in mitochondrial content in this population. Overall this thesis advances our basic understanding of the skeletal muscle adaptive response to HIT and the influence of nutrition.

Acknowledgements

First and foremost, I would like to thank my advisor Dr. Martin Gibala for his help, guidance, and patience throughout my graduate training. He has a unique ability to motivate his students to strive for continual improvement and pursue the highest standard of scientific practice. His mentorship has played a significant role in shaping me personally and academically, and will leave a lasting impression upon all of my future pursuits. I would also very much like to thank Dr. Mark Tarnopolsky, who displays the knowledge and work ethic that all graduate students may aspire to. It was a great privilege to work with you and gain your many insights into all aspects of science and physiology. A special thank you must also go to Dr. Lawrence Spriet, who always provided valuable and insightful opinions and advice towards the development of my training and projects. I was very fortunate to have been able to learn from you.

Thank you to all of those members of the Gibala and Tarnopolsky labs who aided my development along the way. Specifically, I would like to acknowledge Jonathan Little, Adeel Safdar, and Michael Percival, without whom I would not be in the position I am today. Special recognition must also be directed towards Todd Prior and Mahmood Akhtar, without whom many projects and degrees in the Gibala and Tarnopolsky laboratories would never be completed. I would further like to thank Jenna Gillen, Naomi Cermak, Sara Thompson, Frank Myslik, and Steven Tricarico, who played key roles in each of these projects. Ultimately, I want to thank all individuals, past and present, who have contributed to my work and education. This includes my fellow students, the

Kinesiology administrative staff, and those professors not directly involved in the present work.

Finally, I would like to thank all of those members of my personal life who have helped me get to this point. To my family, thank you for your unconditional support and love throughout my life and education. You have instilled the values and work ethic that have helped make me the man I am today. Nearly everything I have, I owe to you. I would also like to thank the many friends I have made throughout my studies at McMaster. You have been the source of many great times and memories, and made the time pass more quickly than seemingly possible. I thank you all, and wish you the greatest of successes in years to come. I would also like to acknowledge Amanda, who provided a great deal of support throughout the time we spent together. Lastly, I would like to thank the person who has meant the most to me over the past few years. To Samantha, I cannot say enough about you and what our time together has meant to me. You have an unrivaled ability to turn any moment into entertainment, and you've reminded me of the importance of balance between work and play, and working smart, not only hard. This is a lesson I now value above many others, and something I still aspire to master. You have many exceptionally qualities that make you who you are, and will continue to make you successful in the future. I hope that we can continue to become better in all that we do, and hope that we may continue to do this together. To you, and to all others who have helped me along the way: Thank you!

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Format and Organization of Thesis

This thesis is prepared in the "sandwich" format as outlined in the School of Graduate Studies' Guide for the Preparation of Theses. It includes a general introduction, 3 independent studies prepared in journal article format, and an overall discussion. The candidate is the first author on all of the manuscripts. At the time of thesis preparation, Chapter 2 was under peer-review, while Chapters 3 and 4 were in preparation for submission.

Contribution to Papers with Multiple Authorship

Chapter 2

Contribution

A.J.R. Cochran, J.P. Little, and M.J. Gibala contributed to the design of the study. A.J.R. Cochran and S. Tricarico ran the exercise trials, which were performed in the laboratories of M.J. Gibala and M.A. Tarnopolsky. M.A. Tarnopolsky performed the muscle biopsy procedures, and skeletal muscle analysis was performed by A.J.R. Cochran, M.E. Percival, and N. Cermak. A.J.R. Cochran wrote the manuscript, with input and contributions from M.J. Gibala and all other authors.

Chapter 3

Contribution

A.J.R. Cochran and M.J. Gibala designed the study. A.J.R. Cochran and F. Myslik ran the experimental and training components of the study. M.A. Tarnopolsky performed the muscle biopsies. A.J.R. Cochran, M.E. Percival and D. Bishop performed the muscle analyses. A.J.R. Cochran and M.J. Gibala interpreted the findings. A.J.R. Cochran composed the manuscript, with input from M.J. Gibala and all other authors.

Chapter 4

Contribution

A.J.R. Cochran and M.J. Gibala designed of the study. J.B. Gillen aided A.J.R. Cochran with supplement assignment and distribution. A.J.R. Cochran and S. Thompson ran the exercise training. M.A. Tarnopolsky performed the muscle biopsy procedures, and skeletal muscle analysis was performed by A.J.R. Cochran and M.E. Percival. A.J.R. Cochran wrote the manuscript, with input and contributions from M.J. Gibala and all other authors.

Chapter 1: General Introduction & Review of Literature

1.1. Introduction

Traditional endurance training (ET), characterized by repeated sessions of continuous moderate-intensity exercise over several weeks, induces a host of metabolic adaptations within human skeletal muscle (Hawley, 2002, Holloszy and Booth. , 1976). One of the hallmark adaptations to ET is a robust increase in skeletal muscle mitochondrial content (Gollnick, et al. , 1973), as evidenced by increased protein content and/or maximal activity of representative enzymes such as citrate synthase. Augmented mitochondrial density within the skeletal muscle results in a greater capacity for oxidative energy provision, which in turn contributes to shifts in substrate utilization and reduced by-product accumulation at a given absolute intensity, and improved endurance performance (Holloszy and Coyle. , 1984).

High-intensity interval training (HIT) is an effective alternative to traditional ET for inducing metabolic adaptations within skeletal muscle, at least over the short term, i.e., up to at least several weeks (Burgomaster, et al. , 2008, Gibala, et al. , 2006). HIT is characterized by brief repeated high-intensity work efforts, interspersed with periods of rest or lower-intensity exercise for recovery. This method of training has been shown to increase mitochondrial content to a similar extent as traditional ET despite a markedly lower total work and time commitment (Burgomaster, et al. , 2008, Gibala, et al. , 2006). Gibala and associates (Gibala, et al. , 2006) showed that just 6 sessions of HIT over 2 wk, totalling ~630 kJ over 135 min, induced similar metabolic adaptations to an ET program

that consisted of ~6500 kJ of work over 630 min. Burgomaster and colleagues (Burgomaster, et al. , 2008) thereafter showed similar results when extended over 6 weeks. A number of other HIT studies have reported adaptations normally associated with ET. These adaptations include increases in resting muscle glycogen content, increased fat utilization and reduced glycogenolysis during submaximal exercise, increased glucose and fat transporter content, enhanced skeletal muscle buffering capacity, and improved endurance capacity and performance (Burgomaster, et al. , 2005, Burgomaster, Heigenhauser and Gibala. , 2006, Burgomaster, et al. , 2007, Burgomaster, et al. , 2008). Thus, HIT is a highly potent and time-efficient method for inducing metabolic remodelling in human skeletal muscle. However, a number of fundamental questions regarding HIT remain to be answered, namely 1) what about HIT makes it such a potent stimulus for metabolic adaptation, and 2) can this stimulus be augmented any further through nutritional or other means?

The present thesis was designed to examine the skeletal muscle adaptive response to HIT, with a specific focus on the properties of the acute exercise stimulus, and whether the adaptive response to chronic training could be modified by nutritional means. While a number of acute and chronic exercise responses were examined, the primary focus of this thesis was the adaptive response of skeletal muscle mitochondrial marker content to said interventions. The introductory chapter of the thesis will provide an overview skeletal muscle remodelling to ET and HIT, and focus upon the potential role of nutrition in modulating these responses.

1.2. Skeletal muscle adaptations to endurance training

Regular, moderate-intensity (e.g. ~65% VO_{2peak}) ET, generally consisting of 3-5 sessions lasting ≥60 min per week, induces a wide variety of metabolic adaptations within human skeletal muscle (Hawley, 2002, Holloszy and Booth. , 1976). Among the most notable of ET-induced adaptations is the expansion of the mitochondrial reticulum within the challenged muscle fibres (Holloszy, 1967). This enlargement is generally evidenced by increases in the total protein and/or maximal enzyme activity of specific markers including citrate synthase (CS), cytochrome c oxidase (COX), and β-hydroxyacyl-coenzyme A dehydrogenase (β-HAD) (Carter, Rennie and Tarnopolsky. , 2001, Holloszy, 1967). The resultant effect of an expanded mitochondrial network is an increased oxidative capacity, or ability to utilize oxygen to derive adenosine triphosphate (ATP) from carbohydrates (CHO) and fats. An enhanced oxidative capacity also results in an improved metabolic control such that at a given absolute work intensity, the skeletal muscle can increase ATP production rates to meet demand more quickly and sufficiently, resulting in less accumulation of metabolic by-products (Gollnick and Saltin. , 1982, Holloszy and Coyle. , 1984, Leblanc, et al. , 2004, Phillips, et al. , 1996a). The requisite ATP production may therefore be achieved with an increased reliance on fat oxidation, a slower utilization of muscle glycogen and total CHO, and a lessened accumulation of muscle and blood lactate (Gollnick and Saltin. , 1982, Henriksson and Reitman. , 1976, Holloszy and Coyle. , 1984, Leblanc, et al. , 2004, Phillips, et al. , 1996a). Improved oxidative capacity is therefore believed to be a primary factor underlying training-induced changes in substrate utilization and increases in the lactate threshold (Joyner and Coyle. ,

2008). It must be noted however, that changes in substrate metabolism during submaximal exercise have been reported without observation of improvements in oxidative capacity, suggesting other mechanisms may be at work (Phillips, et al. , 1995).

Other hallmark skeletal muscle adaptations to ET are the increased expression of particular transport proteins relevant to substrate provision and waste removal. Specifically, ET increases the content of glucose transporter isoform 4 (GLUT4), the predominant transporter responsible for both insulin- and contraction-mediated glucose uptake into skeletal muscle (Phillips, et al. , 1996c, Ploug, et al. , 1990). Though less consistent, ET and chronic low-frequency muscle stimulation have also been shown to alter the content/distribution of fatty acid translocase (FAT/CD36) and plasma membrane fatty acid binding proteins (FABPpm; (Bonen, et al. , 1999, Turcotte, et al. , 1999). Together, these alterations increase the potential for glucose and fatty acid uptake during future exercise bouts as well as during recovery, presumably to augment the restoration rate of intramuscular fuel stores with exercise cessation and substrate provision (i.e. muscle glycogen and intramyocellular lipid (IMCL). Indeed, glycogen restoration following depletion exercise has been correlated with muscle GLUT4 content in human muscle (Greiwe, et al. , 1999). While it remains to be shown whether trained individuals replenish their IMCL stores at a faster rate than untrained individuals (Decombaz, et al. , 2001), it is well-established that endurance trained individuals possess greater resting stores of both muscle glycogen and IMCL (Dube, et al. , 2008, Goodpaster, et al. , 2001, Greiwe, et al. , 1999, Leblanc, et al. , 2004, Phillips, et al. , 1996b, Juel and Halestrap. , 1999). ET has also been shown to augment the content of skeletal muscle

monocarboxylate transporters (MCT) 1 and 4 (Juel and Halestrap. , 1999). MCTs are the primary transporters responsible for lactate shuttling, and mediate the majority of hydrogen ion efflux from the muscle fibres (Thomas, et al. , 2012). At present, it is believed that MCT1 is capable of both lactate uptake from circulation and extrusion from muscle (Bonen, et al. , 2000), while MCT4 (also known as MCT3-M) may be more responsible for the expulsion of lactate from glycolytic fibres (Wilson, et al. , 1998). Endurance-trained individuals have greater MCT1 expression than their sedentary counterparts, and also have a tendency for higher MCT4 content as well (Thomas, et al. , 2005). Furthermore, regular ET in previously untrained individuals has been shown to increase the expression of both MCT1 and MCT4 in skeletal muscle (Dubouchaud, et al. , 2000, Pilegaard, et al. , 1999), though augmentations in MCT1 expression tend to be more pronounced (Thomas, et al. , 2012). Altogether, ET-induced increases in glucose, fat, and monocarboxylate transporters enhance the ability of trained muscle to take up fuels and remove lactate and hydrogen ions during subsequent work sessions. These improved abilities, coupled with the ET-induced mitochondrial content and oxidative capacity, culminate in an increased fatigue resistance, endurance capacity and performance.

1.3. High-intensity interval training

HIT is fundamentally characterized by brief, high-intensity work efforts separated by short recovery periods of rest or lower intensity exercise. Within these parameters, there are a wide number of variations on the HIT paradigm, however HIT may generally be separated into two broad categories: 1) "all-out", and 2) "constant-load" (Figure 1.1).

All-out HIT generally consists of 15-30 s work bouts in which subjects pedal at the highest rate possible against a set resistance, often 7.5-10% of body mass. As a result, power output during work bouts begin very high, often in excess of 250% of the absolute work intensity that can elicit maximal aerobic capacity (VO_{2max}) power output (W_{max}), and progressively decreases towards the end of the work bout. All-out HIT also tends to have relatively long rest periods, lasting at least as long as the work bout and up to six times longer. Conversely, constant-load HIT work bouts generally last 1-5 min, with power outputs being set at a constant intensity of 85-110% W_{max}, independent of pedal rate. Constant-load HIT rest periods are also generally the same length or shorter than the work bouts.

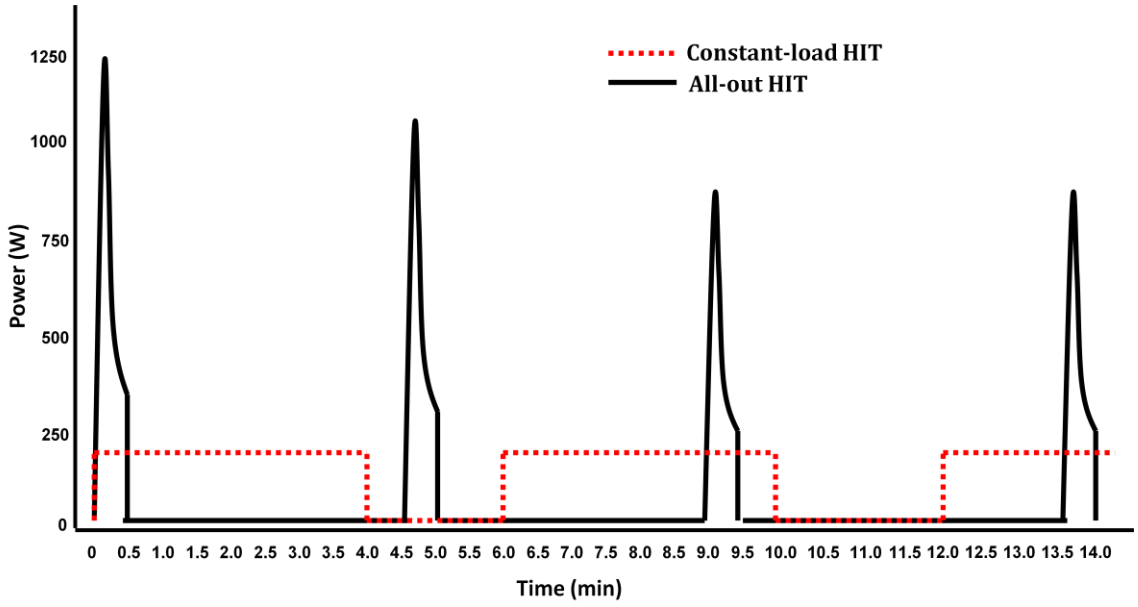

Figure 1.1. A graphical representation of the work profile produced during an acute session of all-out (solid line), and constant-load (dotted line) high-intensity interval training.

1.4. Skeletal muscle adaptations to high-intensity interval training

HIT induces similar skeletal muscle adaptations to those induced by traditional ET. Specifically, all-out and constant-load HIT have each been shown to augment mitochondrial markers in skeletal muscle (Burgomaster, et al. , 2005, Burgomaster, Heigenhauser and Gibala. , 2006, Burgomaster, et al. , 2007, Burgomaster, et al. , 2008, Dawson, et al. , 1998, Gibala, et al. , 2006, Gurd, et al. , 2010, Harmer, et al. , 2008, Little, et al. , 2010b, Little, et al. , 2011, MacDougall, et al. , 1998, Morton, et al. , 2009, Parra, et al. , 2000, Perry, et al. , 2007, Perry, et al. , 2008, Perry, et al. , 2010, Talanian, et al. , 2007). HIT has also been shown to enhance fatty acid oxidation, while reducing total glycogenolysis and CHO oxidation at a given submaximal intensity (Burgomaster, et al. , 2008, Talanian, et al. , 2007), along with reduced metabolite accumulation in the muscle (Burgomaster, et al. , 2008, Talanian, et al. , 2007). Together, these responses suggest an improved metabolic control within the trained muscle.

A number of skeletal muscle transport proteins have also been shown to be augmented by both all-out and constant-load HIT. GLUT4 has been shown to be increased by only three sessions of all-out HIT (Burgomaster, et al. , 2007), while 6 sessions of constant-load HIT increased GLUT4 by over 20% (Perry, et al. , 2008). Similarly, both all-out and constant-load HIT paradigms have been shown to augment skeletal muscle MCT content (Burgomaster, et al. , 2007, Juel, et al. , 2004, Mohr, et al. , 2007, Perry, et al. , 2008, Pilegaard, et al. , 1999) though not all studies have found this (Bishop, et al. , 2008). Skeletal muscle fat transporters are also increased by HIT, though these responses appear to be more varied. For example, the only all-out HIT study to

examine FAT/CD36 and FABPpm failed to observe any changes in content over 6 weeks of training (Burgomaster, et al. , 2007). A number of constant-load HIT studies however have shown increases in FAT/CD36 and/or FABPpm (Perry, et al. , 2008, Talanian, et al. , 2007, Talanian, et al. , 2010) Thus, while all-out and constant-load HIT have each been shown to induce several skeletal muscle adaptations traditionally associated with ET, there may be fundamental differences between the HIT protocols themselves.

1.5. Molecular mechanisms underlying skeletal muscle adaptation to exercise training

While far from fully elucidated, recent advances in molecular physiology have shed some insight on the processes underpinning skeletal muscle plasticity. It is generally accepted that training adaptation is the result of the cumulative changes that occur within the skeletal muscle following each exercise session (Coffey and Hawley. , 2007, Hood, 2001, Mahoney and Tarnopolsky. , 2005). Depending on the mode, intensity and duration, each exercise session creates unique disturbances in the homeostatic environment of the skeletal muscle. These changes include increased ATP turnover, accumulation of metabolic by-products, alterations in calcium homeostasis, increased free-radical production, and increased transmission of mechanical forces through the fibre itself. These disturbances result in the activation of exercise-responsive kinases such as adenosine monophosphate-activated protein kinase (AMPK), calcium/calmodulin-dependent protein kinases (CaMKs), and p38 mitogen-activated protein kinase (MAPK). These kinases modulate the activity of transcription factors and transcriptional coactivators to augment the transcription of target genes relevant to subsequent

adaptation. This increased transcription, coupled with changes in mRNA stability, results in an increased total mRNA expression and greater number of templates available for subsequent translation. Following translation, new proteins may be chaperoned to their destination in the cell where they may be imported and/or assembled into larger functional complexes. Depending on the half-life of each protein, repeated training sessions of similar mode, intensity and duration will result in an increased total content of these proteins, ultimately resulting in a new steady-state. This new steady-state establishes a new functional threshold of the skeletal muscle for tolerating future perturbations of this type (Coffey and Hawley. , 2007, Mahoney and Tarnopolsky. , 2005). On one end of the adaptive spectrum is the hypertrophic response of skeletal muscle to resistance training, which occurs as a result of the accumulation of myofibrillar proteins within the muscle. On the opposing end of the spectrum are the improvements in oxidative capacity and substrate metabolism that occur secondary to the accumulation of new mitochondrial and transport proteins within the muscle. These latter improvements are accomplished to a similar extent by both ET and HIT.

1.5.1. Acute metabolic responses to high-intensity interval training

Due to its high relative work intensity, HIT represents a significant perturbation to skeletal muscle homeostasis. For example, skeletal muscle ATP content, which normally remains stable throughout nearly all exercise scenarios, may be reduced by 25-40% by a single 30 s work interval of all-out HIT (Bogdanis, et al. , 1995, Bogdanis, et al. , 1996, Cheetham, et al. , 1986, Chen, et al. , 2000, Jacobs, et al. , 1982, Jones, et al. , 1985, McCartney, et al. , 1986, Stathis, et al. , 1994, Withers, et al. , 1991, Zhao, et al. ,

2000). Simultaneously, phosphocreatine (PCr) stores may be depleted by 70-90% (Bogdanis, et al. , 1995, Bogdanis, et al. , 1996, Esbjornsson-Liljedahl, et al. , 1999, McCartney, et al. , 1986, Parolin, et al. , 1999, Putman, et al. , 1995, Stathis, et al. , 1994, Withers, et al. , 1991) and glycogen stores by as much as 20-35% (Bogdanis, et al. , 1995, Bogdanis, et al. , 1996, Cheetham, et al. , 1986, Chen, et al. , 2000, Jacobs, et al. , 1982, McCartney, et al. , 1986, Parra, et al. , 2000). Despite the massive reliance upon substrate phosphorylation, ATP demand during such an all-out 30 s effort requires a significant contribution from oxidative metabolism. In fact, Parolin and associates (Parolin, et al. , 1999) showed that during the second half of an all-out 30 s sprint, oxidative phosphorylation (OXPHOS) provides ~50% of the ATP (Figure 1.2). Thus, just a single 30 s all-out effort represents a significant challenge to all of the metabolic systems providing ATP for skeletal muscle work. As each training session of all-out HIT requires 4-6 maximal-intensity efforts lasting 30 s, the demand on skeletal muscle metabolism and ATP turnover is immense. When these 30 s efforts are repeated with just 4 min recovery periods, PCr stores are unable to fully recover (Bogdanis, et al. , 1995, Bogdanis, et al. , 1996) and the significant acidosis (Spriet, et al. , 1987, Spriet, et al. , 1989) that occurs with repeated all-out efforts may also induce a relative shutdown of glycogenolysis (McCartney, et al. , 1986, Parolin, et al. , 1999, Spriet, et al. , 1989). Skeletal muscle adenosine diphosphate (ADP) and monophosphate (AMP) may also be increased by up to 10- and 40-fold, respectively, at some points during the session (Parolin, et al. , 1999). The end result of a reduced ability to utilize the ATP-PCr and glycolytic systems is that the muscle must progressively increase its reliance on OXPHOS for energy provision

during subsequent work bouts (Parolin, et al. , 1999). Parolin and colleagues (Parolin, et al. , 1999) confirmed this showing that during the third of three 30 s all-out efforts, OXPHOS provided ≥75% of the total ATP (Figure 1.2). All-out HIT consisting of ≥3 x 30 s efforts therefore represents a significant challenge to the OXPHOS system.

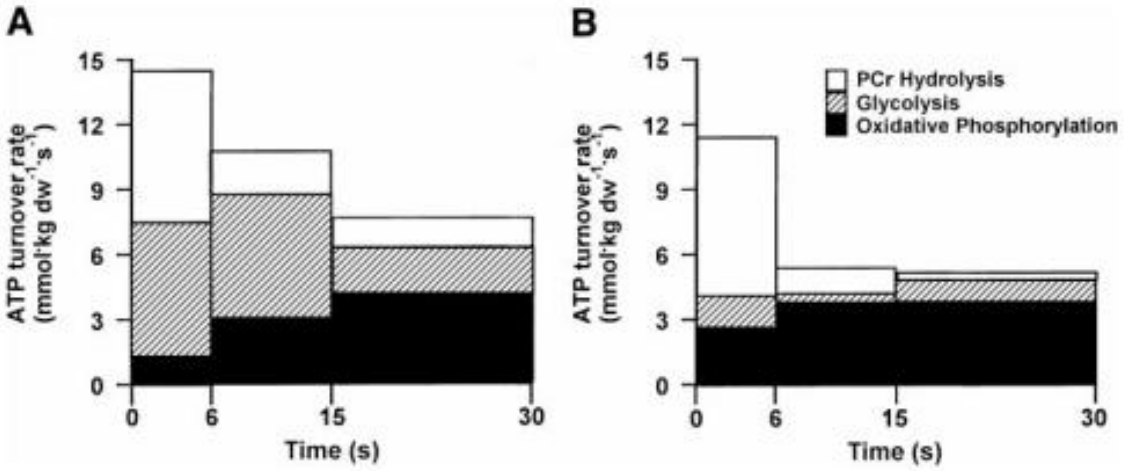

Figure 1.2. Breakdown of total ATP provision from PCr (white), glycolysis (hatched), and OXPHOS (black) sources during a series of three all-out 30 s sprints spaced 4 min apart. A, first sprint. B, third sprint. From Parolin et al., 1999.

The acute metabolic responses to constant-load HIT have been less studied. Furthermore, much more variance exists between the differing constant-load protocols. For example, one method of constant-load HIT involves alternating 1 min work and rest bouts, with work efforts eliciting 100% W_{max}, and the entire workout being completed in 20 min (Little, et al. , 2010b). An alternative model of constant-load HIT prescribes 10 x 4 min work bouts eliciting ~90% VO_{2peak}, separated by 2 min rest periods for a total workout duration of 58 min (Talanian, et al. , 2007). A third involves 6-9 work bouts lasting 5 min performed at 80% W_{max} with 1 min recovery periods, totalling 35-55 min

(Westgarth-Taylor, et al. , 1997). In untrained subjects, 5 min of cycling at 90% VO_{2peak} can reduce ATP levels by ~15%, PCr levels by 80%, and glycogen by 30%, while increasing free ADP by 4-fold and free AMP by 20-fold (Perry, et al. , 2008). Following 5 x 4 min intervals at this intensity, Cochran and associates (Cochran, et al. , 2010) showed that PCr was decreased by ~20-25%, while glycogen reductions remained around the 30-35% range. It would therefore appear that similar, progressive reductions in PCr, as well as similar inhibition of glycogenolysis may occur during constant-load HIT, as it does with the all-out protocols. In support of this, it has been shown that 1-5 min of cycling at 90% VO_{2peak} may reduce muscle pH from ~7.0 to 6.6 (Howlett, et al. , 1998, Perry, et al. , 2008). While the longer duration (e.g. 2-5 min) of each work bout during constant-load HIT is likely to rely largely upon OXPHOS to begin with (McArdle, Katch and Katch. , 2000), it is likely that a similar shift from substrate to oxidative phosphorylation occurs with constant-load HIT, as it does with the all-out paradigms.

1.5.2. Protein signalling responses to acute high-intensity interval training

1.5.2.1. AMP-activated protein kinase

The primary role of AMPK in most eukaryotic cells, including skeletal muscle, is to function as an energy sensor (Hardie, Ross and Hawley. , 2012). More specifically, AMPK detects increases in the intracellular AMP/ATP ratio, which is an indicator of an increased energetic stress within the cell (Hardie, Ross and Hawley. , 2012). In line with this, AMPK has been shown to be activated during exercise in an intensity-dependent manner (Chen, et al. , 2003, Egan, et al. , 2010, Wojtaszewski, et al. , 2000). When activated as a result of this increased energetic stress, AMPK then undertakes a variety of

functions to counteract this condition, including switching on catabolic processes to generate ATP, while switching off anabolic, ATP-consuming pathways (Hardie, Ross and Hawley. , 2012). Among these functions are the acute increases in glucose and fat uptake and metabolism, and the general inhibition of protein synthesis (Hardie, Ross and Hawley. , 2012). AMPK also undertakes more chronic methods of protecting energy homeostasis such as augmenting mitochondrial biogenesis (Bergeron, et al. , 2001, Winder, et al. , 2000, Zong, et al. , 2002) by augmenting PGC-1α activity (Jager, et al. , 2007). In line with the large increases in ATP turnover, as well as the large accumulations of ADP and AMP, both all-out and constant-load HIT induce robust increases in skeletal muscle AMPK phosphorylation (Gibala, et al. , 2009, Little, et al. , 2011, Yeo, et al. , 2008a, Yu, et al. , 2003). Thus, AMPK activation may be one mechanism by which HIT may augment skeletal muscle mitochondrial content.

1.5.2.2. p38 mitogen-activated protein kinase

p38 MAPK is generally considered a "stress-activated" protein kinase because of its activation by environmental stressors, stretch, or inflammatory cytokines (Widegren, et al. , 2000). As previously alluded to, exercise represents a significant stressor to the intramyocellular environment, as exercise may increase energy stress, calcium flux, heat, reactive oxygen species, hydrogen ion production and mechanical stress. It is therefore not surprising that exercise has been shown to activate p38 MAPK in human skeletal muscle, especially when that exercise is of a strenuous nature (Boppart, et al. , 2000, Egan, et al. , 2010, Little, et al. , 2010a, Yu, et al. , 2001). HIT is no exception, as all-out (Gibala, et al. , 2009, Little, et al. , 2011) and constant-load (Cochran, et al. , 2010, Yu, et

al. , 2003) HIT have each been shown to augment human p38 MAPK phosphorylation. As p38 MAPK has been shown to directly phosphorylate the PGC-1α protein resulting in an increased in PGC-1α protein stability and transactivational activity (Puigserver, et al. , 2001), it is no surprise that p38 has been implicated as a signalling molecule relevant to mitochondrial biogenesis (Akimoto, et al. , 2005, Akimoto, Li and Yan. , 2008, Ikeda, et al. , 2008, Pogozelski, et al. , 2009, Puigserver, et al. , 2001, Wright, et al. , 2007a). Therefore, HIT-induced modulation of a p38 MAPK-PGC-1α axis represents another potential mechanism contributing to mitochondrial biogenesis in human skeletal muscle.

1.5.2.3. Peroxisome proliferator-activated receptor γ coactivator 1 α

Multiple lines of evidence implicate the transcriptional coactivator PGC-1α as having an important role in exercise-induced skeletal muscle mitochondrial biogenesis. For example, PGC-1α expression generally coincides with an oxidative metabolic profile, being expressed to a greater degree in highly oxidative tissues such as brain, liver, kidney, heart and skeletal muscle (Handschin, 2010). Within skeletal muscle, PGC-1α also tends to mirror the mitochondrial content of specific muscle fibres, with type I and IIa fibres expressing more PGC-1α as compared with type IIx or IIb (Lin, et al. , 2002). Secondly, PGC-1α overexpression results in mitochondrial biogenesis and oxidative adaptation (Lin, et al. , 2002, Nikolic, et al. , 2012, Wu, et al. , 1999), and these increases, in turn, have been associated with improvements in metabolic functional capacity (Calvo, et al. , 2008, Wenz, et al. , 2009). Conversely, PGC-1α knockout generally results in shifts away from an oxidative metabolic profile (Handschin, et al. , 2007), reduced mitochondrial content (Adhihetty, et al. , 2009), and a reduced exercise capacity (Handschin, et al. ,

2007). Lastly, PGC-1α is induced by interventions which result in augmented mitochondrial content such as cold-exposure in brown fat, fasting in liver, and exercise training in muscle (Handschin, 2010). Altogether, these findings have led PGC-1α to be termed a "master regulator" of mitochondrial biogenesis (Handschin, 2010), and has been purported as a key driving mechanism behind training-induced increases in skeletal muscle mitochondrial content (Geng, et al. , 2010).

The mechanisms by which PGC-1α achieves mitochondrial biogenesis is believed to be via the coordinated regulation of both nuclear- and mitochondrially-encoded gene expression (Scarpulla, 2008). PGC-1α binds to and increases the transactivational activity of a host of transcription factors regulating mitochondrial biogenesis within the skeletal muscle, including the nuclear respiratory factors 1 and 2 (NRF-1, NRF-2), estrogen-related receptors α and γ (ERRα, ERRγ), and the peroxisome proliferator-activated receptors (PPARα, PPARβ/δ, PPARγ). NRF-1 and NRF-2 have been directly linked to the expression of subunits in each of the respiratory chain complexes, mitochondrial import and assembly complexes, and critical factors to mitochondrial DNA transcription and replication, such as mitochondrial transcription factor A (TFAM) (Scarpulla, 2002, Scarpulla, 2008). Knockout of either NRF-1 or NRF-2 generally results in lethality (Huo and Scarpulla. , 2001, Ristevski, et al. , 2004) from a combination of mitochondrial and non-mitochondrial defects (Scarpulla, 2008), indicating the importance of these TFs to mitochondrial function and viability. ERRα and PPARα appear to play overlapping roles in governing the expression of mitochondrial fatty acid oxidation enzymes, however ERRα has been implicated in nearly all aspects of mitochondrial function (Eichner and

Giguere. , 2011), even with the expression of PPARα gene itself (Andersson and Scarpulla. , 2001). ERRγ has also been reported to play a significant role in mitochondrial biogenesis, as overexpression promotes increases in respiratory and fatty acid oxidative enzyme expression, while heterozygotes show oxidative impairment (Narkar, et al. , 2011, Rangwala, et al. , 2010). Another important PGC-1α-regulated TF is the myocyte enhancer factor-2 (MEF2). What is significant about this regulation is that MEF2 has been shown to bind to and augment the expression of the PGC-1α gene promoter itself (Handschin, et al. , 2003). Thus, increases in PGC-1α activity in response to metabolic stress can result in an increased PGC-1α mRNA expression by way of a PGC-1α-MEF2 autoregulatory loop (Handschin, et al. , 2003).

Acute endurance exercise augments PGC-1α mRNA expression in skeletal muscle (Baar, et al. , 2002, Norrbom, et al. , 2004, Pilegaard, Saltin and Neufer. , 2003, Russell, et al. , 2005) and is often associated with increased AMPK and p38 MAPK phosphorylation, which supports their proposed role as stress-activated protein kinases (Akimoto, et al. , 2005, Egan, et al. , 2010, Ikeda, et al. , 2008, Irrcher, et al. , 2003, Little, et al. , 2011, Wright, et al. , 2007a, Wright, et al. , 2007b). As previously mentioned, AMPK and p38 MAPK directly phosphorylate PGC-1α protein, increasing its protein stability and/or its transactivational activity (Jager, et al. , 2007, Puigserver, et al. , 2001). Phosphorylation has also been proposed as a signal for inducing sub-cellular translocation, as recent work has observed that following exercise, PGC-1α protein abundance increases in the nucleus (Little, et al. , 2011, Wright, et al. , 2007b) and mitochondria (Safdar, et al. , 2011) while total protein remains unchanged. Once inside

the nucleus and mitochondria, PGC-1α may coordinate the simultaneous transcription of both the nuclear- and mitochondrially-encoded genes via the TFs listed above, synchronizing increases in mRNA expression for subsequent translation (Safdar, et al. , 2011). As exercise sessions are repeated, these acute cellular responses compound, leading to a higher total volume of mitochondrial protein within the skeletal muscle (Perry, et al. , 2010). Indeed, chronic muscle stimulation, as well as prolonged endurance training have been shown to augment PGC-1α protein in concert with increased mitochondrial markers (Irrcher, et al. , 2003, Russell, et al. , 2003, Taylor, et al. , 2005). These responses therefore fall in line with the theory of training adaptation being the result of cumulative responses following each training session.

Skeletal muscle adaptation to HIT appears to follow similar mechanisms as to ET. All-out HIT and constant-load HIT have each been shown to augment AMPK and p38 MAPK phosphorylation (Cochran, et al. , 2010, Gibala, et al. , 2009, Little, et al. , 2011, Yeo, et al. , 2008a), and these responses have been associated with increased nuclear abundance of PGC-1α in the hours following (Little, et al. , 2011). These increases in nuclear PGC-1α are, in turn, associated with increased PGC-1α, CS, COXII, and COXIV mRNA, which subsequently lead to increased protein and activities 24-72 h later (Little, et al. , 2011, Perry, et al. , 2010). It would therefore appear that the mechanisms underlying skeletal muscle mitochondrial biogenesis to both all-out and constant-load HIT are the same as those regulating ET-modulated biogenesis. The present model of ET/HIT-induced skeletal muscle mitochondrial adaptation is therefore summarized as follows: 1) acute exercise results in perturbations of energy status, calcium homeostasis,

mechanical stretch, reactive oxygen species, heat and hydrogen ions, amongst others, in the working muscle; 2) protein signalling molecules such as AMPK and p38 MAPK detect, and become activated by these disturbances; 3) AMPK and p38 MAPK phosphorylate PGC-1α; 4) PGC-1α is translocated into the nucleus and mitochondrion; 5) PGC-1α binds to and augments the activity of TFs regulating the promoter regions of both nuclear and mitochondrial genome-encoded mitochondrial proteins; 6) the transcription rate of these specific target genes is augmented; 7) target gene mRNA content is increased as a function of transcription rate and mRNA stability; 8) mRNA is translated into protein; 9) new proteins are folded and processed; 10) newly formed mitochondrial proteins are chaperoned to the mitochondria and imported into the target organelle; 11) protein is incorporated and/or assembled into its target functional complex.

It has been hypothesized that by finding interventions that amplify one or more of these processes, we may be able to augment the downstream molecular processes leading to a greater overall training adaptation. The manipulation of an acute training stimulus, or an individual's diet during exercise are two such proposed interventions.

1.6. Nutritional manipulations of the high-intensity interval training stimulus

1.6.1. Carbohydrate manipulation

Carbohydrate has long been known to play a critical role in energy provision for prolonged and intense exercise (Bergstrom, et al. , 1967, Ivy, 1999). Specifically, as exercise intensity increases beyond 75% VO_{2peak}, CHO contributes more than 70% of total ATP, 85% of which is derived from glycogen (Ivy, 1999, Romijn, et al. , 1993, van Loon, et al. , 2001). Beyond this, reliance upon CHO and glycogen increases exponentially, with CHO providing nearly 100% of total ATP as intensity nears VO_{2peak} (Ivy, 1999). CHO and glycogen availability has therefore long been considered a limiting factor for intense work efforts lasting greater than 90 min (Bergstrom, et al. , 1967, Brooks and Mercier. , 1994, Davis, et al. , 1997, Hargreaves, 2004, Karlsson and Saltin. , 1971). This limitation has led to methods such as CHO loading, and CHO ingestion during exercise in order to maximize endurance capacity and performance (Davis, et al. , 1997, Karlsson and Saltin. , 1971, Tarnopolsky, et al. , 1995, Wright, Sherman and Dernbach. , 1991). As all-out and constant-load HIT work bouts are generally performed at intensities exceeding 85% VO_{2peak}, CHO represents a highly important fuel for HIT performance. In support of this, a single session of all-out HIT consisting of three or four 30 s sprints has been shown to reduce glycogen by 20-35% (Bogdanis, et al. , 1995, Bogdanis, et al. , 1996, Cheetham, et al. , 1986, Chen, et al. , 2000, Jacobs, et al. , 1982, McCartney, et al. , 1986, Parra, et al. , 2000). Similarly, a constant-load HIT session consisting of five, 4 min work intervals at ~90% VO_{2peak}, reduced glycogen by 30-35% (Cochran, et al. , 2010). As such, CHO availability has also been shown to affect exercise

performance in shorter, high-intensity events including HIT-like intermittent protocols (Balsom, et al. , 1999, Jenkins, Palmer and Spillman. , 1993), though these findings are less consistent (Hargreaves, et al. , 1998). Due to the importance of CHO for high-intensity work, sports dieticians have long prescribed a relatively high CHO diet for most athletes, ranging from 5-12 g CHO·kg b.w.$^{-1}$·d^{-1}, depending on the specific events (American Dietetic Association, et al. , 2009, Burke, et al. , 2011). The goal of these high CHO diets is to ensure maximal CHO availability to support optimal training and performance intensities, as well as expedite the recovery of fuel stores immediately following in preparation for the exercise session (Burke, et al. , 2011, Jentjens and Jeukendrup. , 2003). The underlying premise for such a strategy is that maximizing CHO availability will allow the athlete to train harder, ultimately resulting in a greater adaptive response (Spriet and Gibala. , 2004).

CHO and glycogen availability appears to play a greater role in skeletal muscle than just fuel provision. Recently, there has a great deal of interest in the ability of CHO to modulate skeletal muscle signalling, and adaptive responses to exercise. Hansen and associates (Hansen, et al. , 2005) were the first to suggest that endurance training with reduced glycogen content may augment metabolic adaptations to training. Utilizing a unique study design, Hansen and colleagues (Hansen, et al. , 2005) had subjects train one leg train once per day, five days per week while the other leg trained twice per day, every second day such that the second training session was initiated with a reduced glycogen content. Although both legs completed the same total amount of training, the leg that trained twice per day achieved greater gains in CS maximal activity and endurance

capacity when compared to its once-daily trained counterpart (Hansen, et al. , 2005) . Yeo et al. (Yeo, et al. , 2008b) used a similar design in highly trained individuals in which one group of subjects performed alternating ET and HIT workouts on sequential days six days per week, while the other group performed these workouts 2 h apart, three days per week. In these highly trained individuals, only the twice per day training group, who began each HIT session with a reduced glycogen content, showed increases in CS, β-HAD, and COXIV (Yeo, et al. , 2008b). These findings indicate that training with a reduced CHO availability may augment the mitochondrial adaptation to training to a greater extent than training with a normal or high glycogen, as traditionally advised. One difficulty in the interpretation of these findings however, is the inability to differentiate between the effects of different training schedule, and those of CHO and muscle glycogen availability *per se*. Morton and associates (Morton, et al. , 2009) attempted to investigate this question by employing three subject groups. The first of these groups trained once per day four days per week, beginning each training session with normal glycogen content and CHO availability (Morton, et al. , 2009). The second and third groups both trained twice per day two days per week, however group 2 consumed a glucose solution before, and throughout the second training session while group 3 consumed a placebo (Morton, et al. , 2009). This study therefore sought to determine the effects of a high CHO availability independent of training schedule. Interestingly, these researchers showed that the activity of succinate dehydrogenase, another mitochondrial marker, was increased in the twice-per-day trained group with low CHO availability to a greater degree compared to the other groups (Morton, et al. , 2009). These findings would appear to confirm the ability of

low-CHO training to augment mitochondrial biogenesis to a greater extent than traditional training, however this was only a single marker of mitochondrial content, and both PGC-1α and COXIV protein were increased to the same extent in all three groups (Morton, et al. , 2009). More research is therefore warranted to determine the role of CHO availability on mitochondrial adaptation.

While it remains to be solidified whether or not reduced CHO training augments mitochondrial biogenesis to a greater degree than training with normal or high CHO, CHO does appear to affect those signalling mechanisms linked to this process. For example, a number of studies have linked CHO and glycogen availability to AMPK activity, depicting an inverse relationship (Akerstrom, et al. , 2006, Steinberg, et al. , 2006, Wojtaszewski, et al. , 2002, Wojtaszewski, et al. , 2003, Yeo, et al. , 2008a). While not all studies have supported this relationship (Lee-Young, et al. , 2006), the presence of a glycogen-binding domain in the β-subunit of AMPK may provide a foundation for these responses (McBride, et al. , 2009, Polekhina, et al. , 2003). CHO may also have a regulatory role in p38 MAPK activity, as two studies have shown CHO-restricted exercise to induce greater increases in p38 MAPK phosphorylation relative to exercise with normal CHO availability (Chan, et al. , 2004, Cochran, et al. , 2010). It would therefore appear that altering CHO availability may have the ability to augment mitochondrial adaptation by way of enhancing upstream protein signalling events.

1.6.2. Manipulating buffering capacity

In the majority of exercise scenarios, skeletal muscle protects its acid-base balance within a tight range approximating a pH value of ~7.2 (Juel, 2008). High-intensity

exercise however, is well-known to present a significant challenge to the skeletal muscles' buffering systems. It has been shown, in fact, that repeated maximal sprints lasting 30 s may drive skeletal muscle pH as low as 6.5 (Bogdanis, et al. , 1996, Parolin, et al. , 1999, Spriet, et al. , 1989). This skeletal muscle acidosis is believed to be at least partially responsible for the relative shutdown of glycogenolysis that occurs during all-out HIT (Parolin, et al. , 1999), as glycolytic flux has been shown to be reduced 40-60% when skeletal muscle pH reaches 6.7 (Spriet, et al. , 1987, Spriet, et al. , 1989). In turn, this skeletal muscle acidosis and glycolytic inhibition has been associated with reduced power output during high-intensity exercise efforts (Parolin, et al. , 1999, Spriet, et al. , 1989). Furthermore, the induction of acidosis has been shown to increase fatigue rates (Jones, et al. , 1977, Spriet, et al. , 1985), while inducing alkalosis appears to have the opposite effects (Jones, et al. , 1977). In line with this, a number of recent studies have examined whether supplementing the body's natural buffering may augment exercise performance.

Perhaps the most popular method of enhancing buffering during high intensity work is via the acute induction of blood alkalosis by way of sodium bicarbonate ($NaHCO_3$) (Carr, Hopkins and Gore. , 2011, Matson and Tran. , 1993, Stellingwerff, et al. , 2007). A number of studies have now shown that the acute ingestion of $NaHCO_3$, generally 0.3-0.5 g/kg b.w. 1-2 h before exercise, has the capacity to augment mean power output and/or fatigue resistance leading to improved performance in short duration, high-intensity efforts (Bishop, et al. , 2004, Bishop and Claudius. , 2005, Driller, et al. , 2012, Edge, Bishop and Goodman. , 2006, Raymer, et al. , 2004, Sale, et al. , 2011). Interestingly, a few studies have suggested that $NaHCO_3$ supplementation

throughout training may have the ability to enhance some training adaptations (Bishop, et al. , 2010, Edge, Bishop and Goodman. , 2006). For example, Edge and associates (Edge, Bishop and Goodman. , 2006) showed that when subjects consumed $NaHCO_3$ prior to each training session during an 8 wk constant-load HIT protocol, that training induced increases in lactate threshold and time to fatigue was significantly greater when compared to those who trained while consuming placebo. Importantly, these two groups performed the same amount of work during training (Edge, Bishop and Goodman. , 2006). A later study by the same group showed that mitochondrial respiratory capacity and time to fatigue was augmented by training to a greater extent in rats who consumed $NaHCO_3$ compared to placebo (Bishop, et al. , 2010). These findings would appear to suggest that the inhibition of acidosis during exercise may have the capacity to not only enhance acute performance, but also augment the adaptive response to chronic training. The proposed mechanisms behind this potential for enhancing training adaptation is an increased lactate production by way of an alkalosis-mediated acceleration of glycogenolysis (or prevention of acidosis-mediated glycogenolytic inhibition) (Hollidge-Horvat, et al. , 2000). This increased lactate production, would then act as a signal for enhancing PGC-1α activity (Hashimoto, et al. , 2007). Indeed, Hashimoto and associates (Hashimoto, et al. , 2007) showed that exposing L6 myotubes to lactate resulted in increased PGC-1α expression, which was, in turn, associated with an increased NRF-2 DNA binding, and COX mRNA and protein expressions. An enhanced skeletal muscle buffering capacity may therefore have the capacity to enhance training-induced mitochondrial biogenesis by way of a) increasing lactate signalling to PGC-1α and downstream TFs, or b) increasing the

capacity of the athlete to train harder during each individual training session (akin to high CHO training), ultimately resulting in greater adaptation (Spriet and Gibala. , 2004).

A potential caveat to training with $NaHCO_3$ supplementation is the finding that acute supplementation with large amounts often results in gastrointestinal distress (Carr, Hopkins and Gore. , 2011, Carr, et al. , 2011, Spriet, Perry and Talanian. , 2008, Stellingwerff, et al. , 2007). More specifically, acute $NaHCO_3$ supplementation has been associated with nausea, diarrhea, vomiting and stomach pain, which manifests approximately 90 min following ingestion (Carr, et al. , 2011). This represents a serious pragmatic issue, as this is time point often directly coincides with the onset of training or competition. Thus, while augmenting the buffering capacity around the skeletal muscle via $NaHCO_3$ may potentially be beneficial for training, the difficulty in adhering to this nutritional regime may make it impractical.

An alternative nutritional method for increasing buffering capacity is by way of β-alanine (β-ALA) supplementation. β-ALA is the rate-limiting precursor to the production of skeletal muscle carnosine, a dipeptide that plays a significant role in intramyocellular buffering (Abe, 2000, Derave, et al. , 2010). Carnosine concentrations in skeletal muscle are generally associated with the glycolytic capacity of the tissue itself. In line with this, higher levels of carnosine are in the skeletal muscle of animals that perform sprint or burst-like activity, with greater concentrations in white compared with red muscle (Abe, 2000). Similarly, type II fibers in human muscle have higher carnosine concentrations relative to their type I counterparts (Derave, et al. , 2010, Hill, et al. , 2007, Kendrick, et al. , 2009). Regular β-ALA supplementation over a period of weeks has been shown to

increase muscle carnosine all fiber types (Kendrick, et al. , 2009) in a near-linear manner (Stellingwerff, et al. , 2012). More specifically, supplementation with 1.6-6.4 g β-ALA per day over a period of 4 wks or greater can increase skeletal muscle carnosine by >40% (Baguet, et al. , 2009, Baguet, et al. , 2010, Derave, et al. , 2007, Harris, et al. , 2006, Hill, et al. , 2007, Kendrick, et al. , 2009, Stellingwerff, et al. , 2012). These β-ALA-mediated increases in muscle carnosine content have, in turn, been associated with improvements in physical performance, specifically in short-duration, high intensity efforts (Baguet, et al. , 2010, Derave, et al. , 2007, Harris, et al. , 2006, Hill, et al. , 2007, Hobson, et al. , 2012, Van Thienen, et al. , 2009). For example, Hill and associates (Hill, et al. , 2007) showed that supplementing with 6.4 g β-ALA/d increased skeletal muscle carnosine by nearly 60% in 4 wks, progressing to ~80% after 10 wks. These increases allowed subjects to increase their time to fatigue and total work production at 110% W_{max} by ~13% and 16% at these respective time points (Hill, et al. , 2007). Similarly, Derave and colleagues (Derave, et al. , 2007) showed that β-ALA supplementation increased skeletal muscle carnosine content by ~40%, and this increase resulted in an improved ability to resist fatigue during repeated bouts of 30 maximal voluntary knee extensions. β-ALA supplementation therefore appears to have the capacity to increase skeletal muscle buffering capacity and exercise performance in a similar manner to $NaHCO_3$ supplementation. In support of this, two studies have now shown that performance may be improved by β-ALA or NaHCO3 supplementation, but that there are no additive effects when these supplements are combined (Bellinger, et al. , 2012, Sale, et al. , 2011). However, no studies have examined the effects of β-ALA supplementation on the

mitochondrial adaptive response to training, and therefore it remains to be determined whether or not β-ALA may have the capacity to augment training adaptation by either increasing work output and/or lactate signalling. From a practical perspective, the only noted side effect of β-ALA supplementation is that large acute dosages in excess 10 mg β-ALA/kg b.w. can result in uncomfortable sensations of paresthesia (Harris, et al. , 2006). These effects therefore necessitated the breakdown of supplementation into ~800 mg doses (8 daily doses to achieve 6.4 g) (Harris, et al. , 2006). However, with the advent of slow-release caplets, these sensations may largely be avoided, along with the number of daily dosages being reduced (Decombaz, et al. , 2012). Therefore, β-ALA supplementation may be a much more practical method than $NaHCO_3$ to achieve improved buffering capacity and greater high-intensity performance.

1.7. Conclusions

The general purpose of the present thesis is to determine the effects of manipulating the high-intensity exercise stimulus on subsequent skeletal muscle adaptation. More specifically, the goal of this thesis was to determine the role of the "interval" in the HIT paradigm, and to determine whether the effectiveness of HIT may be augmented by nutritional means. The specific purpose of Study 1 was to determine whether the intermittent nature of HIT (i.e. alternating work and rest periods) is an obligate component of high-intensity exercise training. We performed two separate investigations to examine this question. The first investigated the acute protein signalling responses to two work-matched exercise sessions performed at all-out intensity with the only difference being that one was performed intermittently while the other was

performed continuously. Thereafter, we had subjects perform six weeks of regular training utilizing the low-volume, continuous model of all-out training to determine whether it could induce similar mitochondrial adaptations to what we have reported previously with all-out HIT, and/or ET. We hypothesized that a designated, low volume amount of work performed at all-out intensity would have activate protein signalling molecules linked to mitochondrial biogenesis to the same extent independent of intermittency or continuity. After establishing that both protocols did elicit similar signalling responses, we hypothesized that six weeks of low-volume, all-out continuous training would result in similar adaptations as six weeks of all-out HIT.

The purpose of Study 2 was to determine whether CHO restriction *per se* during twice-daily HIT could result in an enhanced mitochondrial biogenesis in relative to normal CHO consumption. We have previously shown that CHO restriction between two HIT sessions in the same day resulted in an increased p38 MAPK response to the second HIT session (Cochran, et al. , 2010). These findings, coupled with the proposed effects of lowered CHO availability on AMPK activity and mitochondrial adaptation led us to hypothesis that restricting CHO between HIT sessions over the long term would result in an augmented mitochondrial response relative to when fed CHO. This study was also designed to circumvent the confounding factor of training schedule, while examining a number of markers of mitochondrial biogenesis in attempts to isolate the effects of CHO on HIT-induced adaptation.

Finally, the purpose of Study 3 was to examine the effects of β-ALA supplementation on mitochondrial adaptation to 6 wks of all-out HIT. We hypothesized

that β-ALA supplementation would increase skeletal muscle carnosine, providing an enhanced capacity for work during training, which would ultimately result in a greater adaptation to all-out HIT relative to placebo. This was the first training study examining the effects of β-ALA supplementation on mitochondrial adaptation to exercise.

1.8. References

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Chapter 2: Skeletal muscle remodelling in response to intermittent versus continuous high-intensity exercise: similar acute responses but divergent training adaptations

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Running Head: Intermittent versus continuous high-intensity exercise

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Abstract

High-intensity interval training (HIT) performed in an "all-out" manner (e.g., repeated Wingate Tests) is a time-efficient strategy to induce skeletal muscle remodelling similar to endurance training; however, it remains unclear whether the intermittent or "pulsed" nature of the HIT stimulus is obligatory for skeletal muscle adaptation. In Study 1, we examined whether the acute activation of signalling cascades linked to mitochondrial biogenesis in response to a given volume of high-intensity exercise was dependent on the manner in which the stimulus was applied. Subjects performed either 4 x 30 s Wingate Tests interspersed with 4 min rest (INT) or single bout of all-out continuous exercise (CONT) matched for total work (70 ± 2 kJ). Both protocols elicited similar exercise-induced increases in markers of AMPK and p38 MAPK activation, and PGC-1α mRNA expression (main effects for time, $P \leq 0.05$). In Study 2, we determined whether 6 wk of training (3x/wk) using the CONT protocol would increase skeletal muscle mitochondrial content, similar to what we have previously shown after a 6 wk INT protocol. Despite the similar acute activation of signalling cascades linked to mitochondrial biogenesis observed after both CONT and INT in Study 1, the 6 wk CONT training protocol did not increase the maximal activity or protein content of a range of mitochondrial enzyme markers. We conclude that acute changes in AMPK and p38 MAPK signalling after high-intensity exercise do not necessary predict training-induced adaptations. The intermittent nature of all-out, low-volume HIT may be obligatory for stimulating increases in mitochondrial content.

2.1. Introduction

High-intensity interval training (HIT) — characterized by short bursts of relatively intense exercise interspersed by periods of recovery within a given training session — stimulates mitochondrial biogenesis in skeletal muscle and remodelling towards a more oxidative phenotype (Burgomaster, et al. , 2005, Gibala, et al. , 2006). HIT performed using brief "all-out" or "supramaximal" work efforts (e.g., repeated Wingate Tests) is a particularly potent training stimulus. For example, subjects who trained three days per week using 4-6 x 30 sec bursts of all-out cycling interspersed by 4 min of recovery (for a total of only 2-3 min of intense exercise within a ~20 min session), experienced comparable improvements in mitochondrial content and metabolic adaptation to those who performed 40-60 min of continuous moderate-intensity training per session, 5 d per week (Burgomaster, et al. , 2008). It is therefore possible to stimulate rapid adaptations in skeletal muscle comparable to traditional endurance training with a relatively small volume of HIT, provided the exercise stimulus is very intense and applied in an intermittent manner (Burgomaster, et al. , 2008).

The molecular mechanisms responsible for exercise-induced mitochondrial biogenesis remain to be fully elucidated, but acute HIT appears to stimulate many of the same signalling pathways that are activated after moderate-intensity continuous exercise (Bartlett, et al. , 2012). Specifically, an acute bout of Wingate-based HIT activates AMP-activated protein kinase (AMPK) and p38 mitogen-activated protein kinase (MAPK), in association with increases in the expression of peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1α), a preeminent molecule in exercise-induced mitochondrial

biogenesis (Baar, et al. , 2002, Pilegaard, Saltin and Neufer. , 2003). AMPK and p38 MAPK have been purported to augment the activity of PGC-1α by way of direct phosphorylation (Jager, et al. , 2007, Puigserver, et al. , 2001). This phosphorylation has been associated with PGC-1α translocation to the nucleus (Little, et al. , 2011, Wright, et al. , 2007) and mitochondrion (Safdar, et al. , 2011) where it simultaneously co-activates nuclear- and mitochondrially-encoded genes encoding mitochondrial proteins. The resulting increase in the mRNA expression is ultimately believed to lead to augmented mitochondrial gene expression in the form of protein content (Scarpulla, 2008).

Evidence suggests that exercise intensity plays a key role in the regulation of PGC-1α and its upstream protein regulators. Egan et al. (Egan, et al. , 2010) showed that selected signalling proteins linked to mitochondrial biogenesis were phosphorylated to a greater extent following continuous high intensity exercise (~36 min at 80% VO_{2peak}) compared to a work-matched bout of lower intensity exercise (~70 min at 39% VO_{2peak}). These data are consistent with the hypothesis that higher intensities may be more effective for stimulating mitochondrial biogenesis, at least when a large volume of exercise (~1700 kJ) that simulates traditional endurance training is performed. It would therefore seem likely that the high intensities associated with all-out HIT play a key role in achieving similar mitochondrial adaptations to traditional endurance training with abbreviated time and volume. However, it remains to be determined whether the intermittent or pulsatile nature of HIT (i.e., alternating hard/easy pattern) is critical for skeletal muscle adaptation when total exercise volume is very low.

In the present study, we sought to determine whether the activation of signalling cascades linked to mitochondrial biogenesis in response to low-volume (~70 kJ), all-out exercise was dependent on the manner in which the stimulus was applied. We hypothesized that low-volume all-out exercise would activate signalling cascades linked to mitochondrial biogenesis to a similar extent, regardless of whether the exercise was performed in an intermittent (INT) or continuous (CONT) manner. The INT protocol consisted of 4 x 30 sec Wingate Tests interspersed with 4 min of recovery. The CONT protocol was worked-matched to the INT protocol, and completed as a single all-out effort. After establishing that both protocols elicited similar acute signalling responses (Study 1), we conducted a 6 wk training study (Study 2) to determine if CONT all-out exercise could induce chronic mitochondrial and metabolic adaptations resembling those of our previous HIT studies. We hypothesized that CONT training would improve measures of skeletal muscle mitochondrial content and whole-body substrate metabolism in a fashion similar to what we have previously shown after six weeks of INT training (Burgomaster, et al. , 2008).

2.2. Methods

2.2.1. Ethical approval

All experimental procedures were approved by the Hamilton Health Sciences/Faculty of Health Sciences and McMaster University Research Ethics Board, and conformed in all respects with the Declaration of Helsinki. All subjects completed

routine medical screening and provided written informed consent prior to study participation.

2.2.2. Subjects

A total of 17 subjects volunteered to participate in the two studies (Table 2.1). Eight subjects took part in Study 1, which involved a repeated measures design to evaluate the skeletal muscle metabolic response to an acute bout of INT or CONT high-intensity exercise matched for total work. Nine subjects took part in Study 2, which examined skeletal muscle remodelling in response to a 6 wk training intervention using the CONT protocol. All subjects were young healthy individuals who were habitually active but not specifically trained in any sport.

2.2.3. Study 1

2.2.3.1. Pre-Experimental Procedures

VO_{2peak} and peak aerobic power output (W_{peak}) were initially determined during a ramp protocol to volitional fatigue on an electromagnetically-braked cycle ergometer (Lode Excalibur Sport, Groningen, the Netherlands) using an online gas collection system (Moxus modular oxygen uptake system, AEI technologies, Pittsburgh, PA, USA) as we have previously described (Cochran, et al. , 2010). Specifically, participants began cycling for 2 min at 50 W, followed by a progressive increase in power demand at the rate of 1 W every 2 sec. Thereafter, subjects participated in a minimum of two familiarization trials on separate days using the same electronically-braked cycle ergometer employed during the main phase of the study (Velotron, RacerMate Inc., Seattle, WA) in order to

become acquainted with the exercise protocols. Due to the nature of the experimental design, all subjects performed the INT exercise protocol during their first familiarization visit. This was necessary in order to determine the total amount of work needing to be performed during the CONT exercise protocol for a given subject.

The INT protocol consisted of 4 x 30 sec all-out sprints, performed against a resistance equivalent to 7.5% of body mass (i.e. repeated Wingate Tests), interspersed with 4 min of recovery, as we have previously described (Burgomaster, et al. , 2005). A computer with appropriate software (Velotron Wingate Software v1.0) was interfaced with the ergometer and permitted the appropriate load to be applied for each subject. Total work output, peak power and mean power were calculated and recorded by an online data acquisition system.

For the CONT protocol, subjects performed the same volume of total work as in the INT exercise session, but as a single all-out effort. The ergometer was interfaced with software (Velotron Coaching Software v1.5) that linked power output directly to pedalling cadence, while quantifying total work done in real-time. Subjects were instructed to complete their designated amount of work as quickly as possible by maintaining the highest pedalling cadence possible. Between 50 and 100 rpm, power output corresponded with a range of 75 to 500 W. Cycling was terminated immediately upon completion of the designated amount of work.

Experimental Trials

The main experiment consisted of two trial days separated by at least one week. Trials were conducted in a randomized, counterbalanced manner with half the subjects

starting with the INT protocol and the other half with the CONT protocol. Subjects were instructed to refrain from exercise for 48 h prior to each experimental trial, and to avoid caffeine and alcohol for at least 12 h before the trials. Subjects maintained individual food diaries for the 24 h period preceding the first trial, and replicated their diet during the second trial. Dietary records were subsequently analyzed for total energy and macronutrient content using Diet Analysis Plus (Cengage Learning Inc., Florence, KY).

On the day of each trial, subjects arrived at the laboratory in the morning, 60-90 min after ingesting their habitual breakfast. Food records were collected and subjects then changed into athletic apparel, and rested quietly until trial commencement. A resting needle muscle biopsy sample was obtained from the *vastus lateralis* of one thigh under local anesthesia (1% xylocaine) as previously described (Gibala, et al. , 2006). The muscle sample was immediately frozen in liquid nitrogen and stored at -80 ºC until further analyses. After resting for another 10 min, the subjects moved to the cycle ergometer and completed a standardized warm-up that consisted of 2 min of unloaded cycling followed by 5 min of rest. Subjects then performed the designated exercise protocol. A second muscle biopsy was obtained immediately upon cessation of cycling, and subjects were asked to provide a rating of perceived exertion for the overall exercise protocol, using the Borg scale (Borg, 1974). Subjects then rested quietly in the laboratory for 3 h, at which point a third muscle biopsy was taken. The three biopsies for a given trial were obtained from the same leg through separate incisions >2 cm apart.

2.2.3.2. Muscle Analyses

Western Blotting. Whole cell lysates were prepared by adding ~30 mg wet muscle to ice-cold RIPA buffer (50 mM HCL, 150 mM NaCl, 1 mM PMSF, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease (Complete Mini®, Roche Applied Science, Laval, PQ, Canada) and phosphatase inhibitors (PhosSTOP®, Roche Applied Science, Laval, PQ, Canada). Samples were minced and homogenized on ice (Pro 250, Pro Scientific, Oxford, CT, USA), sonicated, and agitated end-over-end for 15 min at 4^{o}C. Samples were then centrifuged at 15,000 g for 5 min at 4^{o}C. The pellet was then resuspended, and following a second centrifugation at 15,000 g for 10 min, the supernatant was collected for subsequent analysis. Homogenate protein concentrations were determined using a commercial, detergent-compatible, colorimetric assay (BCA protein assay, Pierce, Rockford, IL). Equal amounts of protein (5-20 μg, depending on the protein of interest) were then loaded onto 7.5-12.5% SDS-PAGE gels and separated by electrophoresis for 2-2.5 hours at 100 V. Proteins were transferred to nitrocellulose membranes for 1 hr at 100 V. Ponceau S staining was performed following the transfer and was used to control for equal loading and transfer between lanes. Membranes were blocked using a 5% fat-free milk or BSA solution in TBS-T at room temperature, and incubated overnight with the appropriate primary antibodies diluted in a 3% fat-free milk or BSA in TBS-T, thereafter. Primary antibodies targeting phospho-p38 MAPK, total-p38 MAPK, phospho-acetyl-CoA carboxylase (ACC), were purchased from Cell Signaling Technology (Beverly, MA). Blots were then incubated in the appropriate secondary antibodies for 1 hour at RT, and visualized by chemiluminescence

(Supersignal® West Dura, Pierce). Signal quantification was performed using NIH Image J software.

Real-time RT-PCR. Frozen wet muscle samples (~20 mg) were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was isolated using the RNeasy Mini Kit in conjunction with the RNase-Free DNase Set DNA digestion (Qiagen, Mississauga, ON, Canada). RNA was then reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Carlsbad, CA), aliquoted, and stored at -80^{o}C until further analysis. RT-PCR reactions for PGC-1α mRNA expression were run using forward (5'-CAT CAA AGA AGC CCA GGT ACA-3') and reverse (5'-GGA CTT GCT GAG TTG TGC ATA-3') primers in combination with SYBR green/ROX fluorescence chemistry (PerfeCTa, Quanta Biosciences). Reactions were run on a thermal cycler (Applied Biosystems, Carlsbad, CA), and expression levels were normalized to the housekeeper gene β2-microglobulin (Forward: 5'-GGC TAT CCA GCG TAC TCC AA-3'; Reverse: 5'-GAT GAA ACC CAG ACA CAT AGC A -3'), which was verified to be unchanged in response to our exercise interventions (data not shown).

2.2.4. Study 2:

2.2.4.1. Pre-Experimental Procedures

Subjects initially performed baseline VO_{2peak} testing as described for Study 1. Thereafter, subjects undertook a series of familiarization sessions in order to become accustomed to the testing and training procedures. These sessions included a 250 kJ

simulated cycling time trial (TT), a 60 min steady-state session at ~65% VO_{2peak}, and a practice training session which was modeled after the CONT protocol employed in Study 1. TT familiarizations were repeated at 1 wk intervals until participants could not further improve beyond their previous session. Consistency in performance during familiarizations were verified by t-test ($p = 0.3$), and the latter of two similar results were taken as baseline TT performance. Subjects completed 24 h diet records prior to each of these tests, and diets were replicated over the 24 h period preceding post-training tests.

250 kJ TT: All chronic study participants were instructed to complete, as quickly as possible, a simulated TT consisting of 250 kJ of total work. This test was performed on the same electromagnetically-braked cycle ergometer (Velotron, RacerMate Inc., Seattle, WA) interfaced with software (Velotron Coaching Software v1.5) as training at a standardized gearing. Again, the cycle ergometer was programmed such that power outputs between 75 and 500 W were directly related with pedalling rates, and subjects were instructed to maintain the highest pedalling cadence possible. No feedback was given during the rides with the exception of work remaining, and the test was terminated immediately upon the completion of 250 kJ.

60 min steady-state ride at 65% VO_{2peak}: Subjects cycled continuously for 60 min at an intensity designed to elicit 65% of their peak oxygen uptake. The steady-state ride was conducted on the same cycle ergometer as the VO_{2peak} measurement (Lode Excalibur), and respiratory measurements were made at specific 5 min intervals throughout exercise using the same metabolic cart system described previously (Moxus oxygen uptake system, AEI).

Skeletal Muscle Biopsy: A resting skeletal muscle biopsy was taken approximately one week following performance testing as described for Study 1. Subjects were instructed to record their diet for the 24 h preceding the biopsy, while refraining from exercise for a minimum of 48 h, and abstaining from caffeine and alcohol for a minimum of 12 h pre-biopsy. Muscle samples were immediately frozen under liquid nitrogen, and subsequently stored at -80°C until further analysis. Diets were replicated post-training, and a second resting biopsy was taken 72 h following the last exercise training session.

2.2.4.2. CONT exercise training:

Training was performed 3 d per week for 6 wks, for a total of 18 sessions to align directly with our previous 6 wk INT study schedule. The training intervention was modelled after the CONT protocol employed in Study 1 and each session consisted of a single bout of high-intensity cycling completed as quickly as possible. Based on Study 1 and other pilot work, mean power produced over the course of 4 Wingate tests interspersed with 4 min of recovery in recreationally-active subjects averaged ~1.0 kJ per kg of body mass. Subjects were therefore assigned an initial exercise training load that corresponded to 1.0 kJ per kg body mass. Training load was subsequently increased to 1.25 kJ per kg body mass during the second half of the 6 wk intervention in order to provide progression and maintain the duration of the training session. Workload was self-selected and varied over the training session based on pedalling cadence, with a range of 50-100 rpm corresponding to ~75-500 W. During each training session, heart rate was monitored and ratings of perceived exercise (RPE) scores were obtained based on the Borg scale (Borg, 1974).

2.2.4.3. Post-training testing and procedures:

Post-training procedures were identical in all respects to those conducted prior to training onset, with the exception of order. Subjects first underwent a second resting skeletal muscle biopsy ~72 h post-training. This time point was chosen to evaluate training-induced changes in resting muscle. Steady-state, TT and VO_{2peak} tests took place at 48 h intervals thereafter. All subjects adhered to previously recorded diet records for the 24 h preceding each of biopsy and testing procedures.

2.2.4.4. Muscle Analysis:

Western blotting: Frozen muscle samples of ~30 mg were homogenized and prepared for SDS-PAGE exactly as described for Study 1. Likewise, equal amounts of protein (5-20 μg) were separated by electrophoresis, proteins were transferred to nitrocellulose, and membranes blocked, and probed using primary antibodies targeted against 5 separate mitochondrial protein markers including NDUFA9 (Mitosciences, MS111), Complex II 70 kDa subunit (Mitosciences, MS204), Complex III Core 2 protein (Mitosciences, MS304), cytochrome c oxidase subunit IV (COXIV; Mitosciences, MS408) and the ATP synthase α subunit (Mitosciences, MS507). We also probed nitrocellulose membranes against glucose transporter 4 (GLUT4; Millipore AB1345), and monocarboxylate transporters 1 and 4 (MCT1, Millipore AB3538; MCT4, Millipore AB3316). Thereafter, blots were processed and quantified as in Study 1.

Citrate synthase maximal activity: Approximately 20 mg of wet muscle was homogenized using glass tissue pestles in 10 volumes of buffer containing 70 mM sucrose, 220 mM mannitol, 10 mM HEPES (pH 7.4), supplemented with protease

inhibitors (Complete Mini®, Roche Applied Science, Laval, PQ, Canada). Citrate synthase (CS) maximal activity was then quantified as we have described previously (Gibala, et al. , 2006, Little, et al. , 2010). Homogenate protein content was determined via BCA method using a commercial assay (Pierce, Rockford, IL, USA) and enzyme activity expressed as mmol·kg protein^{-1}·hr^{-1} wet weight.

2.2.4.5. Statistical Analyses:

Exercise data from Study 1 was analyzed via paired Student's t-tests, while all muscle data from Study 1 was analyzed using a two-factor repeated-measured ANOVA, followed where appropriate by a Tukey's HSD post hoc test. All data from Study 2 was analyzed using paired Student's t-tests. The level of significance was set at $P \leq 0.05$ for all analyses and all analyses were conducted using SigmaStat 3.1 software (Systat Software, Chicago, IL). All data are presented as means ± standard deviation (SD).

2.3. Results

2.3.1. Study 1

2.3.1.1. Exercise data

Performance data are presented in Table 2.2. Total work and ratings of perceived exertion were not different between trials (p=0.71, and p=0.81, respectively). Peak power output and mean power output, averaged over the four Wingate tests in the INT trial, was higher than the respective values calculated for the CONT trial. Conversely, total exercise duration in the CONT trial was approximately double that of the INT trial (~4 min versus

2 min, i.e., 4 x 30s), although the latter session required a total of 14 min including recovery between intervals.

2.3.1.2. Muscle data

Muscle glycogen content was reduced by ~25%, and muscle lactate concentration was elevated ~10-fold after exercise, with no difference between protocols (main effect for time, $p<0.01$; Figure 2.1A, 2.1B). Phosphorylation of p38 MAPK and ACC serine-79 increased immediately after exercise by ~3-fold and ~2.5-fold, respectively, with no difference between treatments ($P<0.05$, main effect for time; Figure 2.2A, 2.2B). PGC-1α mRNA expression was increased ~4-fold from rest after 3 h of recovery with no difference between conditions ($P<0.05$, main effect for time; Figure 2.3).

2.3.2. Study 2

2.3.2.1. Exercise data

Subjects completed 99% of the assigned training sessions. Mean total work was 77 ± 12 kJ, completed in an average time of 391 ± 75 s (6:31 ± 00:47 min:sec) at a mean power output of ~212 ± 49 W. Mean heart rate during training sessions was 182 ± 9 bpm, which was equivalent to 95 ± 2% of maximal heart rate. Average RPE values were 18 ± 1. Time required to complete designated training work quotas was increased in association with workload progression, however there were no other significant changes in time to complete training (data not shown). Time to complete 250 kJ of work decreased after training from 26:32 ± 4:48 min:sec to 23:55 ± 4:16 min:sec, a ~9% improvement ($p > 0.001$). During submaximal steady-state cycling sessions at 65% of

pre-training VO_{2peak}, heart rate, respiratory exchange rate, ventilation and absolute oxygen uptake were similar before and after training (Table 2.3).

2.3.2.2. Muscle data

The maximal activity of CS was unchanged after training compared to pre-training (Figure 2.4). Proteins representative of each of the complexes of the electron transport chain were also unchanged after training ($P \geq 0.1$), the one exception being cytochrome c oxidase subunit 4 (COXIV), which showed a 20% increase ($p = 0.014$; Figure 2.5). GLUT4 protein content tended to increase but this was not significant ($p = 0.10$) and similarly, the protein content of MCT1 and MCT4 unchanged by training ($p = 0.52$ and $p = 0.26$, respectively; data not shown).

2.4. Discussion

There were two major novel findings from the present work. First, we showed that an acute bout of low-volume, all-out intensity exercise activates signalling cascades linked to mitochondrial biogenesis similarly regardless of whether it is performed intermittently or continuously. Secondly, we have shown that despite similar acute signalling responses, 6 wks of all-out CONT training is an ineffective training method to increase skeletal muscle mitochondrial content relative to our previous 6 wk studies of all-out HIT (Study 2) (Burgomaster, et al. , 2007, Burgomaster, et al. , 2008). These data suggest that acute AMPK and p38 MAPK signalling responses to all-out exercise do not reflect that exercise session's ability to induce long-term metabolic adaptations. These

findings also suggest that the intermittent nature of all-out HIT may be obligatory for stimulating increases in mitochondrial content with low time and work volume commitment.

The overriding goal of the present study was to try and address the fundamental question: how important is the interval in high-intensity interval training? While low-volume, all-out HIT is a potent stimulus to induce skeletal muscle remodelling towards a more oxidative phenotype (Burgomaster, et al. , 2008, Gibala, et al. , 2006), it is unclear if the intermittent manner in which the HIT stimulus is applied is fundamental to the training adaptation. Our Study 1 signalling findings would appear to suggest that intermittency plays an insignificant role in producing training adaptation to all-out exercise, and that completing a given amount of work at all-out intensity is what is important. This suggestion is corroborated by Wang and associates (Wang, et al. , 2009) who reported similar increases in PGC-1α mRNA content 3 h following 90 min of interval (12 sec at 120 % VO_{2peak} interspersed by 18 sec at 20% VO_{2peak}) or continuous (67% VO_{2peak}) work-matched exercise. Supporting a similar metabolic perturbation between exercise conditions, Wang et al. (Wang, et al. , 2009) also reported similar mean heart rates and ratings of perceived exertion between matched-work trials, which we also observed in Study 1. We also observed comparable levels of muscle glycogen utilization and lactate accumulations between INT and CONT conditions, further supporting similar homeostatic perturbance. This similar metabolic stimulus between INT and CONT conditions may explain our similar ACC and p38 MAPK phosphorylation levels, along with similar PGC-1α mRNA responses. These findings suggest that, for a given volume

of high-intensity exercise, whether the stimulus is applied in an intermittent or continuous manner has little influence on the acute activation of signalling pathways linked to skeletal muscle mitochondrial remodelling.

In contrast to the above interpretation of Study 1, our Study 2 results show that adaptations to all-out intensity training are, indeed, influenced by the manner in which the training stimulus is applied. Specifically, 6 wks of all-out CONT training did not increase skeletal muscle mitochondrial content, as represented by measures of maximal enzyme activity and protein content. While COX subunit IV was increased, this was the only marker that was increased by CONT training while all 5 others remained unaltered. Specifically, CS maximal activity was unchanged following 6 wks of CONT training, and this marker was recently shown to be the one of the best markers of mitochondrial content in human skeletal muscle (Larsen, et al. , 2012). The inability of CONT training to induce positive metabolic adaptation in skeletal muscle is in marked contrast to the robust increases in CS enzyme activity we have observed after 6 wks of INT training in our previous studies (Burgomaster, et al. , 2007, Burgomaster, et al. , 2008). CONT training also had no effect on GLUT4, MCT1 and MCT4 protein contents, which we have shown to be increased by INT training (Burgomaster, et al. , 2007). Furthermore, 6 wks of CONT training had no effects on several cardiorespiratory measures during submaximal, steady-state exercise, while 6 wks of INT training reduces heart rate, respiratory exchange rates, and ventilation (Burgomaster, et al. , 2008). Altogether, our findings suggest that in order to achieve positive skeletal muscle metabolic adaptation utilizing low-volume (~70 kJ), all-out training, it must be performed in an intermittent manner to allow for work to

be performed at the highest possible power output. Thus, the intermittent nature and associated higher power outputs of the HIT stimulus may be very important for eliciting skeletal muscle remodelling. Furthermore, our data suggests that acute activation levels of selected signalling pathways linked to mitochondrial biogenesis following exercise cannot be used to predict chronic training adaptation.

The primary proposed mechanism for these effects of AMPK and p38 MAPK on metabolic adaptation to exercise is through the regulation of the transcriptional coactivator, PGC-1α. PGC-1α plays a key role in metabolic adaptation, as muscle-specific overexpression of PGC-1α is sufficient to increase mitochondrial enzyme activity and exercise capacity in mice (Calvo, et al. , 2008), while muscle-specific knockout results in exercise intolerance (Handschin, et al. , 2007) and attenuated mitochondrial adaptation to training (Geng, et al. , 2010). Indeed, acute exercise has been shown to increase both AMPK activity (Fujii, et al. , 2000) and p38 MAPKphosphorylation (Widegren, et al. , 1998) in human skeletal muscle, and these molecules are suggested to be key modulators of PGC-1α activity (Jager, et al. , 2007, Puigserver, et al. , 2001). However, while a number of studies have shown that exercise of greater intensity or duration (Egan, et al. , 2010, Stephens, et al. , 2002, Wojtaszewski, et al. , 2000) results in greater activation/phosphorylation of signalling proteins in the muscle, it has never been demonstrated that this greater activation decisively results in greater adaptive change. Indeed, no evidence has been presented that subject-to-subject variability in either AMPK, p38 MAPK or PGC-1α have been correlated with training adaptation in human muscle, it has led some to question the purpose focusing so much research upon upstream

signalling events (Timmons, 2011). Our present findings support the contention that ACC and p38 MAPK phosphorylation status do not provide sufficient information to assume training potency. This supposition is supported by other recent publications showing divergence between protein signalling responses and muscle protein synthetic responses to exercise and nutrition (Areta, et al. , 2013). These findings underscore the fact that changes in mRNA expression do not guarantee a similar change in functional protein or enzyme activity. Presently, too little is known regarding the effects that exercise of different types may have on downstream processes such as mRNA stability and turnover, protein translation, protein import and assembly, mitochondrial fusion/fission, and mitophagy, and therefore any combination of these processes may be responsible for the diversion between mRNA and protein expressions. More work must be done to examine the effects that exercise intensity, duration, and factors such as intermittency may have on the intervening biological processes between mRNA content and functional protein expression.

While previous research has strongly implicated AMPK and p38 MAPK in the metabolic adaptation to exercise and made them the best characterized signalling molecules in the process of mitochondrial biogenesis, a few caveats to our present work must be acknowledged. First, we have measured the gross phosphorylation of ACC and p38 MAPK in whole muscle. Recent research has suggested that specific subunits of AMPK and p38 MAPK may play distinctive roles in the adaptive response to exercise (Birk and Wojtaszewski. , 2006, Pogozelski, et al. , 2009) and therefore it is possible that subtle differences in activation could not be resolved by our Western Blotting techniques.

We also made our measurement at the whole muscle level, which may have been less sensitive than if we had examined sub-cellular localizations of the same molecules (Little, et al. , 2010). Lastly, it must be acknowledged that these are only two of a vast network of protein signals within the muscle fibre which may play a role in muscle adaptation to all-out exercise. Nonetheless, our divergent findings between signalling and training responses reiterate the need for studies examining both acute and training responses within the same individuals. Only by conducting these investigations may we determine whether the within-subject variation in acute protein signalling and mRNA expression can in any way be predictive of long-term adaptive responses in humans. Furthermore, more work must be done downstream of mRNA expression to determine how exercise may differentially modulate the ultimate expression of functional mitochondrial protein. Another potential limitation of the present work is that the acute study examined only men, while the chronic study examined a different cohort of men and women. The signalling responses observed in those who completed Study 1 could, therefore, be different than those who completed Study 2. However, no previous HIT studies have revealed any sex-specific responses with regards to training-induced augmentations in mitochondrial content, including our own HIT versus endurance training study that recruited equal numbers of men and women (Burgomaster, et al. , 2008, Perry, et al. , 2008). Thus, while possible that men and women may adapt differently to high-intensity, short-duration training, there is presently little evidence to support this, and it must be concluded that CONT training was simply an insufficient stimulus for mitochondrial adaptation.

Why might the intermittent nature of the exercise stimulus be obligatory for skeletal muscle adaptation to low-volume HIT? It is possible that the repeated, high-amplitude challenges to energy state and metabolic homeostasis may alter the way in which the signal is translated through to the transcriptional and translational machinery. In support of this, Edge and associates (Edge, et al. , 2013) recently showed that manipulating the length of the rest interval in two work-matched HIT training programs had no bearing on the resulting adaptations. Thus, it may be the number of repeated challenges faced by the muscle that allows HIT to be effective in achieving adaptation at the mitochondrial and whole-body level. Alternatively, the durations of the sessions themselves may alter downstream processes of functional protein production. In interest of maintaining time efficiency, CONT training session duration was limited to 10 min. However, nearing 6 wks of Wingate-based HIT, training sessions approach 27 minutes in length (when including recovery intervals). These HIT sessions therefore last ~3 times longer than CONT training sessions. This difference in overall duration of metabolic challenge may play a role in the ability of HIT to induce metabolic adaptation with low work volumes. Thus, it is possible that signal duration may be equally or more important than the amplitude or strength of the signal activation itself for downstream metabolic adaptation to occur.

An interesting finding of the present study was that 6 wk CONT training improved time to complete 250 kJ of work, without increasing any appreciable change in mitochondrial or transport markers. One possible contributing factor could be improvements in cardiovascular function, as VO_{2peak} during whole body exercise is

primarily limited by central factors in young healthy individuals (Bassett and Howley. , 2000). However, while we could only obtain post-training measures of VO_{2peak} in six of our subjects due to technical difficulties, we did not show any significant improvements in VO_{2peak} following our six-week protocol. Cardiovascular improvements may therefore not be the greatest contributer to our observed performance improvements. Other possible contributors include improvements in buffering capacity, and other peripheral metabolic changes that were not measured in the present study. Indeed, endurance performance is determined by a wide array of factors that could not be entirely addressed by the present study (Coyle, 1995).

In summary, we have shown that performing a given low-volume of work at all-out intensity results in similar increases in AMPK and p38 MAPK signalling pathways activation and PGC-1α mRNA expression regardless of whether that work is performed intermittently or continuously. However, these responses provide insufficient information regarding the ability of an acute exercise session to affect long-term metabolic adaptation, as performing <10 min of continuous all-out intensity exercise 3 times per week did not enhance mitochondrial content or steady-state metabolism in recreationally-active individuals. It would therefore appear that intermittency may play a key role in the ability of all-out HIT to induce similar skeletal muscle metabolic adaptations to endurance training with minimal time commitment and work volume.

Acknowledgements

We would like to thank our subjects for their commitment and effort, as well as Todd Prior, Adeel Safdar, and Mahmood Akhtar for their technical assistance.

Grants

This project was supported by operating grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) to MJG and MAT. AJRC was supported by a NSERC PGS-D scholarship and JPL held a NSERC CGS-D scholarship. MP held NSERC CGS-M, while NC held a NSERC PGS-D, and JG held NSERC CGS-D. The authors have no conflicts of interest to declare.

2.5. References

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Table 2.1. Subject characteristics for those completing acute INT versus CONT high-intensity exercise, and those completing 6 weeks of CONT-based training.

Variable	Acute Study	Chronic Study
Participants	8 men; 0 women	5 men; 4 women
Age (years)	22 ± 1	22 ± 2
Weight (kg)	78 ± 8	78 ± 11
Height (cm)	181 ± 5	173 ± 9
VO2peak (mL·kg-1·min-1)	48 ± 7	47 ± 5

Values are mean ± S.D.

Table 2.2. Performance characteristics for the acute INT and CONT high-intensity exercise sessions.

	INT	CONT
Total work (kJ)	66.8 ± 6.8	67.0 ± 6.8
Peak power output (W)	824 ± 126	510 ± 101*
Mean power output (W)	557 ± 90	281 ± 46*
Work duration (min:s)	2:00 ± 0:00	4:02 ± 0:26*
Ratings of perceived exertion	18.1 ± 1.2	18 ± 1.8

Values are means ± SD, n = 8 subjects. INT, intermittent; CONT, continuous trial. *$p \leq 0.05$ versus INT.

Table 2.3. Cardiorespiratory data during cycling exercise at 65% VO_{2peak} before and after 6 weeks of CONT-based training

	PRE-TR	POST-TR
Heart rate (beats·min^{-1})	158 ± 14	157 ± 16
Respiratory Exchange Rate	0.87 ± 0.04	0.86 ± 0.02
Ventilation (L·min^{-1})	55.6 ± 8.1	54.2 ± 6.2
VO_2 (L·min^{-1})	2.14 ± 0.41	2.07 ± 0.39
VO_2 (mL·kg^{-1}·min^{-1})	30.8 ± 4.8	29.8 ± 4.4*

Values are means ± SD, n = 9 subjects. PRE-TR, pre-training; POST-TR, post-training; VO2, oxygen uptake; CONT, continuous trial. *$p \leq 0.05$ versus PRE-TR.

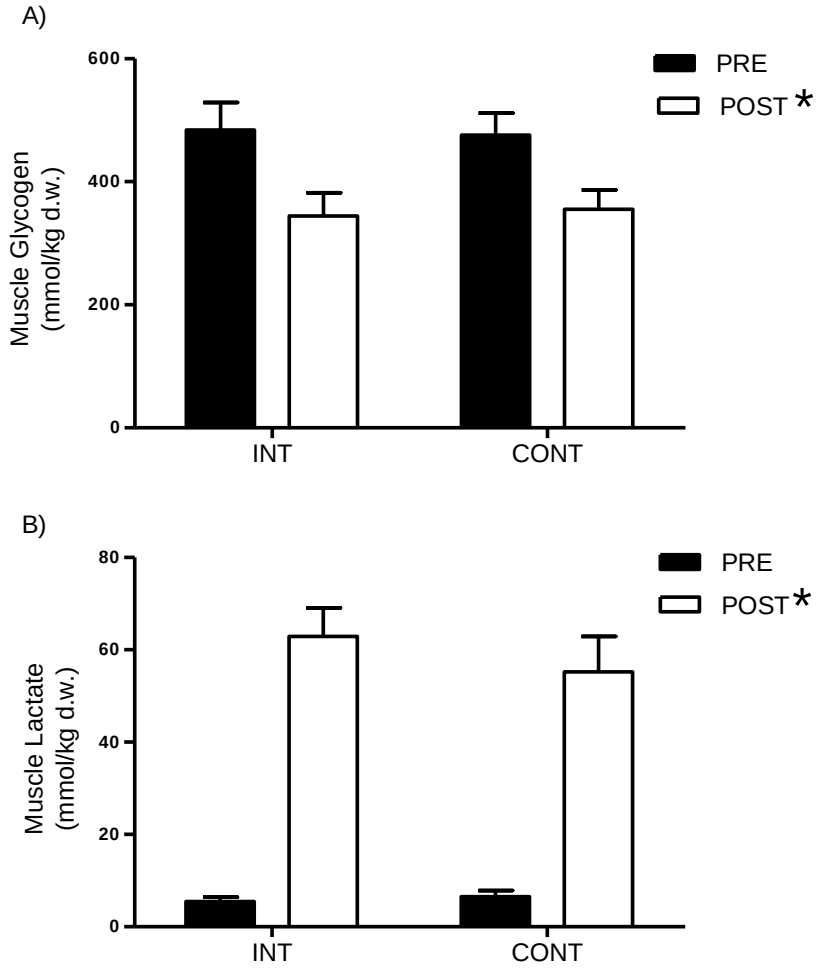

Figure 2.1 Muscle glycogen (A) and lactate (B) concentrations measured before (PRE) and after (POST) performing ~67 kJ of work intermittently (INT) or continuously (CONT) at maximal effort. Values are means ± SEM for 8 subjects. *P<0.05, main effect for time.

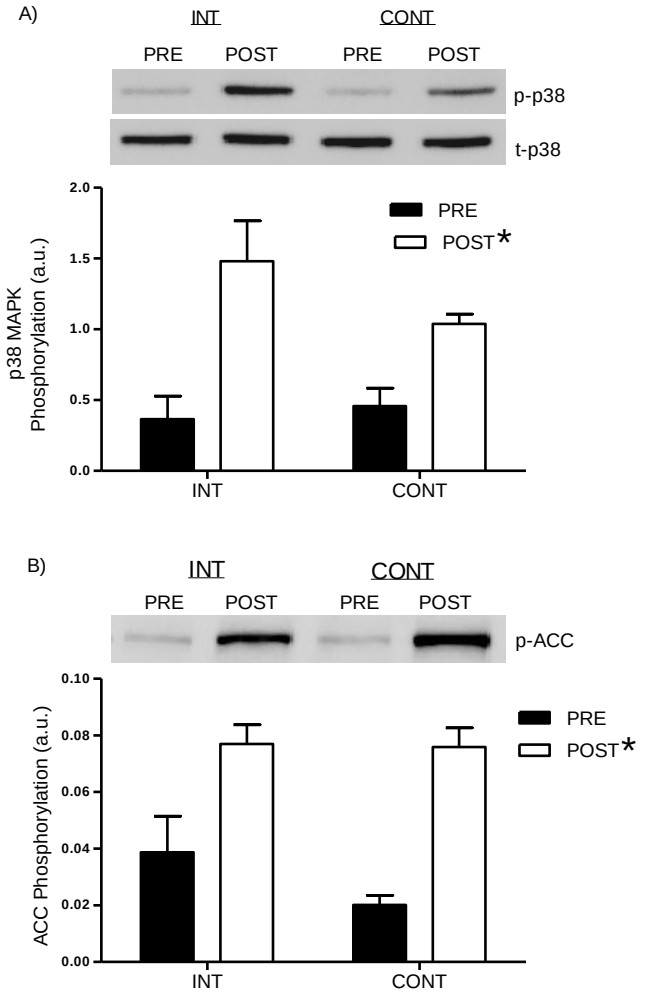

Figure 2.2. Changes in protein phosphorylation of p38 MAPK (Thr180/Tyr182; A), ACC (Ser79; B) before (PRE) and after (POST) ~67 kJ of intermittent (INT) and continuous (CONT) exercise at maximal effort. Values are means ± SEM for 8 subjects. *P<0.05, main effect for time.

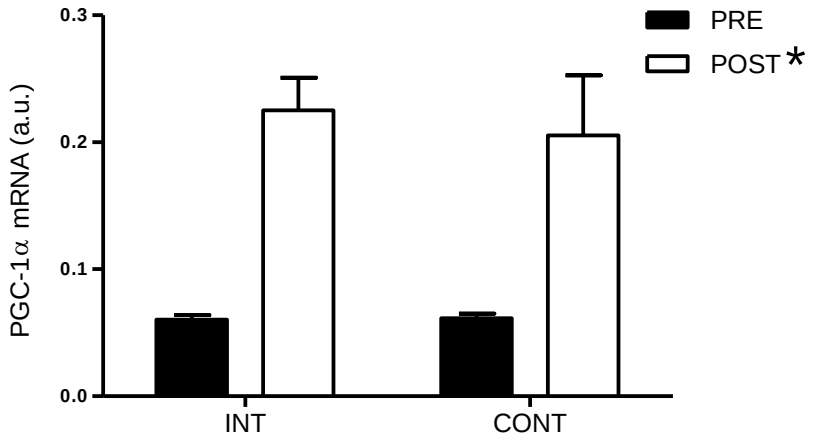

Figure 2.3. PGC-1α mRNA expression before (PRE), and after 3h of recovery (3h POST) from ~67 kJ of work performed intermittently (INT) or continuously (CONT) at maximal effort. Values are means ± SEM for 8 subjects. *P<0.05, main effect for time.

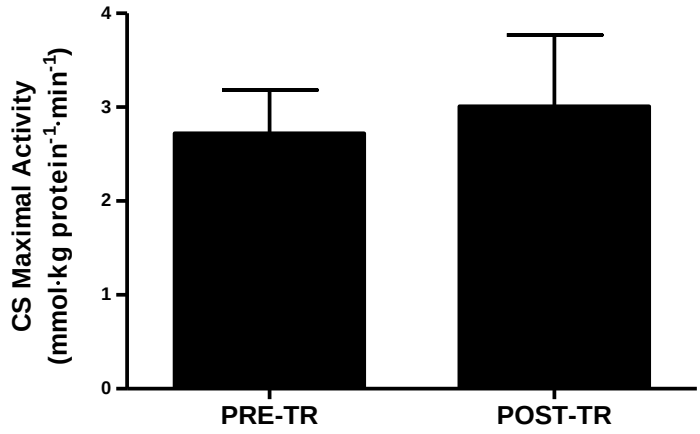

Figure 2.4. Maximal activity of citrate synthase (CS) measured in resting muscle biopsy samples before (PRE-TR) and after (POST-TR) 6 wks of low-volume, all-out CONT training. Values are means ± SD for 9 subjects.

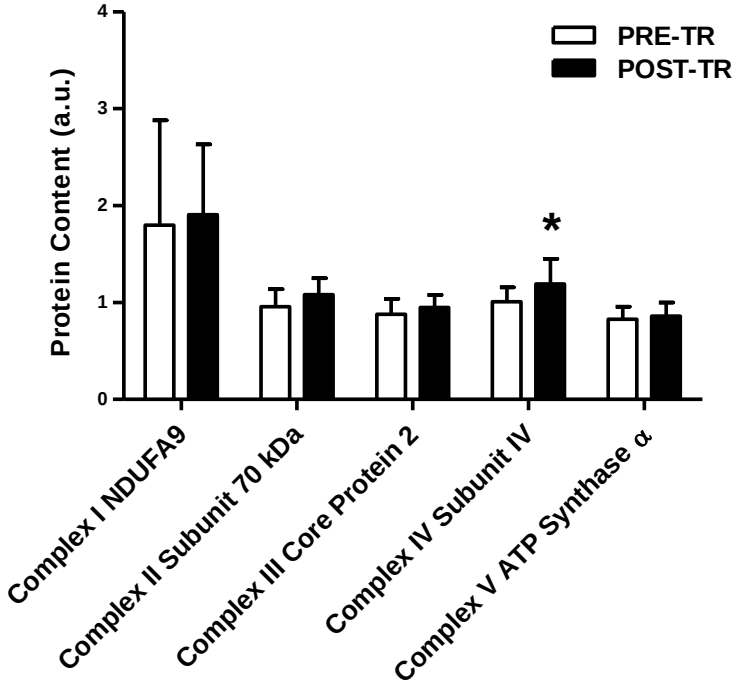

Figure 2.5. Mitochondrial protein content before (PRE-TR) and after (POST-TR) 6 wks of low-volume, CONT training at maximal effort. Values are means ± SD for 9 subjects. NDUFA9, NADH dehydrogenase 1 alpha subcomplex subunit 9; Subunit 70 kDA, Succinate Dehydrogenase 70 kDa flavoprotein subunit; Core protein 2, ubiquinol-cytochrome c reductase assembly protein; subunit IV, cytochrome c oxidase subunit 4; ATP synthase α, catalytic α-subunit of ATP synthase. *P≤0.05 vs pre-training.

Chapter 3: Carbohydrate restriction between twice-daily sessions of high-intensity interval exercise does not augment training-induced adaptations in mitochondrial content

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Running Head: Carbohydrate effects on mitochondrial adaptation to training

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Abstract

Carbohydrate (CHO) is the key supportive macronutrient for high-intensity exercise. Recent evidence has suggested however, that training with reduced CHO availability may augment mitochondrial adaptation to a greater degree than training with high CHO availability. Many of these studies however, cannot differentiate between the roles of training schedule, or CHO availability per se. The purpose of this study was to isolate the role of CHO on the mitochondrial response to high-intensity interval training (HIT). We hypothesized that CHO restriction between two, same-day HIT sessions would augment mitochondrial content to a greater extent compared to when large amounts of CHO were consumed. Eighteen subjects (21 ± 1 years; 44.2 ± 9.0 $mL \cdot kg^{-1} \cdot min^{-1}$) performed two HIT sessions per day, 3 $d \cdot wk^{-1}$, for 2 wk where one group (HI-LO) restricted CHO intake between HIT sessions, while the other received >2 g CHO/kg b.w. (HI-HI). Twice-per-day HIT increased citrate synthase (CS) maximal activity, as well as the protein content of CS and cytochrome c oxidase (COX) subunit 2 and 4 ($p < 0.005$), with no differences between groups. Similarly, 250 kJ time trial performance was increased to a similar extent in both groups (HI-LO: 21:28 ± 7:46 to 18:35 ± 6:20; HI-HI: 21:42 ± 5:20 to 20:18 ± 5:22 min:sec; main effect for time $p<0.002$). These results indicate that CHO restriction between twice-per-day training sessions offers no greater adaptive benefit compared to when CHO is consumed in recreationally-active individuals. Future investigations need to examine the specific roles of CHO availability and muscle glycogen per se in the mitochondrial adaptive response to training.

3.1. Introduction

Carbohydrate (CHO) is well-known to be the key macronutrient supporting ATP production during high-intensity exercise. As it is generally believed that greater training work outputs translates into greater physiological adaptation, athletes are typically advised to consume a high CHO diet in order to maximize CHO availability and high-intensity work capacity for each training session (American Dietetic Association, et al. , 2009). Interestingly, several investigations have recently suggested that training with low CHO availability may be a more effective training paradigm. Using a unilateral leg-extension training model, Hansen and associates (Hansen, et al. , 2005) were the first to report greater increases in citrate synthase (CS) activity when one leg performed 50% of training with reduced muscle glycogen content, while the other leg performed 100% of training with normal glycogen content. Using a cycling model that more closely simulates normal athletic competition, Yeo and associates (Yeo, et al. , 2008b) showed increases in CS and β-hydroxy-acyl-CoA-dehydrogenase (β-HAD) maximal activities when highly-trained cyclists performed 50% of their training with reduced glycogen content, while no increases were shown in those who always trained with high glycogen content. These findings have led to the suggestion that periodic training with reduced CHO availability, i.e., the 'train low, compete high' paradigm (Hansen, et al. , 2005), may result in greater augmentation of mitochondrial content in response to training.

A proposed mechanistic foundation for the train low, compete high model is that CHO restriction augments and/or prolongs exercise-induced increases in protein signalling and metabolic gene transcription (Pilegaard, et al. , 2002, Pilegaard, et al. ,

2005). For example, Yeo et al., (Yeo, et al. , 2008a) showed that exercise-induced AMP-activated protein kinase (AMPK) phosphorylation was greater when high-intensity exercise was undertaken with reduced muscle glycogen content. Similarly, we have shown greater p38 mitogen-activated protein kinase (MAPK) phosphorylation during the second of two high-intensity interval training (HIT) sessions when CHO intake is restricted between sessions, as compared to when CHO is fed (Cochran, et al. , 2010). These findings are significant as AMPK and p38 MAPK have each been shown to regulate the activity of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) (Jager, et al. , 2007, Puigserver, et al. , 2001), a critical regulatory factor in the process of skeletal muscle mitochondrial biogenesis.

One confounding factor of many of the aforementioned low-CHO training studies is the use of contrasting training schedules. For example, in order to create divergent CHO status, both Hansen et al. (Hansen, et al. , 2005) and Yeo et al. (Yeo, et al. , 2008b) had the low-CHO leg/group exercise twice per day every other day, while the high or normal glycogen condition exercised once daily. Therefore, one cannot rule out training schedule *per se* as a contributing factor to the divergent adaptive responses. We sought to help clarify this issue by conducting an investigation in which two groups trained on identical training schedules, with only CHO availability being different between conditions. We hypothesized, based on our previous findings (Cochran, et al. , 2010), that the group that restricted CHO intake between training sessions would experience greater increases in mitochondrial content as compared to a group that consumed large amounts of CHO between training sessions.

3.2. Methods

3.2.1. Subjects

Twenty-two healthy men and women volunteered to participate in the study, however 4 subjects chose to withdraw from the study at various time points for personal reasons. Final data reported herein are therefore based on the remaining 18 participants (Table 3.1). The subjects were recreationally active and participated in regular exercise 2-3 times per week, but were not specifically trained in any one particular form of exercise. All subjects provided written informed consent prior to participation. The experimental protocol was approved by Hamilton Health Sciences/Faculty of Health Sciences Research Ethics Board.

3.2.2. Pre-Experimental Procedures

The experimental design is outlined in Figure 3.1. Following medical screening, subjects underwent an incremental test to volitional fatigue on an electronically braked cycle ergometer (Lode Excalibur v2.0, Groningen, the Netherlands) to determine peak oxygen uptake (VO_{2peak}) using and peak aerobic power output (W_{peak}). Gas exchange analysis was performed using an online gas collection system (Moxus modular oxygen uptake system, AEI technologies, Pittsburgh, PA, USA). Following a 2 min warm-up at 50 W, work intensity was increased in a ramp fashion at a rate of 1 W every 2 s until volitional fatigue. VO_{2peak} was defined as the highest value measured over 30 s and corresponded to 42.8 ± 9.4 $mL \cdot kg^{-1} \cdot min^{-1}$ for the combined group. Subjects also

performed several familiarization trials in order to become oriented to the exercise equipment, training protocols and performance tests as described below. Familiarization included a practice interval session in which the appropriate training intensity was determined, a 250 kJ time trial (TT), and a repeated sprint test, with each test conducted on separate days. Following familiarization, subjects were matched based on sex, baseline fitness and performance values and divided into two groups: a low CHO group, designated the "HI-LO" group, and a high CHO training group, deemed "HI-HI".

Time Trial. Subjects completed 250 kJ of work as quickly as possible on the same ergometer that was used for the VO_{2peak} test (Lode). The ergometer was programmed such that power output was directly related to pedalling rate, and subjects were instructed to complete the work as fast as possible. Subjects were strongly encouraged throughout the test, but were not given any verbal, temporal, or physiological feedback other than total work completed.

Repeated sprint test. Subjects performed a repeated sprint test on an ergometer (Lode) that consisted of five maximal intensity sprints lasting 15 s each against a resistance of 7.5 $g \cdot kg^{-1}$. The sprints were interspersed by 15 s rest periods. Strong verbal encouraged was given throughout the test, but subjects did not receive any form of feedback. Peak power output (PPO), mean power output (MPO), and total work produced during the sprints was collected.

*Skeletal muscle biops*y. A sample was obtained from the vastus lateralis of one thigh under local anesthesia (1% xylocaine) as previously described (Gibala, et al. , 2006). The muscle was immediately frozen at -80°C until further analysis.

3.2.3. Training

Both groups trained a total of 6 d over 2 wk, with a minimum of one day of rest between training days. During each training day, participants completed two identical HIT sessions separated by 3 h of recovery. Each HIT session consisted of a standardized warm-up (2 min at 50 W) followed by 5 x 4 min work intervals with 2 min rest between bouts. Absolute exercise intensity was determined during familiarization and designed to elicit ~95-100% of maximal heart rate reserve at the end of the third, fourth and fifth work bout. This load corresponded with ~60-65% W_{peak}. All training was performed on a cycle ergometer (LifeFitness Model 95Ci, Schiller Park, IL, USA) in a mode in which power output was maintained constant over a pedal cadence that could vary from 60 to 120 rpm. On each training day, the first HIT session was performed approximately 2 h following the participants' habitual breakfast. Upon cessation of the first HIT session, subjects were provided with food and drinks in a blinded manner that were designed to deliver either a relatively low or high amount of CHO for the HI-LO and HI-HI groups, respectively (see below). The nutritional intervention was designed such that subjects in the HI-LO group performed the first HIT session under normal CHO availability, and their second HIT session 3 h later with reduced CHO availability. Conversely, it was intended that the HI-HI group performed both HIT sessions with high CHO availability. Nutrition was provided in a blinded manner, such that participants were unaware of which training group they were apart of.

3.2.4. Post-training procedures

The post-training procedures with regards to muscle sampling and performance testing were identical to those that were performed pre-training. A biopsy sample was obtained approximately 72 h following the last training session. The 250 kJ time-trial and repeated sprint test were performed on separate days 48 h after the biopsy was obtained.

3.2.5. Nutritional Interventions

On each training day, subjects received 1 L of fluid and a snack bar to be consumed between HIT sessions. The HI-LO group received 1L of artificially sweetened water that provided no energy and a snack bar containing 100 kcal, 17 g of CHO, 1 g of protein, and 3 g of total fat (Quaker Oats Company, Chicago, IL, USA). The HI-HI group received a 1 L drink that contained 157 g of CHO (Carb Boom Sports Nutrition Inc., Toronto, ON, Canada and a bar that contained 250 kcal, 38 g CHO, 15 g of protein, and 5 g of fat (Gatorade, Chicago, IL, USA). Thus, in the interim between training sessions, subjects in the HI-LO group received a total of 17 g of CHO (~0.3 g CHO/ kg b.w.) whereas the HI-HI group received 195 g (~2.3 g CHO/kg b.w).

3.2.6. Muscle Analyses

Enzyme Analysis: A ~20 mg piece of frozen muscle was homogenized using a glass-on-glass homogenizer, and protein concentrations of the homogenates was determined via the bicinchoninic assay method (BCA Protein Assay Kit, Thermo Fisher Scientific Inc., Rockford, IL, USA). The maximal activity of CS was determined as previously described by Carter and associates (Carter, Rennie and Tarnopolsky. , 2001), with absorbance recordings being taken every 30 s at 412 nm.

Western Blotting: A second piece of muscle (~30 mg) was homogenized in RIPA buffer for Western blot analyses using techniques described previously (Gibala, et al. , 2006). Protein concentrations of the muscle homogenates were determined via BCA protein assay (Thermo Fisher Scientific, Rockford, IL). Equal amounts of protein were prepared in 4x sample buffer and heated to 95°C for 5 minutes. Samples were then separated by 7.5%-12.5% SDS-PAGE and electrotransferred to nitrocellulose membranes. Ponceau S staining was performed following transfer to visualize equal loading and transfer. Following 1 h blocking in 5% fat-free milk Tris-buffered saline 0.1% Tween® 20 (TBS-T), membranes were incubated in primary antibodies directed against cytochrome *c* oxidase (COX) subunit II (MitoSciences, MS405), COX subunit IV (Mitosciences, MS408), and CS (kind gift from Dr. Brian Robinson, The Hospital for Sick Children, Toronto, CANADA) in 3% fat-free milk TBS-T or 3% BSA TBS-T depending on previously determined optimization conditions. After 3 x 5-min washes in TBS-T, membranes were incubated in the appropriate species-specific secondary antibody diluted (1:15,000) in 3% fat-free milk TBS-T for 1 hr at RT, washed in TBS-T for 3 X 15-min, and visualized by chemiluminescence (SuperSignal West Dura, Pierce) using a FluorChem® SP Imaging System (Alpha Innotech Corporation, San Leandro, CA, USA). ImageJ software (NIH) was used to quantify the optical density of protein bands.

3.2.7. Statistical Analyses

All data were analyzed using a 2-factor mixed analysis of variance with the between factor group and repeated factor time. Significance level was set at $p \leq 0.05$. Values are presented as means ± SD.

3.3. Results

3.3.1. Muscle data

The maximal activity and protein content of CS increased after training in both HI-LO and HI-HI with no differences between groups ($p<0.005$, main effect for training; Figure 3.2A and 3.2B). Similarly, the protein content of COX subunit 2 (COXII) and COXIV increased after training with no difference between groups, although the change tended to be greater in the HI-HI group ($p<0.001$; main effects for time; $p = 0.06$ interaction effects; Figure 3.2C and 3.2D).

3.3.2. Exercise performance

Time trial performance data are shown in Figure 3.3. Time to complete 250 kJ of work was improved after training in both groups ($p<0.002$, main effect for time) and this corresponded to an improved mean power output (HI-LO: 211 ± 66 to 244 ± 75 W; HI-HI: 203 ± 53 to 219 ± 60 W; $p<0.001$, main effect for time). While there was a trend towards a greater improvement in the HI-LO group in comparison to the HI-HI group in time trial performance, this did not reach statistical significance ($p=0.13$ for greater improvement in HI-LO). Training also improved mean power output during the repeated

sprint test (HI-LO: 531 ± 136 to 573 ± 160 W; HI-HI: 507 ± 132 to 531 ± 132 W; $p<0.001$, main effect for time) with no difference between groups. Consequently, total work completed during the 5 x 15 s maximal sprints was also increased similarly between groups with training (HI-LO: 39.9 ± 10.2 to 43.0 ± 12.0 kJ; HI-HI: 38.0 ± 9.9 to 39.8 ± 9.9 kJ; $p<0.001$, main effect for training).

3.4. Discussion

The primary finding from the present study was that the carbohydrate restriction between twice-daily sessions of HIT did not augment training-induced gains in mitochondrial content or performance when compared to when large amounts of CHO was consumed.

3.4.1. Carbohydrate-restricted training effects on mitochondrial biogenesis

It has long been suggested that endurance athletes consume large amounts of CHO throughout their preparatory and competitive seasons in order to optimally support their training and adaptive response. The underlying premise is that high CHO availability would allow the athlete to "train harder" and produce greater training benefits (Burke, 2007). The goal following each of these sessions was therefore to consume large amounts of CHO in order to fully replete muscle glycogen and be prepared for the next training session (Burke, 2007, Burke, et al. , 2011). Recent evidence however, has suggested that performing exercise in the state of reduced CHO availability (e.g. low endogenous CHO stores, or CHO restriction between training sessions) may augment

skeletal muscle adaptation to a greater extent than when training is continually supported with CHO (Hansen, et al. , 2005, Morton, et al. , 2009, Yeo, et al. , 2008b). Contrary to some of these findings (Hansen, et al. , 2005, Morton, et al. , 2009, Yeo, et al. , 2008b) we have shown no effect of CHO-restricted training on skeletal muscle mitochondrial adaptation or performance responses to 2 wks of HIT. Specifically, we have shown that the restriction of carbohydrate between multiple sessions of HIT within the same day does not appear to further augment the maximal activity of CS, or the protein content of a number of mitochondrial markers including CS, COXII, or COXIV compared to when CHO is provided. Interestingly, those trends that did arise regarding differences in some of our mitochondrial markers were in favour of high-carbohydrate training.

One possible factor explaining our divergent findings from the aforementioned studies supporting the train low, compete high paradigm may be muscle glycogen. We showed in our previous work (Cochran, et al. , 2010) that an initial HIT session consisting of 5 x 4 min intervals at 90% VO_{2peak} was sufficient to lower muscle glycogen levels by ~30%, the short period of time between training sessions did not allow the large amounts of provided CHO to create marked differences in muscle glycogen content. Thus, while the HI-LO and HI-HI groups were training under markedly different CHO availability conditions (i.e. different glucose availability), muscle glycogen levels were likely similar between groups. It is also possible that a 30% reduction in muscle glycogen content may not be sufficient to significantly alter the exercise stimulus during the second session, despite our previous indications (Cochran, et al. , 2010). In support of this, Yeo et al. (Yeo, et al. , 2008b) reduced total muscle glycogen by 50-60% prior to the performance

of the second training session of the day. Similarly, the participants in the study by Hansen and colleagues (Hansen, et al. , 2005) began their second training session with approximately one-third of their resting muscle glycogen levels. While not measured directly, Hulston and colleagues (Hulston, et al. , 2010) utilized nearly the exact same training paradigm as Yeo and associates (Yeo, et al. , 2008b), and therefore likely had similar reductions in muscle glycogen (Yeo, et al. , 2008b). Conversely, Morton and associates (Morton, et al. , 2009) reported that their subjects began their second training session with approximately 35% lowered muscle glycogen content, and reported no differences between any of their groups for COXIV or PGC-1α protein content, despite providing large amounts of CHO to their Low+Glu group. These findings would appear to support our present results, and suggest that endogenous CHO stores may need to be lowered by more that 40-50% during the first training session in order to reap the benefits of twice-daily, or CHO-restricted training. Muscle glycogen may therefore be the key factor in modulating the training stimulus during the second of two training sessions within a single day.

An alternative explanation for our differing results may be training schedule between low and normal/high CHO conditions. Indeed, Hansen et al. (Hansen, et al. , 2005), Yeo et al.(Yeo, et al. , 2008b), and Hulston et al. (Hulston, et al. , 2010) all used different training protocols between groups/legs in order to alter CHO availability and showed positive effects of reduced CHO on mitochondrial markers, while our participants all trained utilizing the exact same schedule with only CHO delivery being different. Morton et al. (Morton, et al. , 2009) directly examined this question using 3 separate

groups of subjects. Specifically, 2 of 3 groups trained using the same schedule with only CHO delivery being altered between sessions, while the third group trained using the one session per day method. In the end, Morton and associates (Morton, et al. , 2009) showed training-induced increases in PGC-1α and COXIV protein in all three groups, and no effects of nutritional manipulation in these measures. Thus, training schedule may not play a majority role in the differential findings between Hansen (Hansen, et al. , 2005), Yeo (Yeo, et al. , 2008b), and ourselves. It must be noted however, that Morton et al. (Morton, et al. , 2009) did show larger increases in succinate dehydrogenase in their Low+Pla group in comparison to their Low+Glu, and Norm groups, which may indicate differential roles of muscle glycogen and CHO availability, depending on the marker chosen to represent mitochondrial content within the muscle.

3.4.2. Carbohydrate-restricted training effects on performance

The present study shows that restricting CHO intake between two HIT sessions spaced 3 h apart does not statistically improve time-trial performance to a greater extent than performing the same training while consuming high amounts of CHO between sessions. Instead, we have shown a collective 10% improvement in time to complete 250 kJ of mechanical work between both groups performing multiple HIT sessions per day. These findings are in accordance with those of both Yeo et al. (Yeo, et al. , 2008b), Morton et al. (Morton, et al. , 2009), Cox et al. (Cox, et al. , 2010), and Hulston et al. (Hulston, et al. , 2010) who all showed no differences in performance between those who trained with low or high CHO availability. In fact, Hansen and colleagues (Hansen, et al. , 2005) were the only group to report greater improvements in performance in the low,

compared to high CHO condition. These differences may be related to the duration of the study, as Hansen et al. (Hansen, et al. , 2005) trained subjects for 10 weeks, whereas the other studies lasted 6 weeks or less (Morton, et al. , 2009, Yeo, et al. , 2008b) (present study). Alternatively, differences in performance may be related to the mode of exercise used in the investigations, as Hansen et al. (Hansen, et al. , 2005) utilized a unilateral leg-extension training model, whereas the present and other studies utilized whole-body performance measures such as cycling or running. Hansen et al. (Hansen, et al. , 2005) also used leg-kick time to exhaustion as their measure of performance, whereas all of the other studies utilized time-trials. Therefore, the mode and method of performance testing may also have played a role.

3.4.3. Perspective and limitations

One potential mechanism underlying the train low, compete high paradigm is the ability of CHO to modulate signalling molecules linked to mitochondrial biogenesis in skeletal muscle. We have previously shown greater activation of p38 MAPK during the second of two HIT sessions when CHO was restricted during the intervening rest period (i.e. the same protocol used in the present study) (Cochran, et al. , 2010). p38 MAPK has been identified as being important in exercise-induced mitochondrial biogenesis (Akimoto, et al. , 2005) by way of PGC-1α (Puigserver, et al. , 2001). It appears however, that only the γ subunit of p38 is required for driving the mitochondrial adaptive response to exercise (Pogozelski, et al. , 2009). As our previous investigation examined the gross phosphorylation of all the p38 isoforms together and not those of the isolated p38 MAPK subunits, this may be one explanation for the differences between our acute and chronic

investigations (Cochran, et al. , 2010). It is also possible that increased p38 MAPK signalling alone in humans, in absence of other enhanced signals, may be insufficient to augment the entire downstream adaptive program. Interestingly, Yeo and colleagues (Yeo, et al. , 2008a) showed greater exercise-induced activation of AMPK when exercise was initiated with ~50% of resting muscle glycogen concentrations. This enhanced signalling may play a role in the greater mitochondrial responses reported by Yeo et al.(Winder, et al. , 2000, Yeo, et al. , 2008b). It appears however, that our previous findings of greater p38 MAPK activation during a second HIT session with reduced CHO availability (but no differences in muscle glycogen or AMPK signalling) was not a sufficient stimulus to carry through to a greater mitochondrial adaptation in the long-term. Future research is required to determine the importance of these signalling molecules and the methods by which nutritional manipulation may alter the skeletal muscle adaptive response to exercise training.

In conclusion, we have found that CHO restriction between multiple HIT sessions of within the same day offers no greater benefit with regards to mitochondrial biogenesis, or cycling time-trial performance in recreationally-active individuals. While previous research has shown CHO restriction between multiple training sessions per day to augment the activity of upstream signaling pathways related to mitochondrial biogenesis, this increased signalling alone does not appear sufficient to carry through to an increased mitochondrial content over the course of 2 weeks. Future research should focus specifically on the role of muscle glycogen in governing the adaptive response to training,

and define further the role of protein signalling molecules in predicting long-term training responses in humans.

Acknowledgements

We would like to thank our subjects for their commitment and effort, as well as Todd Prior, Adeel Safdar, and Mahmood Akhtar for their technical assistance.

Grants

This project was supported by operating grants from the Natural Sciences and Engineering Research Council of Canada (NSERC). AJRC was supported by a NSERC PGS-D scholarship. MJG was supported by an NSERC Research Discovery Grant.

Disclosures

The authors have no conflicts of interest to declare.

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Table 3.1 Subject characteristics for the HI-LO and HI-HI groups.

Variable	HI-LO	HI-HI
Men; Women	4; 5	5; 4
Age, years	21 ± 1	20 ± 1
Weight, kg	75.1 ± 15.4	70.1 ± 13.6
VO_{2peak}, L·kg-1	3.3 ± 1.1	3.1 ± 1.2
VO_{2peak}, mL·kg-1·min-1	42.0 ± 8.6	43.4 ± 10.6
Peak Aerobic Power, W	313 ± 55	318 ± 63

Note: Values are mean ± S.D.

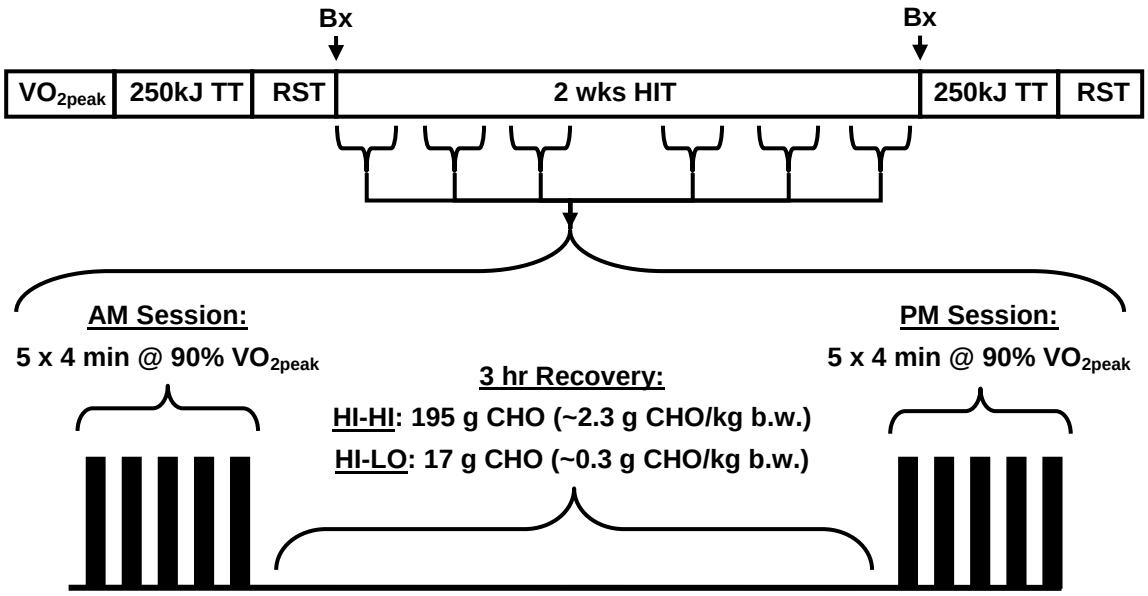

Figure 3.1. Outline of study design. VO_{2peak}, peak oxygen consumption; 250 kJ TT, 250 kJ time trial; RST, repeated sprint trial; Bx, skeletal muscle biopsy; HIT, high-intensity interval training; HI-LO, carbohydrate-restricted group; HI-HI, carbohydrate-fed group.

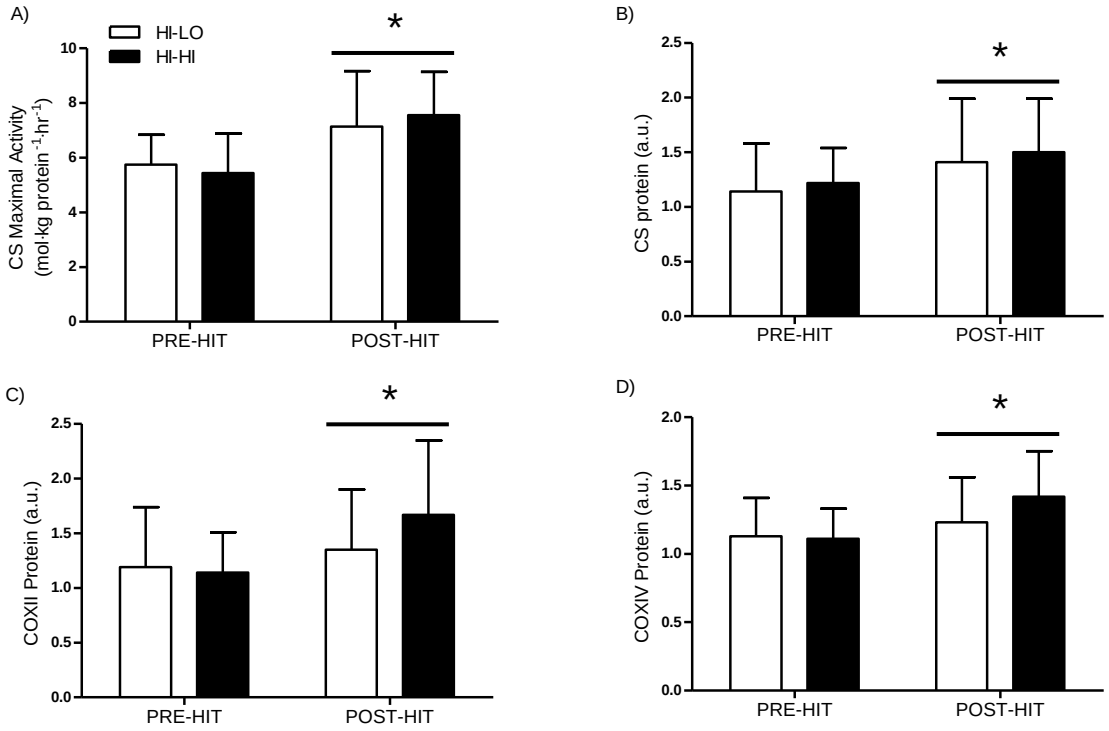

Figure 3.2. Muscle data depicting skeletal muscle mitochondrial marker content before (PRE-HIT) and after (POST-HIT) 2 weeks of twice-daily HIT with (HI-HI) and without (HI-LO) carbohydrate intake between sessions. A, citrate synthase (CS) maximal enzyme activity; B, CS protein content; C, cytochrome c oxidase (COX) subunit II content; D, COX subunit IV content. Values are means ± standard deviation (SD). *, different from PRE-HIT time point ($p<0.005$, main effect for time).

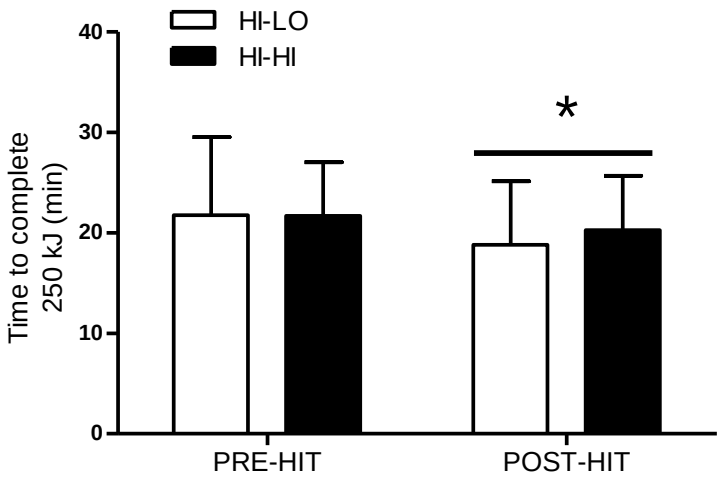

Figure 3.3. Time trial data depicting time to complete 250 kJ of mechanical work before (PRE-HIT) and after (POST-HIT) 2 weeks of twice-daily HIT with (HI-HI) and without (HI-LO) carbohydrate between sessions. *, different from PRE-HIT time point ($p<0.005$, main effect for time).

Chapter 4: Effects of β-alanine supplementation on the skeletal muscle adaptive response to high-intensity interval training

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Abstract

Low-volume high-intensity interval training (HIT) stimulates skeletal muscle and performance adaptations similar to traditional high-volume endurance training. Oral ingestion of β-alanine (β-ALA) has been shown to improve skeletal muscle buffering capacity by increasing skeletal muscle carnosine content and enhance acute high-intensity exercise performance. We hypothesized that β-ALA supplementation would augment work capacity during all-out HIT and lead to enhanced chronic training adaptations. Twenty-four men supplemented with 3.2 g β-ALA/d or placebo (PLA) for a total of 10 wk. Following 4 wk of supplementation, subjects completed a 6 wk HIT intervention. Each session consisted of 4-6 repeated 30 s maximal sprints (repeated Wingate Tests). Before and after HIT, a resting muscle biopsy was obtained and subjects completed a 250 kJ time-trial. Muscle carnosine content was increased by 33 and 52% after 4 and 10 wks of β-ALA supplementation, respectively, but was unchanged in PLA. HIT increased various markers of mitochondrial content including cytochome c oxidase (COX) and β-hydroxyacyl-CoA dehydrogenase maximal activities, and COX subunits II and IV protein contents, with no differences between treatments. Similarly, there was no difference between groups in time trial performance, although both groups improved as a result of training (β-ALA: 21:20 ± 3:20 to 18:46 ± 3:34 min:sec; PLA: 20:21 ± 2:34 to 18:30 ± 2:44 min:sec; $p < 0.001$). These findings indicate that β-ALA does not augment the increase in skeletal muscle mitochondrial content or performance adaptation to 6 wk of all-out HIT in recreationally-active men. It is possible that HIT-induced improvements may have overwhelmed any subtle influence of nutritional in mediating these effects.

4.1. Introduction

High-intensity exercise represents a significant metabolic and homeostatic perturbation to skeletal muscle. For example, a single, 30 s bout of "all-out" cycling (i.e. a Wingate test) can reduce intramyocellular stores of PCr and glycogen by >75% and ~25%, respectively owing to a large reliance on non-oxidative energy provision. The exceptional demand for ATP hydrolysis which exceeds the capacity for oxidative energy delivery also results in a marked accumulation of hydrogen ions, which is associated with a reduction in skeletal muscle pH (Parolin, et al. , 1999, Spriet, et al. , 1989). Acidification of the local skeletal muscle environment is believed to play a role in the rapid fatigue and performance decrement that occurs during all-out exercise (McCartney, et al. , 1983). One possible mechanism involved is pH-related inhibition of enzymes such as glycogen phosphorylase (Parolin, et al. , 1999) and phosphofructokinase (Trivedi and Danforth. , 1966) which interferes with glycogenolytic flux (Parolin, et al. , 1999, Spriet, et al. , 1985, Spriet, et al. , 1989). As a result, power output rapidly declines as the sprint progresses culminating in fatigue indexes >50% over 30 s of maximal work (McCartney, Heigenhauser and Jones. , 1983). Other proposed inhibitory effects of skeletal muscle acidosis include the disruption of PCr resynthesis (Trivedi and Danforth. , 1966), and direct interference with the muscle contractile function (Donaldson, Hermansen and Bolles. , 1978, Fabiato and Fabiato. , 1978).

In attempts to combat this acidosis and the associated performance reductions, buffering agents have become common nutritional aids used by competitive and recreational athletes alike. One such method of combating acidosis is the use of acute

sodium bicarbonate ingestion. A number of studies have shown that acute ingestion of sodium bicarbonate 1-2 h before exercise can induce acute alkalosis of the blood, and enhance performance in high-intensity, short-duration efforts (Bishop, et al. , 2004, Bishop and Claudius. , 2005, Carr, Hopkins and Gore. , 2011). Chronic use of sodium bicarbonate during training has even been associated with improved mitochondrial adaptation in animals (Bishop, et al. , 2010). However, sodium bicarbonate ingestion also commonly coincides with gastrointestinal distress, including nausea and diarrhea, which detracts from its practicality as a training and performance aid (Carr, Hopkins and Gore. , 2011, Stellingwerff, et al. , 2007). As a result, athletes have sought out alternative means to improve buffering capacity and high-intensity performance. One such method is β-alanine supplementation, which enhances skeletal muscle buffering capacity by way of increasing skeletal muscle carnosine (Harris, et al. , 2006). A number of studies have now shown that chronic ingestion of β-alanine increases skeletal muscle carnosine content, and these increases have been associated with improved high-intensity exercise performance (Baguet, et al. , 2010, Derave, et al. , 2007, Hill, et al. , 2007). However, there are presently no studies that have determined whether chronic β-alanine may affect skeletal muscle adaptation to high-intensity training.

High-intensity interval training (HIT) performed in an "all-out" manner (e.g., repeated Wingate Tests) is a potent strategy to enhance skeletal muscle oxidative energy provision and exercise performance. For example, several weeks of Wingate-based HIT increases the maximal activity and protein content of mitochondrial enzymes and endurance capacity to the same extent as a much higher volume of traditional endurance

training (Burgomaster, et al. , 2008, Gibala, et al. , 2006). Such repeated maximal efforts however, can result in skeletal muscle pH values as low as 6.5 (Spriet, et al. , 1989) and a near complete inhibition of glycogenolysis (Parolin, et al. , 1999). The result is a rapid and progressive reduction in work output (Spriet, et al. , 1989) as the number of sprints progress, along with an increased reliance upon oxidative phosphorylation for ATP production (Parolin, et al. , 1999). It remains to be determined however what effects the enhancement of skeletal muscle buffering capacity may have work capacity during training, and the resultant training response. It was the purpose of the present study to determine whether β-alanine supplementation may alter the skeletal muscle response to all-out HIT. We hypothesized that an increased total work production throughout training, secondary to β-alanine supplementation, would ultimately lead to a greater overall mitochondrial adaptive response.

4.2. Methods

4.2.1. Subjects

Twenty-four subjects were initially recruited to take part in the study. All subjects were young healthy individuals who were habitually active 2-3 times per week, but not specifically trained in any sport. Two subjects were unable to complete the full experimental protocol owing to issues unrelated to the study and therefore an additional two subjects were recruited in order to achieve two experimental groups with n=12 each. Descriptive data for the final 24 subjects are presented in Table 4.1. All experimental procedures were approved by the Hamilton Health Sciences/Faculty of Health Sciences

and McMaster University Research Ethics Board, and conformed in all respects with the Declaration of Helsinki. All subjects completed routine medical screening and provided written informed consent prior to study participation.

4.2.2. Experimental Design

The experimental design consisted of 1) baseline familiarization and biopsy, 2) a 4-wk supplementation period, 3) post-supplementation biopsy and physical test battery, 4) a 6-wk training period, and 5) a post-supplementation + training supplementation biopsy. A schematic of the experimental design is presented in Figure 4.1.

4.2.2.1. Baseline familiarization and testing

VO_{2peak} and peak aerobic power output (W_{peak}) were initially determined during a ramp protocol to volitional fatigue on an electromagnetically-braked cycle ergometer (Lode Excalibur Sport, Groningen, the Netherlands) using an online gas collection system (Moxus modular oxygen uptake system, AEI technologies, Pittsburgh, PA, USA) as we have previously described (Cochran, et al. , 2010). Subjects thereafter completed a series of familiarization trials on separate days an electronically-braked cycle ergometer (Velotron, RacerMate Inc., Seattle, WA) in order to become acquainted with the exercise protocols. Specifically, subjects were familiarized with a 250 kJ simulated time trial, and a repeated-sprint test consisting of four, 30 s "all-out" cycling efforts (i.e. Wingate tests). These repeated sprints were separated by 4 min rest periods in which subjects rested or cycled lightly against no load. For the 250 kJ time trial participants were instructed to complete, as quickly as possible, a simulated TT consisting of 250 kJ of total mechanical work. This test was performed on the same electromagnetically-braked cycle ergometer

(Velotron, RacerMate Inc., Seattle, WA) interfaced with software (Velotron Coaching Software v1.5) at a standardized gearing. No feedback was given during the rides with the exception of work completed. Familiarization sessions were repeated at 7 d intervals until subjects were unable to improve upon their previous sessions. Subjects completed 24 h diet records prior to each of these tests for replication post-supplementation, and post-supplementation+training. One week following the final familiarization session, a resting needle muscle biopsy sample was obtained from the *vastus lateralis* of one thigh under local anesthesia (1% xylocaine) as previously described (Gibala, et al. , 2006). Subjects were instructed to refrain from exercise for a minimum of 48 h, and to avoid caffeine and alcohol for at least 12 h before the biopsy. Subjects maintained individual food diaries for the 24-36 preceding the biopsy for replication later in the study. Muscle samples were immediately frozen in liquid nitrogen and stored at -80 ºC until further analysis. Data from the final familiarization sessions along with baseline measures of fitness was used to pair-match subjects, who were separated into two groups of 12. Matched pairs of subjects were then randomly allocated to either the β-alanine (β-ALA) supplementation or placebo (PLA) supplementation group.

4.2.2.2. Supplementation

Supplementation began immediately following the baseline biopsy, and continued for ~12 wks in a double-blind fashion. Subjects ingested 3.2 g β-ALA/d in two 1.6 g dosages via slow-release tablet (High Intensity Sustained Release Beta Alanine, PowerBar®, Nestlé, Switzerland) or a similar dosage of PLA (Nestlé). This

supplementation regime was carried out throughout the entire 4-wk supplementation period, as well as throughout all testing and training periods thereafter.

4.2.2.3. Post-supplementation biopsy and testing

Following 4 wks of supplementation, a second resting biopsy was taken from the *vastus lateralis* as described above. Subjects adhered to the same exercise and dietary controls as they did in the 48 h preceding the baseline biopsy. Time trials and repeated-sprint tests were repeated as described above at three and five days following this second biopsy. Training began for all subjects 48 h following the repeated-sprint test.

4.2.2.4. Training

All subjects took part in a 6 wk training protocol consisting of three sessions per week of all-out HIT. Each session of all-out HIT consisted of 4-6 x 30 s work bouts performed at all-out intensity (i.e. repeated Wingate tests) on an electronically-braked cycle ergometer (Velotron, RacerMate Inc.). These work bouts were separated by 4 min of rest. Four all-out intervals per session were completed in the first two weeks of training, 5 were completed in weeks 3 and 4, and 6 were performed in weeks 5 and 6.

4.2.2.5. Post-supplementation+training biopsy and testing

Final resting biopsies were obtained 72-96 h following the final training sessions. All subjects refrained from exercise for a minimum of 48 h, and repeated their diets as before each of the previous biopsies. Final time trials, repeated sprint tests, and VO_{2peak} assessments were performed at 48 h intervals thereafter as described above.

4.2.3. Muscle Analyses

4.2.3.1. Carnosine analysis

Carnosine analysis was performed by way of ultra-performance liquid chromatography (UPLC) using the MassTrak Amino Acid Analysis Solution (Waters Corporation, Milford, Massachusetts, USA). Initial cell lysate preparation took place as described below for Western Blotting analysis. Thereafter, samples were prepared by combining 20 μL of sample with an equal volume of 10% sulfosalicylic acid and 250 μM norvaline standard. Following thorough mixing and centrifugation (5 min at 13,000 rpm), 10 μL of supernatent was combined with 70 μL of borate/NaOH buffer, and 20 μL of Waters MassTrak derivitization reagent containing 6-aminoquinolyl-N-hydroxysuccinimydyl carbamate. The solution was mixed and heated at 55°C for 10 min and analyzed using a Waters Acquity UPLC with a 2.1 x150 mm MassTrak AAA column as per the manufacturer's instructions.

4.2.3.2. Enzyme activities

Approximately 20 mg of wet muscle was homogenized using an automated homogenizer (FastPrep®-24, MP Biomedicals, Solon, OH) in 10 volumes of buffer containing 70 mM sucrose, 220 mM mannitol, 10 mM HEPES (pH 7.4), supplemented with protease inhibitors (Complete Mini®, Roche Applied Science, Laval, PQ, Canada). Cytochrome c oxidase (COX) and β-hydroxyacytl-CoA dehydrogenase (β-HAD) maximal activities were then quantified as we have described previously (Carter et al., 2001; Gibala 2006; Burgo 2008). Homogenate protein content was determined via BCA

method using a commercial assay (Pierce, Rockford, IL, USA) and enzyme activity expressed as mol·kg protein^{-1}·hr^{-1} wet weight.

4.2.3.3. Western Blotting

Whole cell lysates were prepared by adding ~30 mg wet muscle to ice-cold RIPA buffer (50 mM HCL, 150 mM NaCl, 1 mM PMSF, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease (Complete Mini®, Roche Applied Science, Laval, PQ, Canada) and phosphatase inhibitors (PhosSTOP®, Roche Applied Science, Laval, PQ, Canada). Samples were homogenized via automated homogenizer (FastPrep®-24, MP Biomedicals, Solon, OH) and agitated end-over-end for 1h at 4^{o}C. Samples were then centrifuged at 15,000 g for 5 min at 4^{o}C, and the supernatant was collected for subsequent analysis. Homogenate protein concentrations were determined using a commercial, detergent-compatible, colorimetric assay (BCA protein assay, Pierce, Rockford, IL). Equal amounts of protein (5-20 μg, depending on the protein of interest) were then loaded onto 7.5-12.5% SDS-PAGE gels and separated by electrophoresis for 2-2.5 hours at 120 V. Proteins were transferred to nitrocellulose membranes for 1 hr at 100 V. Ponceau S staining was performed following the transfer and was used to control for equal loading and transfer between lanes. Membranes were blocked using either fat-free milk or BSA solutions of differing concentrations in TBS-T at room temperature, and incubated thereafter with the appropriate primary antibodies diluted in a fat-free milk or BSA in TBS-T. Primary antibodies targeting cytochrome *c* oxidase (COX) subunit II (MitoSciences, MS405), COX subunit IV (Mitosciences, MS408), NDUFA9

(Mitosciences, MS111), Complex II 70 kDa subunit (Mitosciences, MS204), Complex III Core 2 protein (Mitosciences, MS304), cytochrome c oxidase subunit IV (COXIV; Mitosciences, MS408) and the ATP synthase α subunit (Mitosciences, MS507). Blots were then incubated in the appropriate secondary antibodies for 1 hour at RT, and visualized by chemiluminescence (Supersignal® West Dura, Pierce). Signal quantification was performed using NIH Image J software.

4.2.4. Statistical Analysis

All data were analyzed using a 2-factor mixed analysis of variance with the between factor group and repeated factor time. Significance level was set at $p \leq 0.05$. Values are presented as means ± SD.

4.3. Results

4.3.1. Skeletal muscle carnosine content

Skeletal muscle carnosine content was not different between groups at study onset (β-ALA: 6.40 ± 1.26, PLA: 6.35 ± 1.76 mmol/kg w.w., $p = 0.194$). Following 4 wks of supplementation, muscle carnosine was increased by 33% in the β-ALA group, while remaining unchanged in the PLA group (β-ALA: 8.53 ± 1.65, PLA: 5.91 ± 1.14 mmol/kg w.w., $p < 0.001$). After supplementing for further 6 wks, this divergence was magnified, with the β-ALA achieving a skeletal muscle carnosine content that was 52% greater than baseline, while the PLA group remained at baseline levels (β-ALA: 9.74 ± 1.60, PLA: 6.21 ± 1.72 mmol/kg w.w., $p < 0.001$; Figure 4.2).

4.3.2. Training and exercise performance

Subjects completed an average of 17 ± 1 training sessions, with all subjects completing a minimum of 16 sessions. Mean total work production increased in coincidence with training volume progression ($p < 0.001$, main effect for time; Figure 4.3), however, there was no divergence in total work production between groups across time ($p = 0.37$). Time trial performance improved similarly between groups after training (β-ALA: 21:20 ± 3:20 to 18:46 ± 3:34 min:sec; PLA: 20:21 ± 2:34 to 18:30 ± 2:44 min:sec; $p < 0.001$, main effect for training; Figure 4.4A). Though the β-ALA group did appear to have a slightly greater improvement (β-ALA: -2:34 min:sec vs. PLA: -1:50 min:sec), this did not reach statistical significance ($p = 0.20$). Mean power output across 4 repeated Wingate tests was increased after training in both groups, with no differences between groups (β-ALA: 628 ± 87 to 659 ± 92 W, PLA: 578 ± 90 to 602 ± 88 W; Figure 4.4B). Finally, VO_{2peak} also increased by ~9% with no difference between groups (β-ALA: 48.1 ± 6.9 to 52.2 ± 4.3, PLA: 50.6 ± 6.0 to 55.4 ± 8.9 $mL \cdot kg^{-1} \cdot min^{-1}$; $p < 0.001$, main effect for time).

4.3.3. Skeletal muscle mitochondrial activities and protein contents

COX maximal activity was increased by training in both groups by ~40%, with no differences between groups (β-ALA: 5.6 ± 2.1 to 7.3 ± 1.8, PLA: 5.2 ± 2.3 to 7.8 ± 1.9 $mol \cdot kg^{-1} \cdot hr^{-1}$; $p < 0.001$, main effect for time; Figure 4.5). In line with this, COXII and COXIV protein contents were increased by ~33% and ~22%, respectively, with no differences between groups ($p < 0.05$, main effect of time; Figure 4.6A and 4.6B). β-HAD activity was also increased by ~19% by all-out HIT, but there were no differences

between groups (β-ALA: 5.6 ± 1.8 to 6.1 ± 0.4, PLA: 5.5 ± 1.7 to 7.2 ± 1.5; $p < 0.005$, main effect of time; Figure 4.7). Interestingly, a trend did arise for a larger improvement in β-HAD activity for the PLA group ($p = 0.07$), however this did not reach significance. Protein content of the core protein 2 of complex III was increased by training ($p < 0.05$), while ATP synthase tended to be increased ($p = 0.08$), with no differences between groups. However, NDUFA9 and the 70 kDa protein of complex II remained unchanged throughout the training and supplementation (data not shown).

4.4. Discussion

The major novel finding of the present study was that β-ALA supplementation did not augment skeletal muscle mitochondrial or performance adaptation to all-out HIT in recreationally-active individuals. While several studies have examined the effects of β-ALA supplementation on acute exercise performance, to our knowledge this is the first study to investigate the effects of β-ALA supplementation on skeletal muscle metabolic adaptation to HIT.

4.4.1. Effect of β-ALA supplementation on work output during training

Several studies have shown that chronic ingestion of β-ALA can acutely increase high-intensity exercise performance (Baguet, et al. , 2010, Derave, et al. , 2007, Hill, et al. , 2007, Sale, et al. , 2011, Van Thienen, et al. , 2009), though this finding is not universal (Bellinger, et al. , 2012, Smith-Ryan, et al. , 2012, Zoeller, et al. , 2007). Based off of these findings, we hypothesized that β-ALA supplementation would increase the work

capacity of those individuals performing all-out HIT, and that this increased work capacity throughout training may translate into greater metabolic and performance adaptations. Contrary to our hypothesis, and despite differences in skeletal muscle carnosine content between groups, there were no differences between groups with regards to total work output throughout training. It is possible that the increases in skeletal muscle carnosine induced by our 3.2 g β-ALA/d regimen was insufficient to achieve those marked differences in carnosine that in turn augment training output. In line with this, many of the previous investigations which showed improvement in total work output in acute exercise tests supplemented with >4 g β-ALA/d (Baguet, et al. , 2010, Derave, et al. , 2007, Hill, et al. , 2007, Stout, et al. , 2007, Van Thienen, et al. , 2009), though some have shown performance improvement with as little as 2.4 g β-ALA/d (Stout, et al. , 2008). Our 3.2 g/d prescription was, however, consistent with the recommended dosage of the commercial supplement used for this investigation. Nonetheless, it would appear that greater increases in muscle carnosine than 33% and 52% after 4 and 10 wk of supplementation, respectively, may be required to affect marked improvements in training and performance outputs under our study conditions. Greater daily dosages, or a longer pre-training supplementation period, may therefore have been more effective.

An alternative explanation for the lack of divergence between training outputs may be that the work efforts were too short in order to allow for significant differences to arise in total work output. A recent meta-analysis examining β-ALA supplementation and exercise performance failed to find any marked performance improvement for high-intensity exercise bouts lasting <60s (Hobson, et al. , 2012). Conversely, ergogenic

effects were found to exist for those events lasting 60-240 s and those lasting >240 s when median β-ALA consumption was 179 g or greater. As our participants in the β-ALA group would have consumed ~220 g over the entire study period, it is possible that the work bouts were simply too short for a marked difference to emerge. Our findings would therefore suggest that in order to augment total work output during training, daily dosages must be in excess of 3.2 g β-ALA/d and/or work intervals must be longer than our present 30 s repeats.

4.4.2. Effect of β-ALA supplementation on skeletal muscle metabolic adaptation to all-out HIT

This is the first study to directly examine the effects of β-ALA supplementation on skeletal muscle metabolic adaptation to exercise training. We found that β-ALA supplementation did not enhance metabolic adaptations induced by all-out HIT in recreationally-active individuals. To our knowledge only one other study has examined the effect of β-ALA supplementation on training adaptation. Smith and associates (Smith, et al. , 2009) showed that when individuals undertook 3 wks of constant-load HIT (i.e. work intervals clamped at a designated power output) at a 2 min:1 min work:rest ratio, that improvements in VO_{2peak}, ventilatory threshold, and time to exhaustion at 110% VO_{2peak} were similar between those consuming 3-6 g β-ALA per day (6 g/dfor 4 wks, then 3 g/d throughout training), and those that consumed PLA. Our present findings support those of Smith et al. (Smith, et al. , 2009) in that our β-ALA and PLA groups showed nearly identical increases in VO_{2peak}, suggesting no further benefit of β-ALA supplementation on whole-body cardiorespiratory training adaptation, but also no

detriment. Unfortunately, Smith and colleagues (Smith, et al. , 2009) were unable to attain muscle samples from their participants, and thus we are unable to make direct comparisons with our intramuscular metabolic adaptations. Our findings would therefore suggest that athletes may supplement with up to 3.2 g β-ALA/d without any detriment to those metabolic adaptations normally induced by training. The present study cannot rule out, however, that larger dosages may have hindering effects or, as was our original hypothesis, augment training adaptation further.

4.4.3. Effect of β-ALA supplementation on performance adaptation to all-out HIT

The present study shows that supplementing with 3.2 g β-ALA/d does not appear to effect performance with regards to repeated sprint ability, or time trial performance. Again, these findings may be attributable to the daily dosage being too low to achieve those pronounced differences in muscle carnosine that would permit such differences. Interestingly, our data did implicate a trend for the β-ALA group to have a greater improvement in time to complete 250 kJ of mechanical work relative to those taking placebo (β-ALA: -2:34 min:sec vs. PLA: -1:50 min:sec; $p = 0.20$). It is possible that those differences imposed by β-ALA supplementation alone were lost in the marked improvements induced by all-out HIT. Our previous work has shown all-out HIT to improve 250 kJ time trial performance by approximately 1:40 min:sec, or ~ 9.5% (Burgomaster, Heigenhauser and Gibala. , 2006). These findings are directly in line with those noted here in the PLA group, with an improvement of 1:50 min:sec, or 9%. Our β-ALA supplementation group, however, showed a ~12.3% improvement in their time to

complete 250 kJ. As Hobson and associates (Hobson, et al. , 2012) indicate in their meta-analysis of β-ALA supplementation, the median improvement in exercise performance with β-ALA was ~2.85% compared with a placebo with a median β-ALA intake of 179 g. Our participants consumed ~220 g β-ALA over the course of the study, and appeared to have an ~3% greater improvement than the PLA group, however this difference did not reach statistical significance. It is therefore possible that the larger performance improvements associated with training alone overwhelmed the more subtle effects of the supplementation. This possibility of the all-out HIT-induced improvements overwhelming the effects of β-ALA also exists in our repeated-sprint performance however, as pointed out by Hobson et al. (Hobson, et al. , 2012), β-ALA appears to have a lesser effect in events lasting <60 s. It is therefore possible that the combination of sub-optimal β-ALA dosage, and the marked effects of all-out HIT alone, contributed to our lack of performance findings.

In summary, we have shown that supplementation up to 3.2 g β-ALA/d has no detrimental effects upon the metabolic adaptation induced by all-out HIT. Furthermore, it would appear that 3.2 g β-ALA/d is an insufficient dosage to see marked improvements in time trial or repeated sprint performance in recreationally-active individuals beyond those which can be induced by all-out HIT alone. β-ALA may therefore offer some benefit to those undergoing intensive exercise training, but these effects will not outweigh those that can be induced by intensive training alone in recreationally-active individuals. Future work should examine the effects of larger β-ALA dosages on skeletal muscle metabolic adaptation to training in already well-trained athletes.

Acknowledgements

We would like to thank our subjects for their commitment and effort.

Grants

This project was supported by operating grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) to MJG and MAT. AJRC was supported by a NSERC PGS-D scholarship. MP held NSERC CGS-M, while JG held NSERC CGS-D. The authors have no conflicts of interest to declare.

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Table 4.1. Subject characteristics of β-Alanine and Placebo groups at study onset

Variable	β-Alanine	Placebo
Participants	12	12
Age (years)	23 ± 2	22 ± 2
Weight (kg)	86.2 ± 13.0	80.4 ± 15.7
Height (cm)	181 ± 8	177 ± 6
VO2peak (mL·kg-1·min-1)	48.1 ± 6.9	50.6 ± 6.0
Peak wattage (W)	332 ± 41	326 ± 38

Values are mean ± S.D.

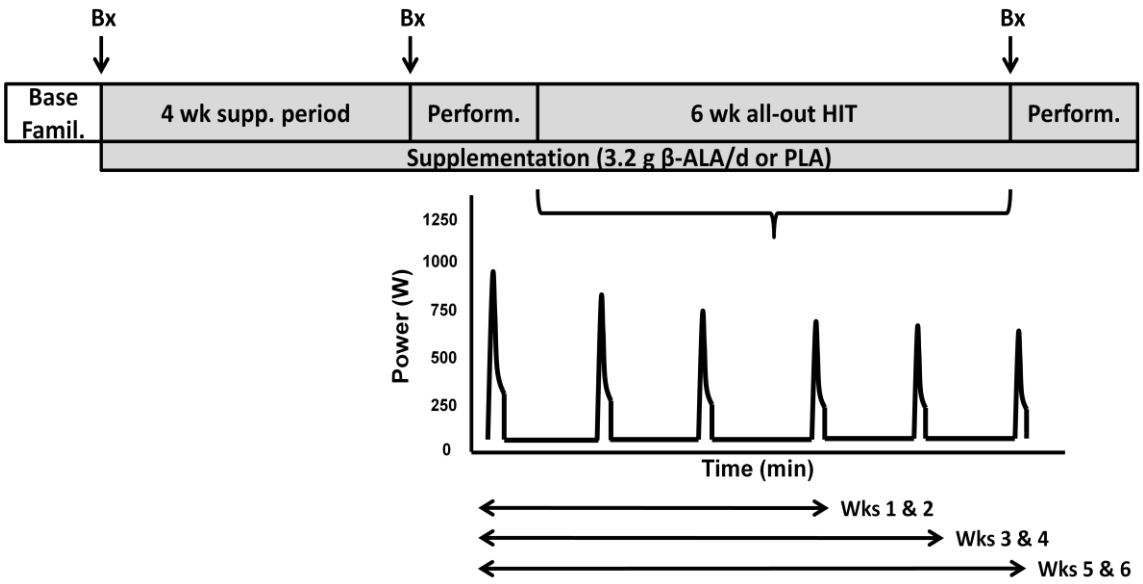

Figure 4.1. An outline of the study protocol. Base Famil., baseline familiarization and subject allocation to groups. Supplementation consisted of 3.2 g β-alanine (β-ALA)/d or placebo (PLA) beginning immediately following baseline testing and continued throughout the remainder of the study. Perform., performance testing consisting of a 250 kJ simulated time trial and repeated sprint test composed of four, 30 s all-out sprints spaced at 4 min intervals. Bx, skeletal muscle biopsy. All-out HIT, high-intensity interval training consisting of repeated, 30 s all-out sprinting efforts spaced at 4 min intervals. Training consisted of 4 repeated sprints in weeks 1 and 2 of training, and was progressed to 5 and 6 every 2 wks.

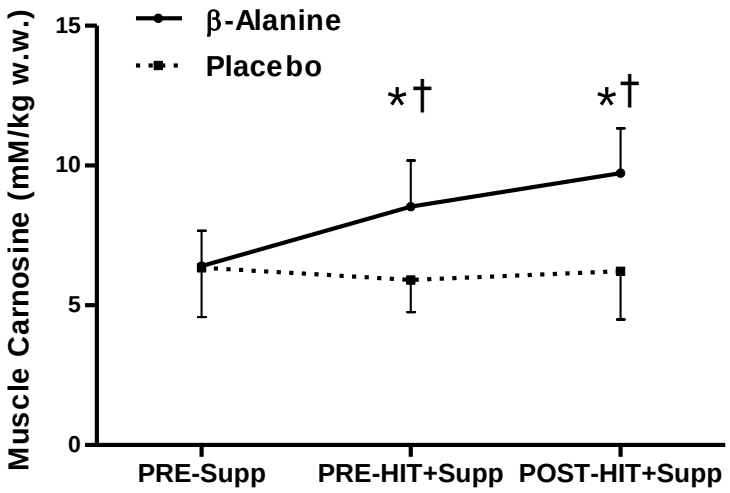

Figure 4.2. Skeletal muscle carnosine content before the start of β-Alanine or Placebo supplementation (PRE-Supp), after 4 wks of supplementation (PRE-HIT+Supp) and after 6 wks of all-out HIT training with continued supplementation (POST-HIT+Supp). Values are means ± S.D. for 24 subjects. *$p < 0.001$, main effect for time. †$p < 0.001$, difference between groups at given time.

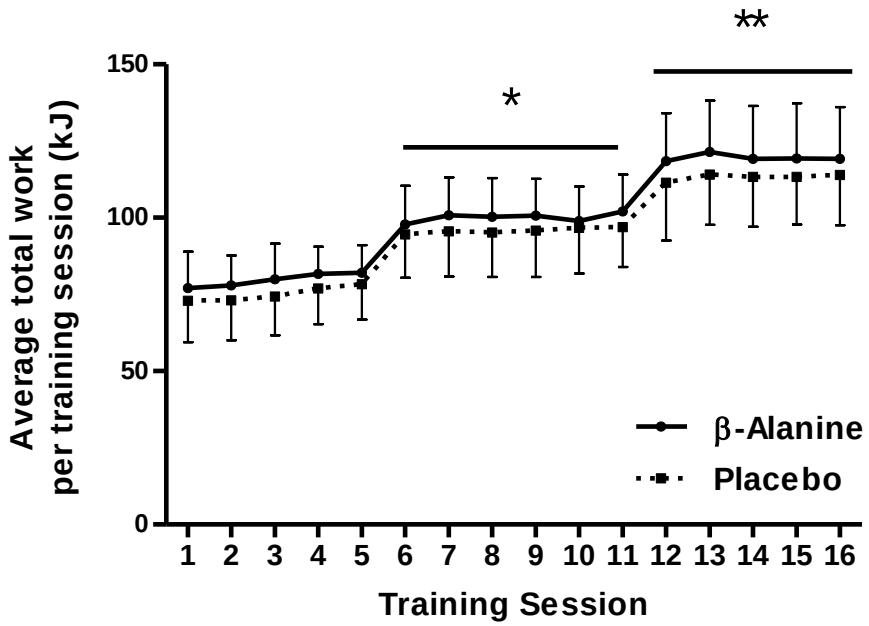

Figure 4.3. Summary of the average total work produced each training session by those in the β-ALA and PLA groups over the 6 wks of all-out HIT. Values are means ± S.D. for 24 subjects *$p < 0.001$, different from weeks 1 and 2; **$p < 0.001$, different than weeks 1, 2, 3 and 4.

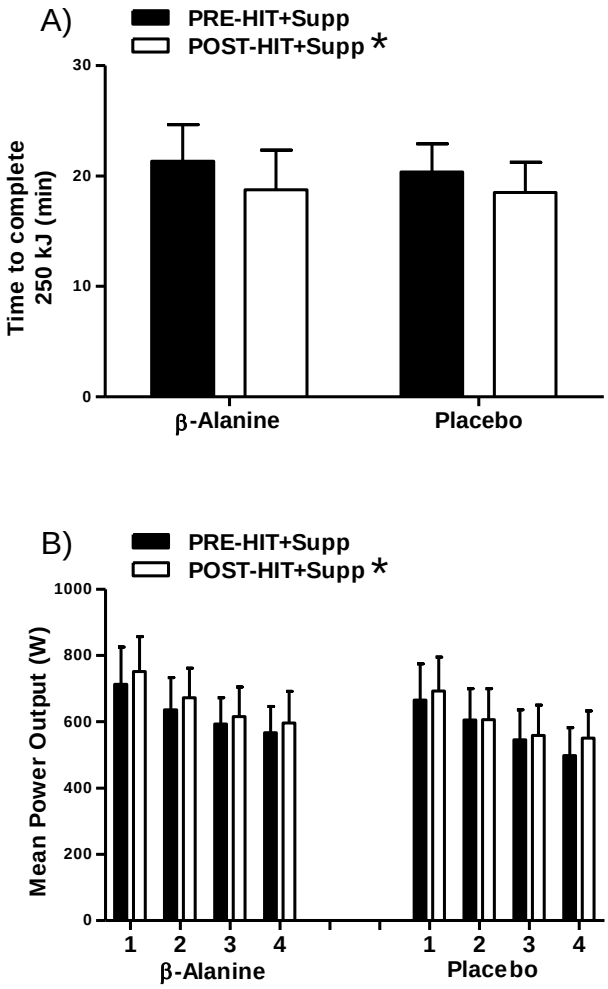

Figure 4.4. Time to complete 250 kJ of mechanical work (A) and average mean power output over four repeated 30 s all-out sprints (B) before (PRE-HIT+Supp) and after (POST-HIT+Supp) 6 wks of all-out HIT in subjects supplementing with β-Alanine or Placebo. Values are means ± S.D. for 24 subjects. *$p < 0.001$, main effect for time.

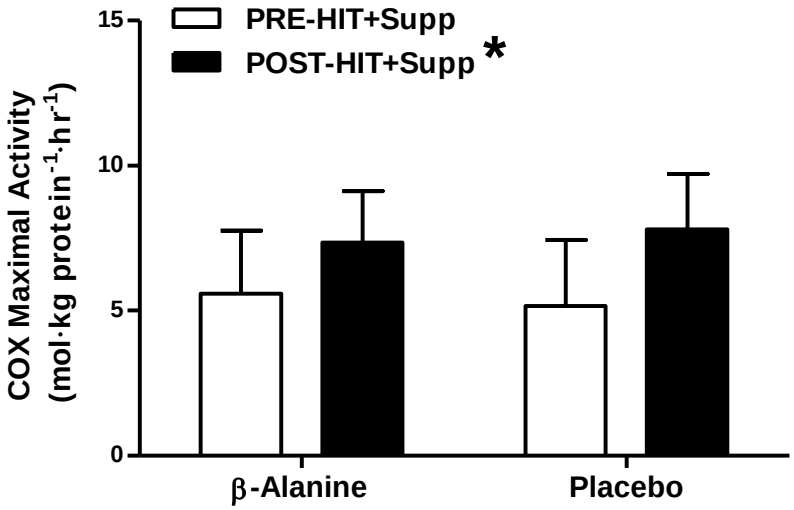

Figure 4.5. Maximal activity of the COX enzyme before (PRE-HIT+Supp) and after (POST-HIT+Supp) 6 wks of all-out HIT in subjects supplementing with β-Alanine or Placebo. Values are means ± S.D. for 24 subjects. *$p < 0.01$, main effect for time.

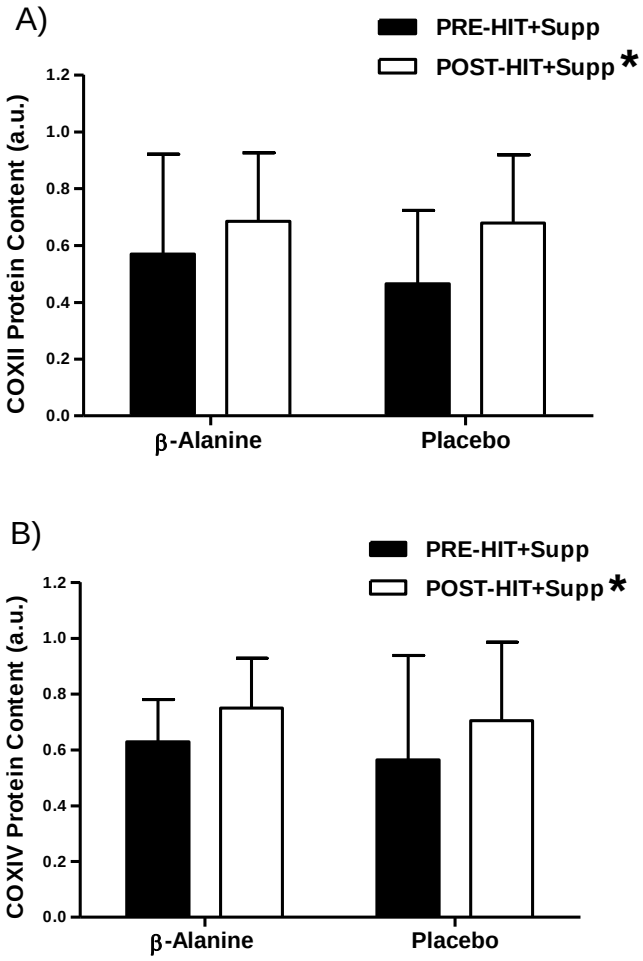

Figure 4.6. Protein contents of COX subunit 2 (COXII; A) and 4 (COXIV; B) before (PRE-HIT+Supp) and after (POST-HIT+Supp) 6 wk of all-out HIT in subjects supplementing with β-Alanine or Placebo. Values are means ± S.D. for 24 subjects. *$p < 0.05$, main effect for time.

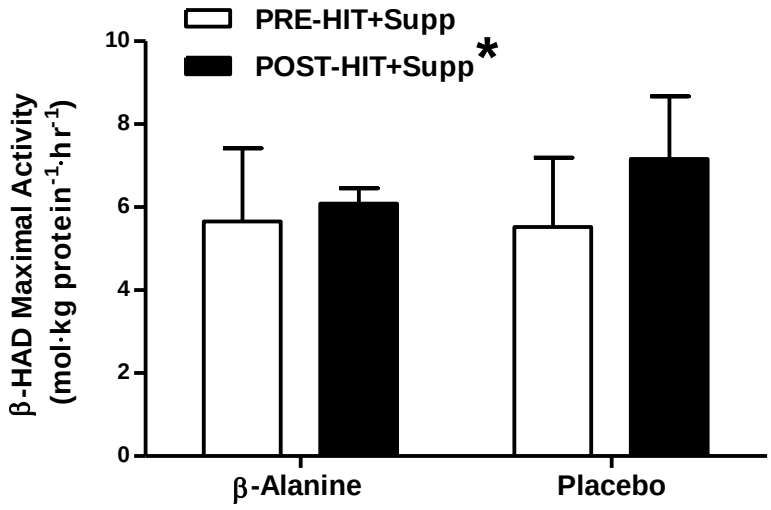

Figure 4.7. Maximal activity of the β-HAD enzyme before (PRE-HIT+Supp) and after (POST-HIT+Supp) 6 wks of all-out HIT in subjects supplementing with β-Alanine or Placebo. Values are means ± S.D. for 24 subjects. *$p < 0.01$, main effect for time.

Chapter 5: General Conclusions

5.1. Introduction

This thesis investigated the acute and chronic responses of human skeletal muscle to high-intensity exercise and the potential for nutrition to manipulate the adaptive response in recreationally active subjects. Study 1 (Chapter 2) examined whether the manner in which a high-intensity exercise stimulus was applied altered the activation of key signalling molecules linked to mitochondrial adaptation, and whether these acute responses were reflective of longer-term training adaptations. It was shown that the acute activation of AMPK and p38 MAPK in response to a work-matched bout of all-out exercise was similar regardless of whether the bout was performed in an intermittent or continuous manner. However, the acute response to the continuous protocol was not reflective of the chronic response, as we found no increase in mitochondrial content after 6 wk of this type of training. Study 2 (Chapter 3) examined the mitochondrial adaptive response to high-intensity interval training with reduced CHO availability over a period of 2 wk. This study showed that restricting CHO between twice daily HIT sessions did not alter the training response as compared to when a large amount of CHO was consumed between sessions. Finally, Study 3 (Chapter 4) examined the effects of β-ALA supplementation on skeletal muscle mitochondrial content after 6 wk of all-out HIT. Similar to Study 2, we found no effect of nutritional manipulation on HIT adaptation. The present chapter is intended to integrate and these various findings and place them in

context within the broader field. The potential limitations of the various experimental approaches, unanswered questions, and future directions will also be considered.

5.2. Manipulation of the all-out intensity exercise stimulus

Previous work from our laboratory established that mitochondrial biogenesis could be stimulated in human muscle with a very small volume of total work, provided that the stimulus was applied with an all-out effort and in an intermittent manner (Burgomaster, et al. , 2005, Burgomaster, Heigenhauser and Gibala. , 2006, Gibala, et al. , 2006). The goal of Study 1 (Chapter 2) was to specifically evaluate the importance of the interval, or intermittent application of the exercise stimulus, in stimulating these adaptations. Our findings suggest that, in order to stimulate increases in mitochondrial protein content and maximal enzyme activity, low-volume all-out work needs to be performed in an intermittent manner. This is based on the observation that 6 wk of all-out CONT training did not increase any of the various markers of skeletal muscle mitochondrial content we have previously shown to be increased with 2-6 wk of all-out HIT. We also found no evidence of changes in other traditional markers of training adaptation such as reduced heart rate, ventilation rate, or respiratory exchange rates at at given submaximal absolute work intensity. Our findings from Study 1 would therefore also question the potential for acute protein signalling responses to predict longer-term training adaptations, as all-out CONT training failed to induce those traditional all-out HIT-induced adaptations, despite very similar acute metabolic and protein signalling responses. Intermittent application of the high-intensity exercise stimulus may thus play an important role in modulating role in the relationship between exercise intensity, duration, and training adaptation.

5.3. Role of protein signalling in determining the adaptive response to training

It is well-established that regular and progressive exercise training increases skeletal muscle mitochondrial content (Holloszy, 1967). However, we still do not fully understand the underlying mechanisms that govern this process, and the ways in which different exercise stimuli may modulate the adaptive response. The discovery of PGC-1α (Puigserver, et al. , 1998), along with the association of AMPK and p38 MAPK in its regulation (Fan, et al. , 2004, Jager, et al. , 2007, Puigserver, et al. , 2001), have marked significant milestones in our understanding of mitochondrial biogenesis over the past two decades. Indeed, robust increases in the activation and expression of these proteins in response to exercise strongly suggest a role in mediating the mitochondrial adaptive response (Akimoto, et al. , 2005, Baar, et al. , 2002, Boppart, et al. , 2000, Chen, et al. , 2000, Fujii, et al. , 2000, Irrcher, et al. , 2003, Norrbom, et al. , 2004, Pilegaard, Saltin and Neufer. , 2003, Widegren, et al. , 1998, Winder, et al. , 2000, Wojtaszewski, et al. , 2000, Yu, et al. , 2003). Moreover, there appears to be an intensity-dependent regulation of the AMPK and p38 MAPK pathways and PGC-1α mRNA expression (Egan, et al. , 2010, Wojtaszewski, et al. , 2000). As PGC-1α is believed to self-regulate its own transcription via autoregulatory loop (Handschin, et al. , 2003), it may be argued that a similar PGC-1α mRNA expression between two conditions could represent similar gross activation and/or translocation to the nucleus of PGC-1α.

Based off the present model of PGC-1α-mediated mitochondrial biogenesis, similar activation of PGC-1α by two distinct exercise conditions would imply that the long-term adaptations resulting from these two exercise conditions should also be similar.

However, our Study 1 findings suggest otherwise and indicate that some aspect of the intermittent application of the high-intensity training stimulus makes it important for enhanced skeletal muscle adaptation. Moreover, the acute measurement of ACC and p38 MAPK phosphorylation, in addition to PGC-1α mRNA expression, cannot predict long-term adaptation. In support this contention, there are presently no studies reporting a correlation between individual AMPK or PGC-1α responses and chronic mitochondrial response to exercise (Timmons, 2011). This may be related to the fact that much of the insight regarding AMPK and p38 MAPK in the adaptive process to exercise has been garnered via cell culture or animal models (Akimoto, et al. , 2005, Bergeron, et al. , 2001, Irrcher, et al. , 2003, Jager, et al. , 2007, Winder, et al. , 2000) which, while insightful, do not necessarily translate to humans, or *in vivo*. Moreover, genetic knockout models can only help to determine if a specific protein is *essential* to mitochondrial biogenesis, and not necessarily determine whether that protein *contributes* to that process (Gurd, Little and Perry. , 2012). Given that the acute activation of PGC-1α was similar between INT and CONT conditions, our data would therefore also suggest that the failure of the CONT training to produce functional mitochondrial biogenesis would lie somewhere downstream of PGC-1α regulation. Little is presently known regarding how exercise may alter processes such as post-transcriptional splicing and modification, translation, protein folding and transport, protein import, and/or assembly into functional protein complexes. Thus, more work must be done to examine these downstream processes, and determine how exercise of different intensities and durations may affect each.

There are a number of caveats that preclude our ability to discount protein signalling events in predicting long-term adaptation. For example, AMPK and p38 MAPK have a number of different isoforms which may play differing roles within the cell and signal translation. Birk and Wojtaszewski (2006) showed there are 12 possible heterotrimeric combinations of AMPK, though only the α1/β2/γ1, α2/β2/γ1, and α2/β2/γ3 forms appear to be expressed in human muscle. Of these, it is suggested that only the α2/β2/γ3 molecule is responsive to exercise, despite it having been demonstrated that AMPKα1 can be activated in human muscle in response to exercise (Chen, et al. , 2003, Gibala, et al. , 2009, Wojtaszewski, et al. , 2000). In previous studies in our laboratory, we have measured AMPK threonine-172 and acetyl-CoA carboxylase (ACC) serine-79 phosphorylation as markers of AMPK activity in human muscle in attempts to gauge mass AMPK activation in response to exercise (Cochran, et al. , 2010, Gibala, et al. , 2009, Little, et al. , 2010a, Little, et al. , 2011). ACC phosphorylation may represent a more sensitive *in vivo* marker of AMPK activity than AMPK phosphorylation itself, as it may be more reflective of the total covalent and allosteric regulation of AMPK (Chen, et al. , 2003). However, while correlated with AMPK activity (Birk and Wojtaszewski. , 2006), it is possible that subtle differences in AMPK activity exist that are not decipherable by our present measures. p38 MAPK possesses three different isoforms: α, β, and γ, and recently, Pogozelski and associates (Pogozelski, et al. , 2009) utilized muscle-specific gene-deletion in mice to show that only the γ subunit was required for endurance exercise-induced mitochondrial biogenesis. It was demonstrated that the overexpression of a negative form of the γ subunit blocked stimulation-induced PGC-1α

transcription, indicating a key role in the regulation of the PGC-1α promoter region (Pogozelski, et al. , 2009). While we have repeatedly shown exercise to augment p38 MAPK phosphorylation, our measures represented the phosphorylation of all p38 MAPK subunits, as there were no commercially-available methods for measuring the isolated γ-subunit phosphorylation at the time of study. This is an important caveat relevant to the present work, and learning how exercise intensity and duration may modulate the activation of this subunit will represent an important avenue for future research. Further work needs to be done to characterize the roles of the independent AMPK and p38 MAPK isoforms on downstream signalling to PGC-1α and molecular machinery responsible for adaptation.

Along the same lines, PGC-1α has three isoforms: a, b and c (Miura, et al. , 2008). These isoforms vary in the coding for the N-terminus amino acid sequence, which may affect the way the isoform binds targeted transcription factors or its transactivational activity (Sadana and Park. , 2007). In mice, exercise appears to augment PGC-1α-b and PGC-1α-c, while PGC-1α-a appears unaffected (Miura, et al. , 2008). In fact, Miura and associates (Miura, et al. , 2008) showed that, while at rest, PGC-1α-b and PGC-1α-c expression make up less than 10% of the total PGC-1α mRNA expression in skeletal muscle (Miura, et al. , 2008). However, after a single exercise session, PGC-1α-b and PGC-1α-c mRNA increased 28% and 41% increase, respectively, while PGC-1α-a remained largely unchanged (Miura, et al. , 2008). These isoform-specific responses contributed largely to the observed 8-fold increase in total PGC-1α mRNA expression (Miura, et al. , 2008). Thus, it is possible that our INT and CONT interval sessions may

have induced differing patterns of PGC-1α isoform expression that were not detected by our total PGC-1α mRNA analysis. These differing responses may therefore be partially responsible for our divergent findings between acute and chronic findings in Study 1. PGC-1α is accompanied by two other family members, PGC-1β and PGC-1α-related coactivator (PRC), which may play overlapping roles in the process of exercise-induced metabolic adaptation. Presently there is little known regarding how PGC-1α, PGC-1β and PRC may interact to accomplish adaptive response in skeletal muscle in response to exercise. PGC-1β and PRC overexpression does appear to augment skeletal muscle mitochondrial content via some of the same downstream mechanisms as identified to be regulated by PGC-1α (Andersson and Scarpulla. , 2001, Shao, et al. , 2010), and both PGC-1β and PRC are responsive to high-intensity interval exercise (Perry, et al. , 2010, Wang, et al. , 2009). This latter point, however, is somewhat contentious (Mathai, et al. , 2008, Mortensen, et al. , 2007). As we only measured PGC-1α mRNA responses to our acute exercise sessions, we may have missed important information regarding the differences between our INT and CONT signalling responses. Wang and associates (Wang, et al. , 2009) did show similar activations of PRC between work-matched continuous and interval sessions lasting 90 min, however our sessions lasted less than 15 min making direct comparisons difficult. Therefore, there is a possibility that PGC-1β or PRC may be contributing to the divergent responses within our Study 1 investigation.

It is important to acknowledge that AMPK and p38 MAPK represent only two of an ever-growing number of proteins and molecules implicated in the mitochondrial response of skeletal muscle to exercise. For example, calcium represents a key signal

within skeletal muscle governing function and metabolism. It has been proposed that Ca2+ is the key signalling molecule that decodes the neural signal during exercise into not only immediate action, but also subsequent adaptive change (Chin, 2010). This is supported by findings that increasing Ca2+ in myotubes can increase mitochondrial protein markers (Ojuka, et al. , 2003, Wright, et al. , 2007). Ca2+-sensitive secondary messenger systems, such as the Ca2+ calmodulin-dependent kinases (CaMKs) and Ca2+ calmodulin-dependent phosphatase calcineurin (CaN) play key roles in achieving this regulation (Chin, 2010). Indeed, constitutive activation of CaMKIV and CaN have been shown to upregulate mitochondrial marker protein content and PGC-1α expression in skeletal muscle (Jiang, et al. , 2010, Wu, et al. , 2002). However, CaN inhibition has little hindering effect on exercise-induced increases in PGC-1α or a range of mitochondrial proteins in rats (Garcia-Roves, Huss and Holloszy. , 2006), calling into question the role of CaN in regulating mitochondrial adaptation to exercise. Regarding CaMKs, human skeletal muscle does not express CaMKIV or CaMKI, instead expressing only CaMKII (Rose and Hargreaves. , 2003). CaMKII phosphorylation has been shown to be augmented by acute exercise in an intensity-dependent manner (Egan, et al. , 2010, Rose, Kiens and Richter. , 2006) in association with greater PGC-1α mRNA expression (Egan, et al. , 2010), implicating a potential role for CaMKII in exercise-induced mitochondrial biogenesis. It is therefore possible that changes in CaMKII may have existed between our INT and CONT trials, which may have accounted, at least in part, for our divergent findings. Wright and associates (Wright, et al. , 2007) showed however, that Ca2+ and CaMKII requires the p38 MAPK pathway in order to regulate mitochondrial adaptation

(Wright, et al. , 2007). If this is the case, then any CaMKII signalling differences between conditions should have been reflected in our p38 MAPK measurements (keeping in mind our previous discussion of isoform specifics). Therefore, more work needs to be done examining Ca2+ signalling, as well as the relationship between CaMKII and p38 MAPK.

Reactive oxygen and nitrogen species (ROS/RONS) represent another signal that may play a role in adaptive change, as recent evidence suggests that supplementation with large amounts of antioxidants may blunt positive responses in muscle (Gomez-Cabrera, et al. , 2008, Kang, et al. , 2009, Ristow, et al. , 2009, Strobel, et al. , 2011). Irrcher and associates (Irrcher, Ljubicic and Hood. , 2009) showed that ROS exposure in skeletal muscle cells increased PGC-1α mRNA expression, while the addition of an anti-oxidant reduced basal PGC-1α mRNA content. Further analysis revealed that this was accomplished by way of modulating AMPK activity and the PGC-1α promoter by the upstream stimulatory factor-1 (USF-1). Morales-Alamo and colleagues (Morales-Alamo, et al. , 2013) have also suggested that free radical production is critical in the activation of not only AMPK, but also CaMKII, as supplementation with an antioxidant cocktail prior to sprint exercise blunted the exercise-induced increases in AMPK and CaMKII phosphorylation. Similar findings were reported by Kang and associates (Kang, et al. , 2009), who showed that antioxidant administration blunted the exercise-induced responses of p38 MAPK, PGC-1α, and downstream transcription factor elements in rats. These findings together implicate ROS/RONS as an important potential regulator of mitochondrial biogenesis in skeletal muscle and adaptive signalling. However, this signal again appears to act, at least partially, through or in concert with AMPK and p38 MAPK

and thus, any major differences in ROS signalling would again be reflected in our present measurements. However, it cannot be determined with certainty that differences in ROS/RONS did not contribute to our differing training outcomes. To this point, it has been suggested that the oxidation of particular residues in kinases such as p38 MAPK may cause a divergence between that protein's phosphorylation and activity levels (Templeton, et al. , 2010, Gerhart-Hines, et al. , 2007). These findings reiterate the complexity of signal regulation, and the need for more work regarding the role ROS/RONS in the metabolic adaptive response to exercise.

The Sirtuin family of protein deacetylases represent another possible avenue for the modulation of the mitochondrial response to exercise. It has been suggested that SIRT1 could enhance mitochondrial gene expression by way of PGC-1α deacetylation, and that this deacetylation is required for mitochondrial fatty acid oxidation genes to be expressed in skeletal muscle (Gerhart-Hines, et al. , 2007). Conversely, the expression of the acetyltransferase GCN5, or the SIRT1 inhibitor nicotinamide, resulted in PGC-1α acetylation and decreased PGC-1α-target genes (Gerhart-Hines, et al. , 2007). SIRT1 mRNA and protein expressions have been shown to be augmented following exercise in humans (Dumke, et al. , 2009, Guerra, et al. , 2010, Marfe, et al. , 2010, Suwa, et al. , 2008). However, contraction-induced increases in mitochondrial content have been associated with increased (Little, et al. , 2010b, Suwa, et al. , 2008), unchanged (Chabi, et al. , 2009), and decreased (Gurd, et al. , 2009, Gurd, et al. , 2010) SIRT1 protein contents. Gurd and associates (Gurd, et al. , 2011) have asserted that SIRT1 activity and not protein is what is important for upregulating mitochondrial gene expression. However, Philp and

associates (Philp, et al. , 2011) have contended that SIRT1 activity is not necessary for PGC-1α deacetylation or mitochondrial biogenesis, as muscle-specific knock-out of SIRT1 did nothing to hinder increases in mitochondrial content after ~3 wk of wheel-running. These latter findings have recently been corroborated by Menzies and associates (Menzies, et al. , 2013), who also showed that muscle-specific knockout of SIRT1 did not impair exercise-induced mitochondrial biogenesis. It would therefore appear that SIRT1 is not required for skeletal muscle mitochondrial adaptation to exercise under knockout conditions. Interestingly, when resveratrol was added to the exercise intervention a synergistic effect was found on the augmentation mitochondrial biogenesis (Menzies, et al. , 2013). This effect was reliant on the SIRT1 protein, indicating that SIRT1 may play a contributing role to exercise-induced mitochondrial biogenesis (Menzies, et al. , 2013). However, as the addition of resveratrol also was shown to augment exercise-induced AMPK and p38 MAPK phosphorylation, as well as ROS production, more work needs to be done to delineate the role of SIRT1 in this capacity (Menzies, et al. , 2013).

In summary, we have shown that the acute exercise-induced responses of AMPK, p38 MAPK, and PGC-1α mRNA do not necessarily predict the potential for chronic training to stimulate increases in skeletal muscle mitochondrial content. While acknowledging that other signalling mechanisms may be at work, we believe that the primary source of divergence between our acute observations and chronic adaptations likely occurs downstream of PGC-1α. This contention is supported by the finding that our PGC-1α mRNA responses were similar between exercise conditions. As PGC-1α activation formulates an auto-regulatory loop with its own promoter via MEF2

(Handschin, et al. , 2003), it may be suggested that PGC-1α activation was equivalent. While the initial signalling events regulating PGC-1α shall remain an exciting avenue for future research, it is only one of many areas that needs to be further investigated, and a great deal regarding the effects of exercise on the downstream biogenic processes remain to be established. It is no longer sufficient to believe that an upregulation of PGC-1α activity or expression will automatically result in mitochondrial biogenesis. A recent report suggesting that PGC-1α is actually dispensable in the process of exercise-induced mitochondrial biogenesis underscores this concept (Rowe, et al. , 2012).

5.4. Dietary Manipulation of the Training Response to High-Intensity Interval Training

Nutrition plays a critical supporting role in exercise performance and adaptation. CHO in particular is the primary macronutrient fueling high-intensity exercise, and is considered to be particularly limiting for prolonged exercise at 60-75% VO_{2peak} (Ivy, 1999). As a result, athletes have traditionally been advised to consume a high CHO diet prior to training in order to support the necessary high intensity and work volumes, and make the consumption of high CHO immediately following training a fundamental priority in order to prepare for the next training session (Burke, et al. , 2011). In recent years however, the alternative method of training with low CHO availability has become a popular. Research in this area was stimulated in large part by the findings of Hansen and associates (Hansen, et al. , 2005) who showed that training twice per day with restricted CHO intake, thus resulting in 50% of training being performed in a low CHO state, resulted in greater increases in specific markers of mitochondrial biogenesis and

muscle fatigue resistance. These authors coined the term "train low, compete high", asserting that training with reduced CHO availability would result in a greater mitochondrial biogenesis and performance improvements (Hansen, et al. , 2005). The findings by Hansen et al. (2005) have since been replicated by Yeo and associates (2008b) and Hulston et al. (2010) who collectively showed, using a similar training schedule in highly-trained cyclists, that CS and COXIV, as well as whole-body fat oxidation were elevated only in the "train low" group. The proposed mechanism underlying this effect is an enhanced AMPK phosphorylation following the second training session (Yeo, et al. , 2008a). Together, these findings suggest that performing 50% of training with reduced CHO availability in the form of muscle glycogen augments metabolic adaptation to a greater extent than when CHO is readily available. However, a potential confounding factor in these investigations is the fact that the training schedule between groups was different.

We designed a study in which two groups of individuals trained using the same schedule and varied only CHO intake between those sessions. In an acute investigation using this protocol, we showed that restricting CHO between two sessions of HIT resulted in an increased p38 MAPK phosphorylation following the second session (Cochran, et al. , 2010). Altogether, it would appear that evidence from both training and signalling investigations suggest an important role for CHO in the augmentation of mitochondrial biogenesis. However, contrary to the findings of Hansen (Hansen, et al. , 2005), Yeo (Yeo, et al. , 2008b), and Hulston (Hulston, et al. , 2010), we found that when subjects performed two HIT sessions per day, 3 d per week, varying only CHO intake between

sessions, there were no differences in mitochondrial adaptation between groups (Study 2). Beyond supporting our findings in Study 1 suggesting that protein signalling holds little bearing on long-term adaptation, these findings would support training schedule, rather than CHO availability, as the important mediating factor in magnifying mitochondrial adaptation to exercise training. However, a number of possible caveats remain which preclude a firm conclusion in this regard.

The first factor which may be playing a particularly important role in the modulation of training adaptation is muscle glycogen. One common factor in those studies finding greater augmentation in metabolic adaptation to training (Hansen, et al. , 2005, Hulston, et al. , 2010, Yeo, et al. , 2008b) is that the first session of daily training resulted in a 50% or greater reduction in muscle glycogen. In contrast, other examinations finding similar mitochondrial adaptations despite altered CHO availability utilized interventions which reduced muscle glycogen levels in the vicinity of 30-35% (Study 2) (Morton, et al. , 2009) differences in muscle glycogen. Other studies finding little difference in mitochondrial adaptation with altered CHO availability primarily manipulated the availability of exogenous CHO or blood glucose, and not endogenous muscle glycogen stores (Akerstrom, et al. , 2009, De Bock, et al. , 2008, Stannard, et al. , 2010). Only Van Proeyen et al. (2011) have shown marked differences in CS and βHAD increases between a CHO-fed and CHO-restricted group over a 6 wk training period in absence of a noted difference in pre-training session muscle glycogen content. It would therefore appear that muscle glycogen may be an essential factor determining

mitochondrial training response, and not necessarily exogenous or blood-borne CHO availability.

Morton and associates (2009) have performed the only systematic analysis that may shed some light on the potential importance of muscle glycogen. These researchers employed 3 groups to determine the role of CHO availability on mitochondrial adaptation to training (Morton, et al. , 2009). One group (Norm) trained one session per day, 4 d per week, thus beginning each session with normal levels of muscle glycogen. The other two groups trained twice per day, 2 d per week, thus beginning every afternoon session with reduced muscle glycogen content. However, one of these groups consumed CHO before and through each of these second sessions, thereby possessing a source of exogenous glucose (Low+Glu group), whereas the other group was denied CHO before and during the second session (Low + Pla group) (Morton, et al. , 2009). Therefore, the Norm group began each training session with normal muscle glycogen levels, the Low + Glu group performed 50% of their training with lowered muscle glycogen (35%) but elevated exogenous CHO availability, and the Low + Pla group performed 50% of their training with low endogenous and blood glucose availability (Morton, et al. , 2009). After 6 wk of training, elevations in succinate dehydrogenase, another mitochondrial marker, was greatest in the Low + Pla group, indicating a potential for reduced CHO to augment mitochondrial adaptation (Morton, et al. , 2009). However, PGC-1α and COXIV were elevated to a similar extent in all three groups, making the findings equivocal (Morton, et al. , 2009). Altogether, further research is required to clarify the role that CHO plays in the modulation of mitochondrial training effect, however it would appear that muscle

glycogen may need to be reduced >50% prior to training to make a marked long-term effect on mitochondrial training adaptation.

β-ALA represents another nutritional manipulation which may have the potential to alter long-term training responses in skeletal muscle. A number of studies have shown that β-ALA can increase skeletal muscle carnosine content, which can subsequently increase work capacity and performance (Derave, et al. , 2007, Harris, et al. , 2006, Hill, et al. , 2007, Van Thienen, et al. , 2009). Therefore, akin to the idea that high CHO may support greater training intensities for longer durations, β-ALA may also be able to support greater training intensity and work output, especially if training intensities would normally induce significant acidosis. We have thus performed the first training study examining the effects of β-ALA on the mitochondrial adaptive response to all-out HIT. As repeated all-out efforts lasting 30 s may reduce skeletal muscle pH to as low as 6.5, and decreased pH is associated with reductions in work output (Spriet, et al. , 1989), it was hypothesized that β-ALA might have the capacity to augment training work output, ultimately resulting in greater mitochondrial adaptation. However, contrary to our hypothesis, we found (Study 3) that β-ALA did not alter either total work output, or the mitochondrial adaptive response in response to 6 wk of all-out HIT. These findings would appear to indicate that β-ALA does not augment work capacity during all-out HIT training and has no effects on the resultant mitochondrial biogenesis that occurs over 6 wk during this type of training. While it appears that β-ALA provides no overt effects on the skeletal muscle adaptive response or performance following all-out HIT, it is also

important to note that there is also no inhibitory effect of the supplement on training adaptation. This is an important practical note for training.

While we did not find any beneficial effect of β-ALA on training adaptation, there are few mitigating factors that must be noted. Our dosage protocol consisted of 3.2 g β-ALA/d for 4 wks prior to the beginning of training, and continued supplementation throughout training. This was the exact dosing prescribed by the label on the commercial β-ALA product that we used. However, those studies that have reported the most profound increases in muscle carnosine and performance have used a dosage of 6.4 g β-ALA/d (Harris, et al. , 2006, Hill, et al. , 2007). These researchers saw an increase in skeletal muscle carnosine of 60-65% over the course of 4 wks of β-ALA supplementation, whereas our dosage regime achieved only a 33% increase in muscle carnosine content after 4 wks, and ~52% after approximately 11 wks. These differences in β-ALA supplementation and skeletal muscle carnosine content may explain our similar work outputs throughout training, and inability to detect performance differences in our time trial. It is therefore possible that a greater amount of daily β-ALA may have altered our outcomes with regards to work outputs during training, and thus may have altered the resulting training outcomes.

An alternative explanation for the general lack of effect of nutritional manipulation on mitochondrial adaptation to HIT may be related to the population we chose to examine. Our previous investigations of all-out HIT utilizing recreationally-active individuals have shown that 6 wks of such training can increase CS maximal activity by ~25% (Burgomaster, et al. , 2008), COXIV protein by 35% (Burgomaster, et

al. , 2007) and β-HAD by ~30% (Burgomaster, et al. , 2008). Utilizing a very similar training paradigm as we did in Study 2, Talanian and associates (Talanian, et al. , 2007) showed an increase of 20% and 32% for CS and β-HAD in recreationally-active women over a period of 2 wks. Our Studies 2 and 3 induced mitochondrial adaptations very similar to these previous investigations. Specifically, 2 wks of constant-load HIT in our Study 2 produced increases in CS nearing 31%, 11% greater than the increases reported by Talanian et al. (Talanian, et al. , 2007), and Perry and associates (Perry, et al. , 2008) showed that 6 wks of a similar protocol increased COXIV of 18%, compared to our 19% over 2 wks. Similarly, our Study 3 findings show that 6 wks of all-out HIT increases CS and β-HAD activities by 40% and 19%, respectively, along with 20% increases in COXIV protein content. Collectively, these results are an indicator of the potency of both constant-load and all-out HIT, as the increases in mitochondrial markers are relatively large over a short time frame. It is therefore possible that small effects induced by the nutritional manipulations in both Study 2 and Study 3 may been undetectable due to the marked effects of HIT alone. This may mean that, effectively, small effects of nutrition were "washed out" by the large effects of training, especially considering the low baseline fitness of our subjects. For example, both Yeo (2008b) and Hulston (2010) and associates each used highly trained cyclists or triathletes that already had a significant training base and mitochondrial baseline. This may have made the distinct effects of nutrition more readily detectable. Altogether, our study may have been too short to allow the effects of nutrition to rise above the large effects of exercise in these untrained subjects. Future research may wish to examine these manipulations in those subjects closer to their

physiological ceiling, allowing for the effect of nutrition to be more easily differentiated from that of training.

5.5. Future Directions

In the last 15 years, significant advances have been made in our understanding of how human skeletal muscle adapts to exercise with growing interest in HIT as a specific paradigm. Not only has HIT been repeatedly been validated as an effective and potent means to induce mitochondrial biogenesis, but in-vitro and animal research have identified an extensive network of potential mechanisms and contributors to this process. These mechanisms include AMPK, p38 MAPK, and PGC-1α. This being said, much remains to be determined regarding HIT and the in-vivo contribution of each of these proteins towards mitochondrial biogenesis in humans. For example, future work needs to determine whether individual acute responses in protein signalling correlate with within-subject long-term adaptive outcome. Moreover, future work needs to examine the regulation of the numerous cellular processes that occur between the initial protein signal and the production of functional mitochondria. These processes include transcription factor binding, transcription, transcriptional splicing, translation, and protein folding and assembly, to name a few. It is likely that the regulation of these processes by exercise play an important role in determining whether the initial exercise stimulus is propagated into functional outcome, or dissipated completely. Along these lines, a number of questions also remain regarding HIT and what makes it so effective. It would appear, based on our findings, that intermittency may play a key role in achieving mitochondrial adaptation to low volume, high-intensity training. However, it remains to be determined

how other factors, such as resistance, and the length of work and/or rest intervals may affect the training response. For example, it is possible that the load imposed during all-out HIT (e.g., 7.5% body weight as applied in a typical Wingate Test) plays a key role in producing muscle adaptation. Similarly, the length of either the work, and/or rest interval may alter HIT-induced adaptation as well. Interestingly, some muscular adaptations appear to be similar independent of rest interval being either 1 or 3 minutes in length (Edge, et al. , 2013), however this does not rule out the possibility that the rest interval play an important role. Certainly, there are many permutations of HIT that may result in distinct responses within human muscle.

It is well known that muscle glycogen and CHO availability play key roles in supporting exercise at high-intensity. However, despite recent findings having suggested that training with reduced CHO availability can augment mitochondrial adaptation, our results would suggest that training with reduced CHO availability has no greater benefit than training with adequate CHO availability. At least, this would appear to be the case in untrained subjects. However, muscle glycogen may play a greater role in modulating training adaptation compared to CHO availability *per se.* Therefore, future research may wish to isolate the role of muscle glycogen in the regulation of metabolic adaptation to exercise. Furthermore, in order to separate the role of nutrition from exercise, future research may also wish to examine this question using individuals of a higher training status. This may allow a greater chance of deciphering nutrition-specific effects.

5.6. Summary

The present thesis examined the specific role of the interval in stimulating adaptations to high-intensity exercise, and the potential for CHO and β-ALA to modulate skeletal muscle metabolic responses to HIT in previously untrained or recreationally active individuals. We have established that the interval may be a critical component in maximizing the potency of HIT, and to achieving mitochondrial biogenesis with little total time commitment. We have further shown that the measurement of acute AMPK, p38 MAPK, and PGC-1α mRNA responses in human muscle is ineffective in determining long-term adaptation to an exercise program, calling attention to the need for further work examining downstream effectors of mitochondrial protein production and assembly. Lastly, we have shown that dietary manipulation during HIT via CHO restriction or β-ALA supplementation does not appear to markedly affect mitochondrial response in recreationally-active participants. This may be related to subjects' muscle glycogen status, or because greater augmentations in muscle carnosine are required. More likely however, any small effects of nutritional manipulation were overwhelmed by the potency of HIT, which induced marked mitochondrial adaptation in our relatively untrained population. This underscores the fact that nutritional manipulation plays a supporting role for the metabolic adaptation to exercise, and that no nutritional supplement can compensate for a sub-optimal training program. Collectively, this thesis adds to our knowledge base regarding the effects of HIT on skeletal muscle mitochondrial adaptation, our understanding of the underlying mechanisms of those effects, and the modulating role of nutrition in these processes.

5.7. References

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