MOLECULAR SIGNALLING RESPONSES TO HIGH-INTENSITY INTERVAL EXERCISE

MOLECULAR SIGNALLING RESPONSES TO HIGH-INTENSITY INTERVAL EXERCISE: EFFECTS OF CARBOHYDRATE AVAILABILITY

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ABSTRACT

Manipulating carbohydrate (CHO) availability has been shown to alter acute exercise-induced changes in metabolic gene transcription and training-induced changes in oxidative capacity. The present study examined the effect of CHO availability on signalling pathways linked to mitochondrial biogenesis in response to high-intensity interval exercise (HIE). We hypothesized that reduced CHO availability would augment phosphorylation of AMP-activated protein kinase (AMPK), calcium/calmodulin-dependent kinase II (CaMKII), and p38 mitogen-activated protein kinase (p38) in response to HIE. Ten active men performed two experimental trials in random order, separated by ≥1 wk. During each trial, subjects performed two HIE sessions separated by 3 h (AM and PM sessions). Exercise sessions consisted of 5 x 4 min cycling bouts at a workload that elicited approximately 90% VO_{2peak}, with 2 min rest periods. Between sessions, subjects ingested ~1.2 g CHO/kg b.w./h (HI-HI) or a taste-matched, non-energetic placebo (HI-LO). Muscle biopsies and blood samples were obtained before (Pre) and after (Post) the AM and PM HIE sessions. AMPK, CaMKII, and p38 MAPK phosphorylation increased from AM Pre to AM Post ($p<0.01$). During the PM exercise session, p38 phosphorylation increased in the HI-LO condition (~4.5-fold, $p<0.001$), whereas the HI-HI condition remained unchanged. PM HIE significantly increased CaMKII phosphorylation independent of condition, while no exercise or condition-mediated AMPK effects were observed. In summary, restricting CHO availability following an acute session of HIE augmented the exercise-induced increase in p38 phosphorylation during a subsequent HIE session. It remains to be determined whether chronic changes in p38 MAPK signalling are mechanistically linked to altered skeletal muscle remodelling observed after CHO-restricted exercise training.

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CHAPTER 1: REVIEW OF LITERATURE

1.1 INTRODUCTION

It is well known that regular physical activity induces profound alterations within human skeletal muscle tissue. Traditional aerobic endurance training results in a variety of adaptations that improve energetic efficiency and reduce cellular disturbances during subsequent exercise bouts. The result is increased performance secondary to enhanced muscle oxidative capacity, peak oxygen uptake (VO_{2peak}), and the shifting of muscle fibres to a more oxidative phenotype. Mitochondrial biogenesis is a central metabolic adaptation within skeletal muscle as a result of endurance training and while many of the physiological adaptations related to regular physical activity have been well characterized, the mechanisms behind such changes remain to be fully elucidated. It is the directive of this literature review to examine some of the proposed mechanisms of mitochondrial biogenesis, and the role that carbohydrate plays as a potential mediating factor involved in the process of exercise-induced mitochondrial biogenesis.

1.2 THE SKELETAL MUSCLE ADAPTIVE RESPONSE TO EXERCISE

Exercise-induced skeletal muscle plasticity results from the myocytes' altered protein expression. Protein is the functional unit within all human cells and many of the changes and alterations brought about by exercise occur via altered protein expression patterns (36). Exercise results in transient increases in metabolic gene expression in the hours immediately following exercise cessation (138). Thus, it is widely believed that training adaptation is the result of augmented protein accumulation consequent of

enhanced messenger ribonucleic acid (mRNA) transcription and subsequent translation (138). Which proteins are produced in response to exercise, however, and the resulting protein expression is determined by a variety of different inputs such as the exercise modality, intensity, duration, and half-life of the protein in question (36).

1.2.1 Endurance Exercise & Training

Central to the improved energy status that occurs with endurance training (ET) is an enhanced mitochondrial density within the skeletal muscle, also known as mitochondrial biogenesis. It is well documented that regular, intensive endurance exercise is associated with increased mitochondrial reticular volume within the skeletal myocyte in both rodents (76) and humans (79, 80). Secondary to this increased mitochondrial volume, enzymes of the tricarboxylic acid cycle, carbohydrate oxidation, fatty acid oxidation, and electron transport pathways are augmented, culminating in improved oxidative capacity of the muscle (77). Ultimately, ET results in a reduced perturbation to the cellular metabolic homeostasis, enhanced fat oxidation, and co-ordinately reduced carbohydrate oxidation and muscle lactate production in response to the same absolute pre-training exercise stimulus (77). This shift towards a more oxidative muscular phenotype is reflected by a greater endurance capacity of the trained individual (41, 94) or animal (76), and also by a so-called "fast-to-slow fibre transformation", characterized by an increase in the ratio of more oxidative fibres (Type I and IIa) to glycolytic fibres (Type IIx/b; (4, 136). This morphological adaptation is evidenced by the finding that it is nearly impossible to detect IIb fibres in individuals who have undertaken long-term, intensive ET (164).

1.2.2 Resistance Exercise and Oxidative Capacity

While not a direct topic of review, the subject of resistance exercise and oxidative capacity shall be briefly addressed. Resistance exercise is associated with increases in single fibre and whole muscle size (54, 148), resulting in enhanced maximal force production (54), glucose disposal capacity (78, 126), and resting metabolic rate (25). Traditionally, these alterations are thought to occur without significant changes in muscle oxidative capacity. However limited studies have shown that resistance training (RT) may enhance skeletal muscle oxidative potential (40, 182). In fact, a recent publication by Tang and colleagues (182) demonstrated that 12 weeks of RT enhanced the activity of mitochondrial marker enzymes, while concurrently inducing hypertrophy of all fibre types. Thus, the traditional view that RT is incapable of inducing aerobic adaptation within the skeletal muscle may soon re-open for debate.

1.2.3 High-Intensity Interval Exercise & Training

In contrast to traditional endurance training, high-intensity interval training (HIT) involves brief, intense work sessions interspersed with recovery sessions of rest, or lower-intensity exercise. HIT work can generally be placed into one of two categories, 1) Wingate-based, or 2) Constant-Load. The Wingate anaerobic power test involves subjects pedalling maximally against a resistance equivalent to 7.5% of their body mass for a period of 30-seconds (85). Thus, Wingate-based HIT most often involves the repetition of these "all-out" sprinting efforts in which subjects work at maximal intensities (~250% VO_{2peak}) for 30-second time periods with 4 minutes of recovery between work bouts (16-19, 60). This form of HIT is characterized by a progressive

decrease in power output during work bouts despite constant effort, and thus, has alternatively been termed "supramaximal" or "sprint interval training" (SIT). In comparison, constant-load HIT most often involves up to ten, 4-5 minute work bouts in the ~85-100% VO_{2peak} range, interspersed by 1-2 minutes of recovery (177, 178, 181, 211). Work bouts within the constant load modality rely upon standardized power outputs maintained throughout.

Repeated high-intensity, short-duration exercise has been shown to be an effective oxidative training modality in animals and humans for nearly 30 years (40, 73, 74), however HIT has only gained a great deal of attention with regards to oxidative metabolism in the past decade. MacDougall and colleagues (116) showed that 7 weeks of Wingate-based HIT was capable of inducing increases in citrate synthase (CS), malate dehydrogenase, and succinate dehydrogenase, with a non-statistically significant 39% increase in β-hydroxyacyl coenzyme-A dehydrogenase (βHAD). More recent research has further established HIT as a potent stimulus for aerobic metabolism, inducing many of the same skeletal muscle adaptations as traditional ET despite markedly reduced total work outputs (60). With as little as 6-7 training sessions over the course of a 2-week period, HIT has been shown to enhance maximal CS activity (17, 19, 181), maximal capacity for pyruvate oxidation (PDHa) (17), as well as the protein concentration of cytochrome oxidase (COX) subunits 2 (COXII) and 4 (COXIV) (16, 60). HIT has also been shown to increase resting muscle glycogen content (17, 19), while reducing its oxidation during post-training steady state exercise (17, 181).

Gibala and associates (60) made direct comparisons between a 2 week, 6 session protocol of SIT versus ET in which subjects underwent the Wingate-based HIT (SIT) protocol (4-6 work bouts/session), or 90-120 minutes of continuous cycling at ~65% VO_{2peak}. Results demonstrated that despite an ~90% lower total work output during training in the SIT versus ET, both groups gained similar increases in COX maximal activity, protein content of COXII and COXIV, muscle buffering capacity, and resting muscle glycogen concentration. Moreover, both groups gained similar performance enhancements in both 50 and 750 kJ time trials, confirming the value of HIT as a viable method to improve performance during aerobic-based exercise.

One caveat of HIT is that certain skeletal muscle adaptations appear to rely directly upon the modality of HIT chosen, which may be related to the overall volume of training performed. For example, it appears that Wingate-based HIT requires longer training periods in order to manifest improvements in the fat oxidation pathway while the longer, constant load intervals appear to enhance fat oxidation more quickly. For example, Burgomaster *et al.* (17) showed that 2 weeks of SIT was insufficient to increase the maximal activity of βHAD despite significant increases in CS. However, when this training was prolonged to a 6 week period, βHAD activity was indeed increased (18). In contrast, Talanian and colleagues (181) used a 2 week, constant-load HIT protocol in which subjects underwent 7 training sessions involving ten, 4-minute work bouts eliciting ~90% VO_{2peak} and showed not only that βHAD and CS maximal activity was increased, but also that fatty acid binding protein ($FABP_{pm}$) protein content was increased, a change that 2 weeks of SIT has failed to show (16). Talanian *et al.*, (181) subsequently

demonstrated that during a 60-minute steady state ride, total carbohydrate oxidation was decreased, while total fat oxidation was concomitantly increased. Thus, despite HIT being a potent exercise stimulus for augmenting skeletal muscle oxidative capacity, some differences exist between the HIT modalities.

1.3 MECHANISMS OF SKELETAL MUSCLE PLASTICITY

While the occurrence of exercise-induced mitochondrial biogenesis has been well characterized, the mechanisms responsible for its induction remain to be completely elucidated. Upon exercise onset, a variety of cellular perturbations stimulate secondary signalling cascades within the skeletal muscle which ultimately result in the proliferation of the mitochondrial reticulum (Figure 1.1). These primary signals include alterations in intramyocellular calcium (Ca^{2+}), the ratio adenosine monophosphate (AMP) to adenosine triphosphate (ATP), the redox potential of the skeletal muscle (NADH:NAD), as well as the innate mechanical stretch that occurs within the skeletal muscle with exercise (36). Which of these signals and their subsequent signalling cascades play the majority role in initiating mitochondrial protein accumulation within the myocyte remains elusive. It is likely that mitochondrial biogenesis is the result of coordinated interplay between the protein signals, and not due to a single predominant pathway *per se*.

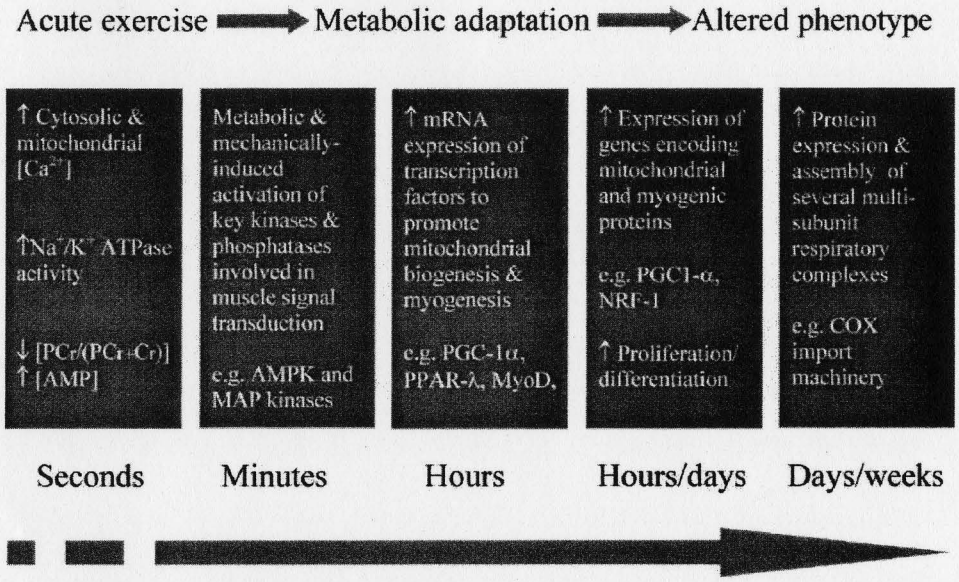

Figure 1.1 – Overview of the various intramyocellular signals relating acute exercise to phenotypic alteration. (71)

1.3.1 Signalling Pathways Directed Towards Mitochondrial Biogenesis

1.3.1.1 Calcium & Calcium/Calmodulin-dependent Kinases

The amplitude and duration of the increases in cytosolic Ca^{2+} is determined by the frequency and pattern of neural stimulation to the working muscle (33). It is believed that the pattern of these Ca^{2+} transients impose downstream regulation of gene expression (33). Indeed, studies that have increased intracellular Ca^{2+}, without inducing muscular contractions or changes in high-energy phosphorylation potential, have shown increased levels or expression of numerous mitochondrial enzymes including aminolevulinate synthase (ALAS), CS, malate dehydrogenase, cytochrome c, COX subunit 1 (COXI), and the β-subunit of F1ATPase (55, 56, 134). Furthermore, treatment of skeletal muscle cells with Ca^{2+} has been shown to increase the protein expression and/or DNA binding of critical mitochondrial biogenetic factors such as peroxisome proliferator-activated

receptor γ coactivator-1α (PGC-1α), mitochondrial transcription factor A (Tfam), and nuclear respiratory factors 1 and 2 (NRF1 and NRF2, respectively) (133, 134). Finally, Ca^{2+} has been linked to increased glucose transporter 4 (GLUT4) expression through the myocyte enhancer factor 2 (MEF2) transcription factor (119, 135).

Calcium release into the sarcoplasm results in the activation of a variety of downstream signalling proteins including the Ca^{2+}-dependent phosphatase calcineurin, Ca^{2+}-dependent protein kinase C, and the Ca^{2+}-calmodulin-dependent protein kinases (CaMKs) (33). Interest in CaMKs as mediators of mitochondrial biogenesis was piqued by the finding that overexpression of the CaMKIV isoform in mice resulted in a vast array of muscular changes including increased percentage of type I fibres, mitochondrial volume, and PGC-1α gene expression, as well as enhanced PGC-1α promoter activity (205). Subsequent research determined however, that CaMKIV is not expressed in human skeletal muscle, and its counterpart CaMKI did not respond as a true CaMK (155, 156). CaMKII remains of great interest in mitochondrial biogenesis, however, as it is expressed in human muscle, is activated in response to exercise (155, 156), and also shows increased expression in response to exercise training (154). CaMKII may enhance mitochondrial content and GLUT4 biogenesis via deactivation of class II histone deacetylase (HDAC) proteins that act as repressors of MEF2 (114). CaMKII phosphorylation of HDAC4 targets it for nuclear extrusion (114) along with HDAC5, which forms protein-protein interactions with HDAC4 (9). The result is relieved inhibition of MEF2 and enhanced transcription of various MEF2-binding promoters, which include PGC-1α (66, 120) and GLUT4 (120, 129, 172). CaMKII has also been

linked to downstream activation of p38 mitogen-activated protein kinase (203), another signalling protein associated with enhanced MEF2 activation and mitochondrial biogenesis (3, 120). Alternatively, CaMK is associated with adenosine monophosphate-activated protein kinase through an upstream CaMK kinase (CaMKK), providing another path through which Ca^{2+} may mediate its effects on GLUT4 and mitochondrial biogenesis (90, 91).

1.3.1.2 Adenosine Monophosphate-activated Protein Kinase

Adenosine monophosphate-activated protein kinase (AMPK) is a heterotrimeric enzyme whose primary function is to monitor intracellular energy status, increasing ATP-producing functions while decreasing ATP-consuming processes in response to energy deficit (39, 95). Thus, AMPK activity has been linked to a wide variety of intramyocellular processes such as glucose uptake, lipid uptake and oxidation, protein synthesis inhibition, as well as mitochondrial biogenesis (95). AMPK is fully activated by phosphorylation of its threonine-172 (Thr^{172}) residue, which resides in the catalytic α subunit (173). Phosphorylation takes place primarily via the upstream AMPK kinase (AMPKK) LKB1-STRAD-MO25 complex which is constitutively active in skeletal muscle (163), but is also accomplished by CaMKK (82, 91). AMP plays a critical role in this phosphorylation/activation as it 1) directly activates AMPK allosterically (27), 2) induces a conformational change within the AMPK structure, relieving autoinhibition of the Thr^{172} site (168), and 3) prevents dephosphorylation of Thr^{172} by endogenous

phosphatases (165). The end result is a hypersensitive system in which relatively small changes in AMP may induce increases in AMPK activity up to 100-fold (39).

AMPK activity increases in response to muscle stimulation in rodents (72, 84), and exercise in both rodents (131) and humans (30, 31, 152, 192, 201). This activation occurs in an intensity, and duration-dependent manner (31, 192, 201), and may be influenced by isoform, fibre-type, and sex-based differences (58, 152, 163). Furthermore, AMPK activation during exercise also appears to take place in a localized manner (106), despite some circulating factors such as leptin (127) and adiponectin (207), as well as interleukin-6 (158) being shown to alter its phosphorylation.

Exercise training has also been shown to alter AMPK activity and expression as 3 weeks of unilateral endurance kicking significantly augmented basal AMPK Thr^{172} phosphorylation by 74%, concomitant with increases in the maximal activities of mitochondrial marker enzymes CS (~37%) and βHAD (~35%) (57). Furthermore, trained individuals have increased AMPK α-subunit expression in comparison to untrained individuals (132). Recent evidence indicates however, that AMPK activation may become more difficult with training, as it has been shown that trained cyclists did not significantly increase their AMPK phosphorylation in response to a 1 hr of cycling at 70% VO_{2peak}, whereas trained power lifters unaccustomed to cycling did activate AMPK (37). Conversely, cyclists did show AMPK activation following a bout of resistance exercise (37). This is consistent with the idea that exercise training results in improved maintenance of metabolic energy status and that training likely results in smaller

disturbances in the AMP:ATP ratio, thus reducing AMPK activation. This is supported by findings that AMPK phosphorylation is blunted in trained versus untrained individuals in response to high-intensity exercise (132). These alterations in the ability to activate AMPK in response to exercise may be mediated by training-induced alterations in the subunit expression of the AMPK heterotrimer (14, 132).

AMPK was first linked to mitochondrial biogenesis by way of animal studies using the pharmacological AMPK agonist 5-aminoimidazole-4-carboxamide 1-4-ribofuranoside (AICAR). Chronic treatment of animals with AICAR *in-vivo* was found to induce alterations mimicking those of exercise training, including increases in CS, succinate dehydrogenase, malate dehydrogenase, ALAS, and cytochrome c activities (198), as well as PGC-1α protein content (180). Further pharmacological evidence for AMPK-mediated mitochondrial biogenesis was garnered by Bergeron and associates (13) who showed that chronic feeding of β-guanidinopropionic acid (β-GPA), a creatine analog, to rats resulted in significantly increased AMPK activity, cytochrome c content, ALAS expression, and DNA binding activity of NRF1, a critical transcription factor in mitochondrial biogenesis. This data has since been supported by transgenic animal experiments whereby the overexpression of a muscle-specific dominant negative form of AMPK abolished all β-GPA-induced adaptations (214). Furthermore, transgenic experiments designed to consititutively activate AMPK results in increased maximal activity of CS and COX, as well as enhanced, or trends toward enhanced mRNA content of a variety of critical mitochondrial biogenetic proteins including Tfam, NRF2, and MEF2C (150, 189). Enhanced AMPK activation also resulted in greater proportion of IIx

and IIa fibres and increased mitochondrial DNA content (mtDNA) (150, 189) indicating that AMPK does appear to play a role in basal level mitochondrial volume.

AMPK may play a role in mitochondrial biogenesis through a variety of mechanisms. Firstly, AMPK has been shown to translocate to the nucleus of human skeletal muscle in response to exercise, providing the opportunity for interaction with transcription factors and cofactors, or potentially interact with DNA directly to enhance mitochondrial protein expression (121). This indeed appears to be the case, as subsequent research has indicated that AMPK may directly phosphorylate PGC-1α, reputed master coordinater of mitochondrial biogenesis (88), and enhance the DNA-binding activity of PGC-1α's downstream transcriptional factor NRF1 (13). This phosphorylation is required for PGC-1α self-mediated gene expression, as PGC-1α is regulated by way of an autoregulatory loop through the transcription factor MEF2 (66). Furthermore, AMPK has been shown to phosphorylate HDAC5, relieving inhibition of the MEF2 transcription factor to enhance GLUT4 transcription (122). AMPK may mediate PGC-1α expression and mitochondrial biogenesis through a similar mechanism, as the PGC-1α promoter also contains a MEF2 binding site (44). However, while much research implicates AMPK in mitochondrial biogenesis, recent isoform-specific knockouts of AMPK indicate that it is not critical to exercise-induced PGC-1α transcription (97), or training-induced increases in mitochondrial marker proteins (96, 150). Therefore, while AMPK may play an important role in basal, and pharmacologically-induced mitochondrial biogenesis, and may play a role in exercise training, it is likely not the only contributor.

1.3.1.3 p38 Mitogen-Activated Protein Kinase

p38 mitogen-activated protein kinase (p38) is one of four identified mitogen-activated protein kinases (MAPKs) within skeletal muscle along with the extracellular signal-regulated kinases 1 and 2 (ERK1/2), the c-jun NH_2-terminal kinases (JNK), and ERK 5 (a.k.a. big MAPK) (105). MAPKs have been linked to a variety of cellular functions such as CHO and fat metabolism, cell proliferation and differentiation, apoptosis, inflammation, as well as gene regulation (105). Each MAPK pathway is thought to consist of a three-tiered method of phosphorylation in which an upstream MAPK kinase kinase (MAPKKK) phosphorylates a MAPK kinase (MAPKK), which phosphorylates the designated MAPK (157). MAPK activation occurs via dual phosphorylation of threonine (Thr) and tyrosine (Tyr) residues within a conserved Thr-X-Tyr sequence located within the activation loop in response to growth factors, cytokines, and cellular stressors (107, 157). Thus, it is believed that the MAPKs may play an important role in coupling cellular stress signals with adaptive responses (105).

Specifically, p38s activating motif consists of a Thr-Glycine-Tyr sequence which is phosphorylated *in-vitro* in response to cellular stresses such as reactive oxygen species (ROS) (100), mechanical stretch (15), and inflammatory cytokines (212). Thus, exercise has been reported to activate p38 MAPK in both rodents (3, 62, 204) and humans (120, 197, 210, 211) as muscle contraction is associated with each of these p38 agonists. Intensity may play a mediating role in this p38 phosphorylation, as more intense perturbations in vitro appear to activate p38 to a greater extent (100, 159, 202) and p38 activation is observed most consistently with high exercise intensities (204, 210, 211).

This activation is not force or tension-dependent, however (46, 117), indicating other signals must mediate phosphorylation as well. Activation frequency may be one such signal, as higher stimulation of isolated rat muscle can enhance p38 phosphorylation independent of force production (159). In line with this, Wright and associates (203) recently proposed CaMKII as an upstream p38 kinase, citing that caffeine-induced Ca^{2+} release induced p38 phosphorylation, while CaMK inhibitors attenuated this activation. Other potential p38 activators include insulin (186), thyroid hormone (86), and AMPK (112, 209). This latter proposal is attractive as it offers p38 as a point of convergence for the AMPK and CaMK pathways to implement their effects on PGC-1α and mitochondrial biogenesis, however, recent findings show dissociation between AMPK and p38 (75).

Exercise modality and training status may also mediate p38 activation. It has been shown that unaccustomed exercise results in more robust activation, despite trained and untrained subjects exercising at the same relative intensity (211). Furthermore, in a study by Coffey and associates (37), well-trained powerlifters who performed 1h of cycling at approximately 70% VO_{2peak}, or 8 sets of 5 maximal isokinetic leg extensions showed a significant increase in p38 phosphorylation only following the cycling exercise but not during the resistance exercise. Conversely, well-trained cyclist experienced increases in p38 phosphorylation following resistance exercise, but not following their familiar endurance activity (37). Thus, it appears that training history may affect p38 activation during exercise.

p38 phosphorylation and mitochondrial biogenesis are linked primarily through the regulation of PGC-1α activity and expression. Nuclear p38 phosphorylation is

increased in response to exercise (120) and is believed to affect PGC-1α in skeletal muscle via 2 distinct mechanisms, whereby p38 1) directly phosphorylates Thr^{262}, serine 265, and Thr^{298} resulting in increased PGC-1α protein half-life (142) and disruption of PGC-1α protein repression by the p160 myb binding protein ($p160^{myb}$) (51, 103), and 2) directly phosphorylates MEF2 (63, 213), and activating transcription factor-2 (ATF2) (145, 146), increasing their transcriptional activity. This is important to mitochondrial biogenesis as both MEF2 and ATF2 transcription factors bind to the PGC-1α promoter, enhancing PGC-1α mRNA expression in response to both voluntary running and motor nerve stimulation (2, 3). Furthermore, PGC-1α has also been shown to regulate its own expression via an autoregulatory loop whereby PGC-1α coactivates MEF2 to enhance its own transcription (66). Thus, derepression of PGC-1α allows coactivation of the MEF2 transcription factor (51), which subsequently acts in conjunction with ATF2 to increase PGC-1α expression (3, 66). An alternative means of p38-mediation of PGC-1α may include alteration of chromatin structure through its downstream target mitogen- and stress-activated kinase 1 (MSK1) (105, 162). Indeed, p38 phosphorylation, MSK1 activity, and histone H3 phosphorylation have all been shown to occur in human skeletal muscle in response to high intensity exercise (211). However, as p38 was not the only MSK1-targeting MAPK activated by exercise, it cannot be said that p38 performs this function exclusively (211). It does appear however, that p38 plays a critical role in PGC-1α activity and expression (208) and that it is the function of p38 and its coordinate signalling proteins, AMPK and CaMK, to maximize potential for PGC-1α-mediated mitochondrial biogenesis (Figure 1.2).

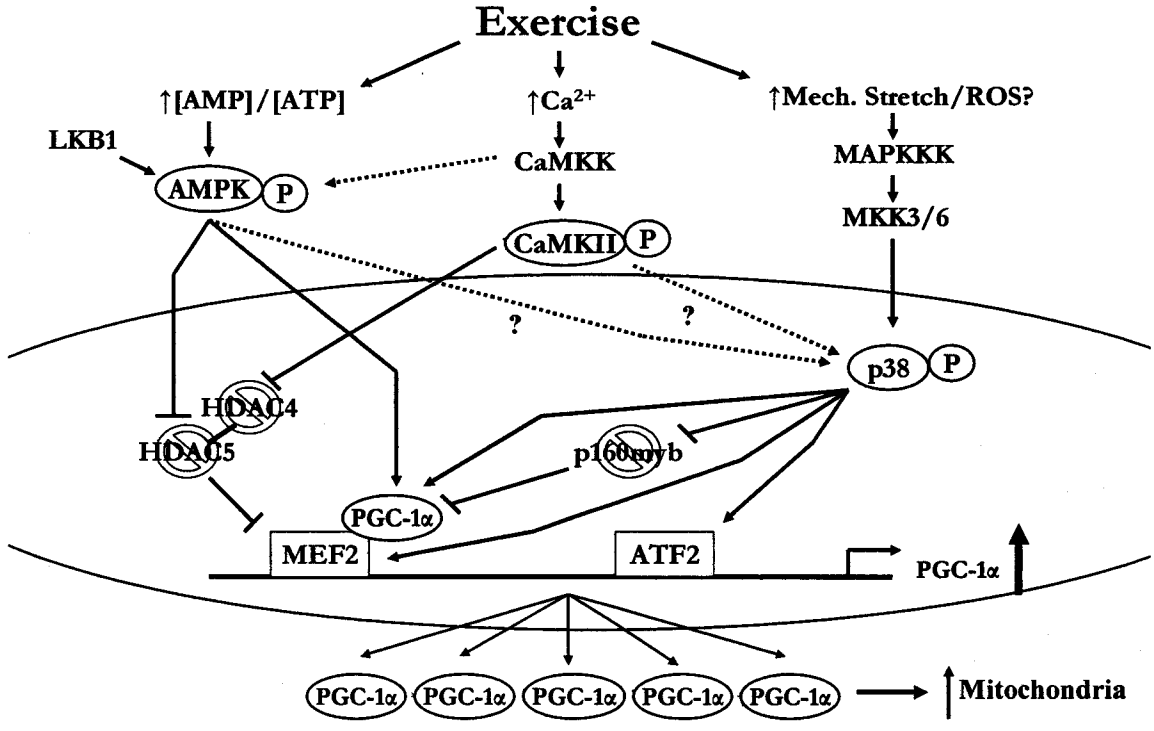

Figure 1.2 – Schematic overview of the potential pathways leading to mitochondrial biogenesis through the PGC-1α transcriptional coactivator. Solid arrows indicate activation or resulting products of activation. Blocked lines indicate inhibition. Circled text indicates proteins of interest, while circled "P" indicates phosphorylation. Boxed text indicates transcription factors. Text overlaying crossed circles indicate inhibitor proteins. Dashed arrows indicate potential interactions between pathways.

1.3.2 PGC-1α & Mitochondrial Biogenesis

Peroxisome proliferator-activated receptor γ coactivator-1α is a transcriptional coactivator expressed predominately in highly oxidative tissues such as brown adipose tissue, heart, kidney, brain, and skeletal muscle (67). As such, PGC-1α is believed to play a primary role in the stimulation of oxidative metabolism, and mitochondrial biogenesis (67). This is supported by the findings that PGC-1α is expressed to a higher degree in

more oxidative muscle fibres, and when overexpressed, can induce fibre type conversion towards a more oxidative phenotype (113). Furthermore, ectopic expression of PGC-1α in adipocytes and skeletal muscle cells results in enhanced markers of oxidative metabolism, including COXII, COXIV, ATP synthase, and cytochrome c (144, 206). Intriguingly, COXII is a mitochondrially-encoded subunit, and PGC-1α overexpression results in an approximate doubling of mitochondrial DNA content, indicating that PGC-1α must co-ordinately induce effects on both the nuclear and mitochondrial genome to augment mitochondrial biogenesis (144).

PGC-1α is a transcriptional coactivator. Thus, its enhancement of mRNA production is independent of direct DNA binding (143). Rather, PGC-1α docks on to specific transcriptional factors (TFs), enhancing their transcriptional activity upon binding to a response elements in a target gene's promoter (143). NRF1 and NRF2 appear to be two critical TFs in this process, as these transactivate many oxidative genes including cytochrome c, and subunits of succinate dehydrogenase, COX, and ATP synthase (166). Furthermore, NRF1 and 2 each transactivate the promoters of Tfam, and mitochondrial transcription specificity factors TFB1M and TFB2M, critical components in mtDNA replication, maintenance, and mitochondrial proliferation (61, 206). This NRF-mediated mechanism may explain PGC-1α's coordinate effects on both nuclear and mitochondrial genomes, as expression of a dominant-negative NRF1 completely ablates ectopic PGC-1α-mediated Tfam promoter activity and subsequent mitochondrial biogenesis in myotubes (206). Further evidence for the importance of PGC-1α/NRF1/Tfam pathway is offered by the finding that ectopic expression of PGC-1α enhances the expression of

NRFs 1 and 2, as well as Tfam, while homozygous knockouts of either NRF1 or Tfam result in completely ablated oxidative metabolism, and embryonic lethality (81, 109).

MEF2 is a critical mediator in PGC-1α-mediated mitochondria biogenesis as alluded to in previous sections. The PGC-1α promoter contains two MEF2 binding sites, both of which are necessary for PGC-1α gene transcription to occur, as mutation of either of these sites significantly blunts PGC-1α promoter activity in cardiac muscle (44). Furthermore, MEF2 itself is coactivated by the PGC-1α protein, resulting in the autoregulatory loop put forth by Handschin and colleagues (66). Thus, activation of PGC-1α by any of its upstream signals results in enhanced PGC-1α gene transcription as well the enhanced transcription of other target genes (66). MEF2 is inhibited by HDACs, and phosphorylation of HDAC proteins results in the formation of a binding site for a 14-3-3 chaperone protein targeting them for nuclear extrusion (123). This nuclear export relieves MEF2 inhibition, resulting in enhanced transcription at the target promoter. Thus, HDAC phosphorylation by the upstream signalling protein AMPK and CaMKII plays a significant role in enhancing PGC-1α expression and subsequent protein content (9, 122).

Other TFs directly coactivated by PGC-1α include estrogen-related receptor α (ERRα), and the family of peroxisome proliferator-activated receptors (PPARs). ERRα and the PPARs α and δ/β are primarily linked to fatty acid (FA) uptake and oxidation as they are most highly expressed in FA oxidative tissues such as heart, kidney, & skeletal muscle (125, 171). Specifically, ERRα has been shown to upregulate fatty acid translocase (FAT/CD36), acyl-CoA oxidase (ACO), medium-chain acyl-CoA

dehydrogenase (MCAD), and carnitine palmitoyltransferase 1 (CPT1). Recent evidence suggests that this upregulation requires PPARα, as PPARα knockout prevents ERRα-induced increases in MCAD, ACO, and CPT1 (83). ERRα has also been linked to other oxidative genes such as COXIV, cytochrome c, and ATP synthase (83, 167) indicating potential interaction with these promoters. It has recently been proposed that PGC-1α coactivates both ERRα and NRF2, which then upregulate their own promoters as well as each others' in a "double-positive-feedback loop" which occurs upstream of NRF1 (83, 128). This may provide a mechanism whereby ERRα may co-ordinately regulate both fat metabolism and electron transport genes. PPARδ/β is an emerging member of the mitochondrial biogeneration pathway, and appears to play overlapping roles with PPARα with regard to fat metabolism (130). Recently however, PPARδ/β has been implicated in a FA-mediated increase of PGC-1α protein and mitochondrial biogenesis (64), as well as transgenic and contraction mediated fibre-type switching (115). This will no doubt be an exciting source of future research, as PPARδ/β is the most highly expressed PPAR isoform in skeletal muscle, its expression is nearly three-fold higher in oxidative versus glycolytic muscles, and it is inducible in an activation frequency dependent manner (115).

PGC-1α is a highly-inducible protein, and its expression has been shown to be augmented by a variety of different factors in a tissue-specific manner. PGC-1α was originally shown to be a cold-inducible factor in adaptive thermogenic tissues such as brown adipose tissue, and skeletal muscle (144), but has since been shown to coactivate various gluconeogenic, and ketogenic genes in the liver in response to fasting (149). Exercise has also been identified as a potent activator of PGC-1α expression in both

rodents (3, 8) and humans (140, 161). Similarly, while exercise has been shown to increase the expression of PGC-1α, increased PGC-1α appears to facilitate greater exercise capacity. In support of this, Calvo and associates (26) recently exposed muscle-specific PGC-1α overexpressing mice to both voluntary and forced exercise conditions. PGC-1α overexpressing mice showed significantly greater exercise capacity than the wild-type mice performing 38% longer during progressive endurance testing, and nearly 45% longer during a VO_{2peak} challenge, reaching speeds 24% and 34% higher than the WT mice during the respective tests (26). Coupled to these results were a 24% greater VO_{2peak}, enhanced expression of various fat metabolism and OXPHOS genes, and significantly greater ability to oxidize fatty acids during exercise challenges, as reflected by respiratory exchange ratio (RER) (26). Conversely, muscle-specific PGC-1α knock-out mice show exercise intolerance, and fibre shifting away from an oxidative phenotype (65). Taken together, these results indicate that PGC-1α plays an important role in skeletal muscle mitochondrial biogenesis, fibre type, and exercise capacity.

Exercise training has been shown to increase PGC-1α protein content and expression within rodent (185) and human (18, 140, 160) skeletal muscle. Russell and colleagues (160) showed that, following 6 weeks of endurance training, PGC-1α expression was significantly elevated, as was downstream expression of metabolic genes such as COXIV, PPARα, GLUT4, and CPT1. PGC-1α protein content also increased in all fibre types in response to ET, with the greatest protein induction occurring in Type IIa fibres (160). These increases in PGC-1α were associated with a shift towards a more oxidative fibre type (160), a finding supported by prior overexpression experiments that

induced fibre type conversion (113). Furthermore, recent evaluation of PGC-1α expression from trained, normally active, and spinal cord injured individuals revealed that PGC-1α expression was 1.9-fold greater in the endurance cyclists than normal individuals, while spinal cord-injured subjects' expression was only 26% of normal (104). This expression was correlated with type I fibre percentage ($r=0.78$), indicating not only that PGC-1α may play a role in slow fibre phenotype, but also that these effects may be governed by activity levels (104). This was further supported by Pilegaard *et al.* (140) who showed that unilateral leg extensor training for 4 weeks resulted in greater basal and exercise-induced PGC-1α transcription, along with greater basal expression of CS, and βHAD. Furthermore, these increases were associated with a performance increases in the trained leg that were 2-fold greater than the untrained leg during an exercise time-to-exhaustion test (140). Burgomaster and colleagues (18) also showed that 6 weeks of SIT or ET alike could induce significant increases in PGC-1α protein content, CS and β-HAD activities, pyruvate dehydrogenase content, and glycogen sparing in response to a similar absolute workload. Taken together with the results of Calvo *et al.* (26), it is clear that PGC-1α plays a critical role in skeletal muscle oxidative and exercise capacity.

The results of the above studies clearly elucidate PGC-1α function in mitochondrial biogenesis, oxidative capacity, fibre type transition, substrate uptake, and exercise capacity in human skeletal muscle. Thus, it is of great interest to discover methods of augmenting PGC-1α expression and protein content for both clinical and athletic pursuits. It has now been shown that exercise training is one important method of inducing this augmentation (140, 160), however, it is important to pursue methods by

which these effects may be further increased, optimized, and perhaps synergized. Recent evidence suggests that nutrient manipulation may be one such option.

1.4 NUTRIENT MANIPUATION AND GENE REGULATION

1.4.1 Carbohydrate Availability and Exercise

Carbohydrate (CHO) has long been recognized as a critical substrate for skeletal muscle energy provision and exercise performance. The reliance upon CHO for ATP provision during exercise is mediated in an intensity and duration-dependent fashion, and is modified by factors such as training status, gender, and substrate concentrations (48). Specifically, CHO reliance at exercise onset increases exponentially as exercise intensity is escalated, contributing <20% total energy to exercise at intensities less than 25% $VO_{2peak,}$ but increasing its contribution to 60-70% of total energy provision at 60-65% $VO_{2peak,}$ and greater than 70% of total energy above 75% VO_{2peak} (87, 153, 190, 191). Within this CHO fraction, plasma glucose plays the predominant role at lower intensities, with muscle glycogen becoming increasingly more critical as exercise intensifies, contributing ~80% or more of the total CHO-derived energy above 60% VO_{2peak} (87, 153, 190). In contrast to CHO, fat oxidation follows an inverse hyperbole pattern, reaching maximal fat oxidation rates at approximately 48% VO_{2peak} for untrained individuals, and 65% VO_{2peak} for trained individuals before decreasing thereafter (191). This roughly coincides with the intensity at which CHO becomes the dominant exercise fuel source (191). Thus, CHO is critical to exercise eliciting greater than 50-65% VO_{2peak} for extended periods of time.

Exercise duration alters substrate oxidation such that sustained exercise below 60% VO_{2peak} results in a progressive decrease in CHO-derived energy, and progressive increase in fat oxidation (153, 195). This decreased CHO oxidation appears secondary to reduced muscle glycogenolysis and pyruvate dehydrogenase (PDH) activation, while plasma glucose oxidation is maintained (153, 195, 196). This decrease in CHO oxidation is compensated for primarily by fatty acids which become progressively more available to the working muscle over time (195). In fact, during prolonged, moderate-intensity exercise, fat may become the major energy provider following 120 minutes of exercise (195). However, as maximal fat oxidation rates appear to occur at approximately 50-65% VO_{2peak} (179, 191), and can provide only 50-60% of total energy required for exercise at this intensity (153, 190), fatigue is often associated with CHO depletion (42, 87).

The CHO-dependence of human skeletal muscle for performance at high intensities for prolonged durations may be altered by exercise training. Specifically, ET and HIT alike have been shown to enhance fat oxidation and co-ordinately reduce CHO reliance at the same absolute exercise intensity following training (17, 18, 28, 110, 181, 184). Furthermore, exercise training appears to augment the intensity that fat may play a majority role in energy provision, as trained cyclists may utilize fat in equal proportions to CHO at 60-65% VO_{2peak} (153, 190), whereas in untrained individuals, CHO becomes the dominant exercise fuel at approximately 48% VO_{2peak} (191). This metabolic adaptation appears to serve in sparing endogenous CHO stores, potentially to allow improved maintenance of intensity and prevent fatigue (99, 147, 183). However, exercise training alone is not sufficient to completely circumvent the importance of CHO to high-

intensity work and thus has prompted a variety of diet-induced interventions by athletes in efforts to maximize their CHO and work efficiency.

1.4.2 Carbohydrate Manipulation and Exercise Performance

Substrate oxidation may be altered in a variety of ways during exercise, including pre- and during-event feeding strategies. These strategies are designed to prevent fatigue, as CHO depletion in the form of muscle glycogen and/or hypoglycaemia have been related to reduced time to fatigue in a number of exercise contexts (11, 42, 118, 183, 193).

Carbohydrate loading refers to the practice of consuming high amounts of CHO and reducing physical activity in an effort to increase muscle glycogen storage prior to an endurance event. This practice has been shown to enhance exercise performance in the form of time to fatigue (183, 193), timed events lasting longer than 60-90 minutes (99, 147), and high-intensity intermittent exercise (11, 89, 151) though others have shown no such benefit (24). The mechanism is tied to greater endogenous stores of muscle glycogen, the most critical fuel for high-intensity exercise lasting greater than a few minutes. Interestingly, increasing muscle glycogen stores results in a faster rate of glycogenolysis (6), which has resulted, in some cases, in similar post-exercise concentrations despite initiating exercise with differing levels of muscle glycogen (147). This has lead to the proposal that muscle glycogen may play a critical role in pace-setting during a race (147), though further investigation is required.

CHO feeding during exercise also increases time to fatigue (10, 38, 42, 45) and time trial performance in events lasting 1 hr or greater (5, 92). The beneficial effects of

CHO ingestion are primarily attributed to the maintenance of euglycaemia (38, 42) and the sparing of liver glycogen (93), although some have shown muscle glycogen sparing (175, 187, 188), or have cited central mechanisms of fatigue prevention (52, 147). Thus, due to their individual effects on endurance performance, both CHO loading and ingestion during exercise have become commonplace for highly-trained and recreational athletes alike, and more commonly, are used in conjunction in efforts of maximising work output.

In general, a greater endogenous or exogenous pool of CHO to feed upon results in greater CHO oxidation during a subsequent exercise bout (6, 43, 194). Conversely, a chronic low CHO diet rich in fat or providing high amounts of fat pre-exercise can result in higher fat oxidation and less CHO usage during exercise (21, 23, 70, 176). This has prompted the investigation of so-called "fat adaptation" diets whereby athletes consume little CHO and large amounts of fat over the course of several days pre-event, in order to enhance their fat oxidation and spare their endogenous CHO stores for improved performance. However, recent research indicates that this CHO-sparing effect may in fact, be an inhibitory effect on CHO metabolism through the suppression of PDH activity (176). Furthermore, performance does not seem to be improved by fat adaptation (21, 23) and may even hinder performance in some cases (69). Thus, the potential inhibitory effects of fat adaptation coupled with the potential performance-enhancing effects of both CHO loading and supplementation has resulted in the suggestion of a high-CHO diet at all times for athletes. This diet is prescribed in hopes that it will allow athletes to "train harder or for longer periods" of time, resulting in greater performance adaptation (20, 22).

There is some recent evidence however, that suggests reduced CHO availability during exercise may not be entirely negative.

1.4.3 Carbohydrate Availability and Metabolic Gene Regulation

Carbohydrate availability has recently been implicated in modulation of gene expression in skeletal muscle. One of the first direct investigations of CHO availability and gene regulation in human skeletal muscle came from Keller and colleagues (102), who showed that 3h of leg-kicking in a low-muscle glycogen versus normal glycogen state resulted in significantly greater exercise-induced transcription of the interleukin-6 (IL-6) gene. Soon thereafter, Febbraio and colleagues (53) showed that reduced muscle glycogen significantly augmented the exercise-induced increase in heat shock protein 72 (HSP72) mRNA and protein following 4-5 hours of prolonged, leg-extensor exercise, indicating that CHO could mediate HSP72 production (53). Reduced CHO availability has since been associated with increased, or sustained expression of numerous metabolic genes within animal and human skeletal muscle, including pyruvate dehydrogenase kinase 4 (PDK4), uncoupling protein 3 (UCP3), fatty acid translocase (FAT/CD36), hexokinase II (HKII), and GLUT4 (7, 32, 34, 35, 59, 137, 139, 174). Conversely, a recent microarray analysis of 316 skeletal muscle genes showed that acute hyperglycaemia resulted in the downregulation of more than 84% of the genes examined (124). These findings reinforce CHO as a significant modulator in skeletal muscle gene expression, and imply a potential role in mediating the adaptive response to endurance training. This hypothesis is further supported by Pilegaard *et al.* (139), who examined the effect of 24-hour CHO restriction following 75 minutes of cycling at 75% VO_{2peak}. These

researchers showed that CHO restriction resulted in the sustainment of exercise-induced PGC-1α expression through 8 hours post-exercise, whereas CHO feeding returned PGC-1α expression to baseline levels within 8 hours. This was the first direct indication that perhaps there may be some benefit to CHO restriction with regards to exercise-mediated mitochondrial biogenesis. However, the direct upstream mediators of this PGC-1α effect were not speculated upon.

1.4.4 Carbohydrate Availability and PGC-1α-directed Protein Signalling

Reduced CHO availability, primarily in the form of muscle glycogen, is associated with enhanced protein signalling in skeletal muscle. Specifically, AMPK possesses a glycogen-binding domain in its β subunit (141), and contraction- and AICAR-induced AMPK activity have each been inversely correlated with muscle glycogen concentrations (47, 199). This effect appears to be mediated not only by β subunit-glycogen binding, but also via the γ3 subunit, as knockout experiments render the heterotrimer unresponsive to glycogen status (12). Many studies have implicated reduced CHO availability with AMPK activity in both fast and slow-twitch muscle in-situ (47, 199) as well as augmenting AMPK activation in human skeletal muscle both at rest, and in response to exercise (200). Similarly, exercise-induced AMPK signalling has been shown to be attenuated by CHO ingestion during exercise (1), though others have demonstrated no such effect (111). Most interestingly, it has recently been shown that initiating 60 minutes of cycling at 70% VO_{2peak} in a low muscle glycogen versus normal muscle glycogen state resulted in greater increases in AMPKα2 activity, nuclear translocation, and GLUT4 gene expression (174). This indicates that manipulating

muscle glycogen or CHO availability may potentially augment PGC-1α expression and mitochondrial biogenesis via AMPK-MEF2 mechanisms (88, 122).

Investigations into the effect of CHO availability on CaMKII and p38 MAPK are less prevalent, though there appears to be some evidence of potential modulation. There have been no direct investigations of CHO availability on CaMKII activity, however recent investigations have shown that reduced muscle glycogen may lead to reduced Ca^{2+}ATPase activity, Ca^{2+} release, and Ca^{2+} reuptake into the SR during exercise (50). Though this effect appears to be unaffected by CHO supplementation (49), CHO availability may alter the kinetics of SERCA-mediated, exercise-induced Ca^{2+} transients. This would alter the activation of secondary messengers such as CaMKII, and potentially modify its potential effects on PGC-1α expression. Similar to CHO-CaMKII investigations, there are few studies directly investigating CHO availability on p38 phosphorylation. However, one recent study showed that when subjects exercised for 60 minutes @ 70% VO_{2peak} with reduced muscle glycogen, nuclear p38 phosphorylation was significantly elevated both at rest, and following exercise in comparison to the normal glycogen condition (29). There was also a trend for an exercise-induced increase in cytosolic p38 phosphorylation in the low glycogen condition only ($p=0.09$) (29). These results indicate that p38 MAPK activity may be augmented by reduced CHO availability, and thus may alter mitochondrial biogenesis via its modulation of PGC-1α. Furthermore, exercise-induced IL-6 gene expression, a downstream effect of p38 activation (29), has been shown to be significantly blunted by CHO ingestion (101). This lends further support for CHO-modulation of gene expression, PGC-1α activity, and potential

mitochondrial biogenesis. Interestingly, slow Ca^{2+} transients have also been linked to enhanced IL-6 mRNA production (98), potentially linking reduced CHO availability, SR alterations, and p38 activities, thus reinforcing the proposed upstream regulation of p38 by Ca^{2+} and CaMKII (203). Taken together, these findings indicate that CHO restriction may play a role in modulating AMPK, Ca^{2+} (and therefore CaMKII), and p38 activity, three signalling proteins implicated as important mediators of mitochondrial biogenesis in human skeletal muscle. Consequently, CHO-restricted exercise may hold potential for augmenting ET and HIT-induced training adaptation.

1.4.5 Carbohydrate-Restricted Endurance Training

Current exercise dogma prescribes the maintenance of a chronic, high CHO diet for the maintenance of training intensity, and the optimization of training adaptation and performance (20, 22). Few studies however, have directly assessed the effects of CHO availability on the muscles' adaptive response to endurance training. Lamb *et al.* (108) investigated the effects of high versus moderate CHO intake during 9 days of intense swim training in collegiate-level swimmers and found that there were no effects of diet on exercise performance at a variety of swim distances. Similarly, the same group tested runners and cyclists over the course of 7 days of training on high or moderate CHO diets and found, despite a significant reduction in muscle glycogen content over the course of training in the moderate-CHO-fed group, there was no difference between groups in ability to complete training sessions, or in a time-to-fatigue performance test post-training (169). Perhaps the best assessment of CHO availability on training came from Simonsen and colleagues (170) who assessed power output during 4 weeks of rowing training in

collegiate-level rowers randomized to high or moderate-CHO diets. Over the course of training, high-CHO fed rowers increased their muscle glycogen stores by approximately 65% whereas the moderate-CHO group maintained muscle glycogen storage (170). In line with this, high-CHO athletes increased their mean training power output by ~10.7% over the course of training, whereas the moderate-CHO group improved a mere 1.6%, leading the researchers to conclude that high CHO feeding during training improved rowing training (170). Therefore, while high CHO would appear to improve training power output, there was no direct measure of performance following training, and very few studies have examined the effects of training with low CHO availability.

Hansen and colleagues (68) were the first to directly assess the potential of low-CHO training. Using a unilateral leg-kicking exercise model, subjects trained for 10 weeks using a schedule designed to alter intramuscular glycogen stores. This study showed that a "low glycogen"-trained leg accrued greater increases in CS maximal activity, and resting muscle glycogen content following training in comparison to the "high-glycogen" trained leg (68). Furthermore, and most startling, was that the low-glycogen leg show a near 2-fold performance improvement over the high-glycogen leg after training in a time-to-exhaustion leg-kicking performance test (68). However, this study was limited by the fact that the legs trained on differing schedules in that the low-glycogen leg trained 2x/day every second day while the high-glycogen leg trained once daily, introducing the possibility that differing adaptations were the result of training schedule, and not muscle glycogen *per se*. Secondly, the researchers did not directly measure muscle glycogen in the "high-glycogen" leg over training bouts to see if, in fact,

glycogen was being resynthesized over the intervening 24h between training sessions. Thus, glycogen may have been progressively decreased over training such that there was no difference in glycogen concentration throughout training making adaptation CHO-independent. Lastly, the use of leg-kicking exercise does not necessarily translate to other athletic contexts, leaving these findings, while intriguing, limited in their application to sporting events. In a more recent publication analysing the same muscle samples however, it was reported that PGC-1α expression both pre-exercise, and 2h following a leg-kicking bout was increased only in the twice-daily trained leg. However, strong trends for a similar pattern were observed in the once-daily trained leg existed (2h post: p=0.074) (68) indicating that further investigation into low-CHO training is required.

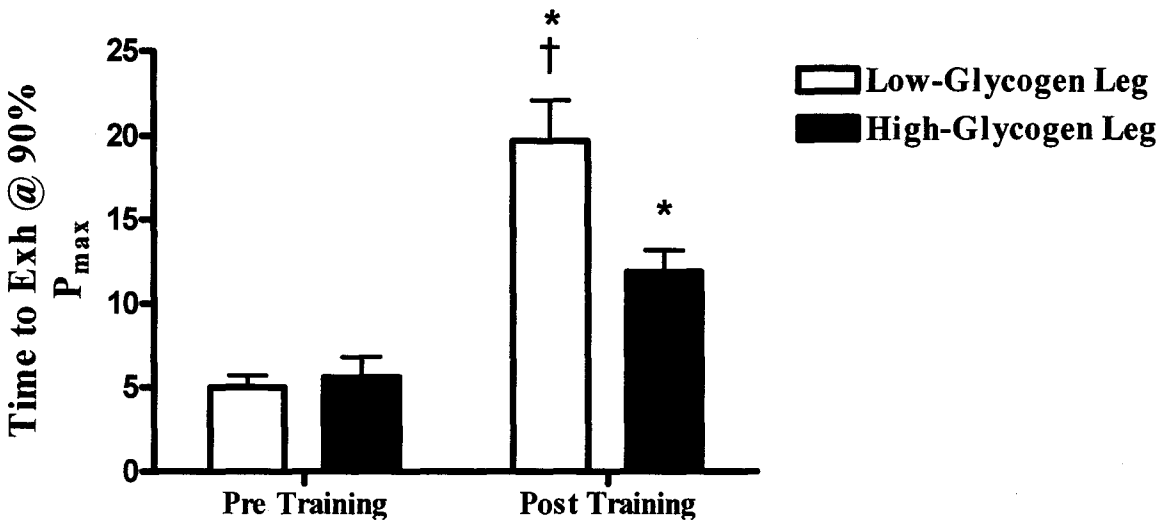

Figure 1.3 – Summary of performance results from Hansen and colleagues (68). Subjects performed leg-kicking exercise to exhaustion at 90% pre-training maximal power output (P_{max}) following 10 weeks of unilateral leg-kicking training. The low glycogen-trained leg trained twice/day every other day, while the high glycogen-trained leg trained once daily. Training volume was matched between legs.

1.5 SUMMARY

It is well known that CHO availability plays a critical role in exercise performance, as its usage often dictates exercise performance and fatigue in high-intensity endurance events. These findings have led many highly competitive and recreational athletes alike to utilize a variety of CHO-manipulating measures such as CHO-loading, CHO ingestion during exercise, and fat adaptation in order to maximize or spare their endogenous CHO storage and optimize their performance. It is for this reason, as well as likely due to the findings of Simonsen and colleagues (170), that a chronic high-CHO diet is prescribed to athletes during training by world-renowned sports nutritionists such as Louise Burke as "there is no evidence that a low carbohydrate diet enhances the outcomes of training" (20). Recent research however, has implicated CHO restriction in skeletal muscle plasticity and response to exercise. It has been shown that reduced CHO availability may enhance metabolic gene expression and protein signalling associated with enhanced mitochondrial biogenesis. Furthermore, CHO availability has been linked to enhanced training adaptation, even though this investigation contained limitations. Thus, these potentially beneficial effects of CHO restriction on metabolic gene regulation, signalling, and adaptation, as well as the proposed interaction of said signalling molecules clearly warrants further investigation into CHO availability and protein signalling.

The primary purpose of the present thesis was to investigate the effects of reduced CHO availability on the acute signalling response of human skeletal muscle to high-intensity interval exercise. It was our secondary goal to devise an experimental protocol whereby CHO availability could be manipulated without the potential confounding

influences of acute fat adaptation and using a training model that simulated normal athletic training. We hypothesized that reduced CHO availability would result in greater phosphorylation of protein signalling molecules associated with mitochondrial biogenesis within the skeletal muscle.

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CHAPTER 2: EFFECTS OF CARBOHYDRATE AVAILABILITY ON THE ACUTE RESPONSE OF HUMAN SKELETAL MUSCLE TO HIGH-INTENSITY INTERVAL EXERCISE

2.1 INTRODUCTION:

Carbohydrate (CHO) has long been known to be a critical substrate for skeletal muscle contraction that directly influences exercise capacity. This knowledge has led to a variety of nutrient manipulation strategies such as CHO loading (62), CHO ingestion during exercise (27, 29), and fat adaptation (66, 67), which are all designed to augment or spare endogenous CHO stores and enhance athletic performance. Furthermore, current training dogma states that athletes should chronically consume a high CHO diet in order to maximize training intensity and duration, thus maximizing subsequent adaptations (14, 15). However, a line of recent evidence indicates that exercising with reduced CHO availability may not necessarily be detrimental to skeletal muscular exercise-adaptation. It has been shown that restricting CHO availability during or following exercise can enhance the transcription of a number of metabolic genes including pyruvate dehydrogenase kinase 4, uncoupling protein 3, fatty acid translocase, hexokinase II, and glucose transporter isoform 4 (GLUT4) (4, 22, 24, 26, 42, 89, 90, 106). It has also recently been shown that acute hyperglycaemia downregulates greater than 80% of 316 examined skeletal muscle genes, further implicating a role for CHO availability in metabolic gene expression (77). Interestingly, Pilegaard *et al.* (90) showed that restricting CHO following a prolonged bout of cycling exercise in untrained subjects sustained the exercise-induced increase in peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α) expression for a greater length of time than when the same

subjects were fed a high-CHO diet. This would indicate that CHO availability may play a significant mediating role in intramuscular PGC-1α expression in the time following exercise.

PGC-1α is a transcriptional coactivator linked to mitochondrial biogenesis in skeletal muscle and many other tissues (46) and is believed to play a critical role in training-induced phenotypic adaptation (13, 55, 91, 99). Furthermore, muscle-specific overexpression of PGC-1α has been shown to significantly increase exercise capacity (16), fast-to-slow fibre-type transition (70), fatty acid oxidation and insulin sensitivity (7). Conversely, PGC-1α knock-out animals display exercise intolerance, fibre shifting away from the oxidative phenotype (45), and reduced PGC-1α content is associated with insulin-resistance and type II diabetes mellitus (79, 88). Thus, PGC-1α appears to be a critical modulator of oxidative metabolism, mitochondrial biogenesis, performance, and disease, and discovering methods of augmenting PGC-1α expression is of utmost importance to both athletic and clinical communities, alike. The upstream regulators of PGC-1α however, are not well defined. Current research implicates a number of protein signalling molecules in the role of PGC-1α mediation, including adenosine monophosphate-activated protein kinase (AMPK) (8, 59, 61, 142), p38 mitogen-activated protein kinase (p38) (2, 3, 54, 134, 137) and calcium/calmodulin-dependent protein kinase isoenzyme II (CaMKII) (23, 81, 83, 133, 134). Interestingly, CHO restriction has also been associated with enhanced activation/phosphorylation of these protein signals directed towards PGC-1α, as reduced muscle glycogen has been linked to enhanced basal, and exercise-induced AMPK and p38 activity (20, 106, 130), as well as reduced calcium

adenosine triphosphatase activity and calcium reuptake during exercise (36). This latter effect may alter the kinetics of exercise-elicited calcium transients and thus, CaMKII activity in response to exercise. Taken together, this indicates that CHO availability may alter the activity of PGC-1α-linked protein signalling, potentially augmenting exercise-induced PGC-1α expression.

Renewed interest in the potential for reduced CHO to augment training-induced gains is also attributable to a recent study by Hansen and associates (47). These authors showed that 10 weeks of leg-kicking training with reduced muscle glycogen in one leg and presumably, normal glycogen levels in the other, induced greater increases in citrate synthase maximal activity, resting muscle glycogen storage, and endurance capacity in the low glycogen leg. In fact, post-training time to exhaustion in the low glycogen-trained leg was nearly double that of the high-trained leg (47). Subsequent analyses revealed that only the "low-glycogen" trained leg had a statistically significant training-induced increase in PGC-1α expression at baseline, as well as 2 hours into recovery from exercise, though there were no significant differences between legs (80). This would indicate that perhaps CHO restriction may induce a more stable increase in PGC-1α expression in response to training. However, these studies are confounded by the fact that the legs trained on differing schedules, introducing the possibility of training adaptations being mediated by schedule, rather than glycogen-content *per* se. Secondly, muscle glycogen was not measured directly in the "high" muscle glycogen-trained leg indicating that there may not have been a difference in CHO availability between legs at all. Thus,

further exploration into the so-called, "train low, compete high" model of CHO availability and training is warranted.

It was our purpose to investigate the effects of reduced CHO availability on the exercise-induced protein signalling responses in human skeletal muscle. We utilized a high-intensity interval exercise (HIE) protocol adapted from Talanian and colleagues (112) in an effort to alter CHO availability, while simultaneously optimizing the potential CaMKII, AMPK, and p38 activation. Furthermore, this protocol was designed to avoid the potential inhibitory effects of prolonged low-CHO, high fat feeding on CHO metabolism (107). We hypothesized that restricted CHO would augment the exercise-induced activation of signalling molecules associated with PGC-1α-mediated mitochondrial biogenesis, and thus, provide a potential explanation for the greater oxidative enzyme adaptation and performance improvements shown by Hansen and colleagues (47).

2.2 METHODS:

2.2.1 Subjects:

Ten healthy men (21 ± 1 years; 180 ± 2 cm; 78.1 ± 3.4 kg) were recruited to participate in the study. Subjects were habitually active 2-3 times per week, but were not specifically trained in any particular exercise modality. All subjects completed a physical activity readiness questionnaire (PAR-Q), medical screening questionnaire, and consent form prior to any exercise testing. This experimental protocol was approved by Hamilton Health Sciences/Faculty of Health Sciences Research Ethics Board.

2.2.2 Pre-Experimental Procedures:

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}) and peak 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). Subjects performed 2 min of resting breathing, following by 2 min of cycling at 50 W. Following this, cycling intensity increased at a rate of 1 W every 2 s until volitional fatigue occurred, as defined as a pedalling cadence <50 rpms. VO_{2peak} was defined as the highest volume of oxygen utilized over a 15 s span and the mean ($\pm$ SE) value for the group was 51.0 $\pm$ 1.6 mL/kg/min. On a separate day, subjects underwent a high-intensity interval exercise familiarization protocol consisting of 5 x 4 min work bouts interspersed by 2 min rest. Cycling intensity was adjusted to elicit approximately 90% of heart rate reserve (%HRR) over the course of the 5 intervals, which corresponded to approximately 60-65% W_{peak}.

2.2.3 Experimental Protocol:

All experimental procedures were completed in randomized and counterbalanced fashion and were separated from familiarization by >1 wk. Subjects were instructed to refrain from caffeine and alcohol for at least 12 and 24 h, respectively, prior to the experimental trials. Subjects arrived at the laboratory approximately 2 h postprandial, were weighed and submitted 24 h diet logs for repetition prior to the second experimental day. Following this, a Teflon catheter was inserted into an antecubital vein and a resting blood sample was obtained. A resting muscle biopsy was also taken from the lateral

portion of the vastus lateralis muscle under local anaesthesia (2% xylocaine). Following resting muscle and blood sampling, subjects moved to the electronically braked cycle ergometer and completed the first of two exercise sessions separated by 3 h (AM and PM sessions, respectively). Exercise sessions consisted of a 2 min warm-up at 50 W, followed by 5 x 4 min work bouts interspersed by 2 min rest periods. This protocol was designed to elicit an intensity corresponding to approximately 90% VO_{2peak}, as adapted from Talanian and colleagues (112). During the 3 h recovery period between sessions, subjects consumed either 1.2 g CHO/kg b.w./hr (HI-HI condition), or a taste-matched, non-energetic placebo (HI-LO condition). Specifically, subjects were provided with 500 mL of the designated beverage and told to finish it over the course of the next hour. At this time, a second 500 mL of the same CHO or placebo beverage was provided. Supplementary water was ingested *ad libitum* during the 2 h CHO or placebo feeding, and only water was ingested during the third hour of rest. Muscle biopsies were obtained immediately before and after each exercise session (AM Pre and Post, PM Pre and Post time points). Muscle samples were dissected free of visible connective tissue, immediately frozen in liquid nitrogen and stored at -80°C for subsequent analysis. Each biopsy was taken from a separate incision site made immediately prior to that muscle sample. AM biopsies were taken from the left leg, while PM biopsies were from the right leg. Blood samples were obtained pre and post each exercise session, as well as immediately following the first work interval of each session (AM Bout 1 and PM Bout 1, respectively). Heart rate monitors were worn throughout exercise for estimation of exercise intensity.

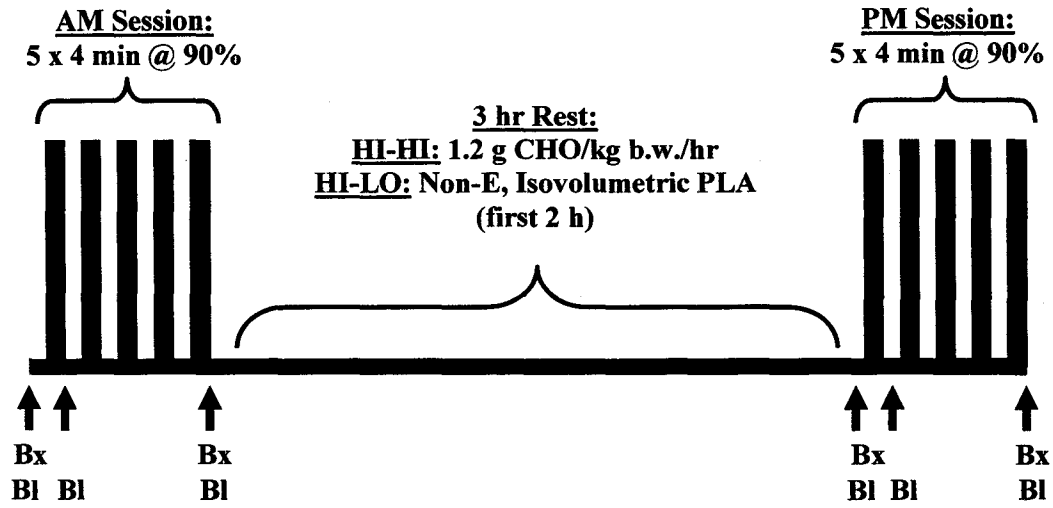


Figure 2.1 – Schematic of Study Design. Bx, Muscle Biopsy; Bl, Blood Sample.

2.2.4 Analyses:

2.2.4.1 Muscle Analyses:

Frozen wet muscle samples were sectioned under liquid nitrogen and stored at -80°C until further analysis. One portion (~30 mg w.w.) was freeze-dried, powdered, and dissected of all non-muscle elements for determination of muscle glycogen and muscle metabolite concentrations.

Muscle Glycogen:

One aliquot of freeze-dried muscle (~2 mg d.w.) was incubated in 2 N hydrochloric acid (HCl) and heated for 2 h at 100°C to reduce glycogen to glucosyl units. The solution was neutralized with 2 N sodium hydroxide (NaOH) and analyzed for glucose concentration via fluorometric assay (Hitachi F-2500 fluorescence spectrophotometer, Hitachi Instuments, Tokyo, Japan), as described by Passoneau and Lowry (87).

Muscle Metabolites:

A second portion of freeze-dried muscle was extracted on ice under 0.5 M perchloric acid (PCA) containing 1 mM ethylenediaminetetraacetic acid (EDTA), and neutralized with 2.2 M potassium bicarbonate ($KHCO_3$). Supernatent was stored at -80°C, and subsequently analyzed for muscle creatine (Cr), phosphocreatine (PCr), and adenosine triphosphate (ATP) concentrations (Hitachi F-2500, Hitachi Instruments, Tokyo, Japan) (87). All metabolites were corrected to highest within-subject total Cr concentration.

Western Blotting:

Activation of protein signalling molecules was assessed by phosphorylation status of the AMPK-Thr^{172}, p38-Thr^{180}/Tyr^{182}, and CaMKII-Thr^{286} residues. Frozen wet muscle samples (~30 mg w.w.) were added to ice-cold RIPA buffer (50 mM HCL, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 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°C. Samples were then centrifuged at 13,000 rpm for 5 min at 4°C, supernatent removed and stored at -80°C for further analysis. Protein concentrations of muscle homogenates were determined spectrophotometrically (Bio-Rad 500-0002, Hercules, CA, USA) by Bradford assay kit (Coomassie Plus, Thermo Fischer Scientific, Rockford, IL). No empirical SDS-interference was found to occur with the Bradford assay. Proteins were separated via 10% SDS-polyacrylamide gel electrophoresis for ~2h

at 110V (Mini Protean Tetra Cell, Bio-Rad Laboratories, Mississauga, ON, Canada). Protein was subsequently transferred to nitrocellulose at 100 V for 1 hr. Protein transfer and loading was verified via Ponceau staining technique (Ponceau S Solution, Sigma-Aldrich, St. Louis, MO, USA), and reversed using 0.1 M NaOH. Membranes were blocked for 1 h at room temperature using 5% non-fat milk or bovine serum albumin (BSA) in tris-buffered saline containing 0.1% Tween-20 (TBST). Membranes were subsequently incubated with monoclonal antibodies for anti-phospho Thr^{172}, Thr^{180}/Tyr^{182}, and Thr^{286} for AMPK, p38, and CaMKII, respectively, as well as total PGC-1α protein at 4°C, overnight (Cell Signalling Technology, Inc., Danvers, MA, USA). Primary antibodies were diluted in 3% non-fat milk in TBST at 1:2000, 1:3000, and 1:1000, for phospho-AMPK, phospho-p38, and total PGC-1α proteins, respectively, while phospho-CaMKII was incubated at a dilution of 1:1000 in 3% BSA. Blots were developed using anti-rabbit horseradish peroxidase-conjugated secondary antibody (NA934, Amersham Biosciences, Laval, PQ, Canada) diluted at a ratio of 1:5000 for 1 h at room temperature. Protein was visualised with an enhanced chemiluminescence kit (Supersignal® West Dura, Thermo Scientific, Rockford, IL, USA) and quantified via spot densitometry using Fluorochem® SP Imaging system and software (Alpha Innotech Corporation, San Leandro, CA, USA). All densitometry readings were corrected for loading against Ponceau, and interblot variability was accounted for by correction to a whole muscle homogenate control lane run on all blots.

2.2.4.2 Blood Analyses:

Blood was collected in 4 mL lithium heparinised, and no-additive tubes (BD Vacutainer®, BD Biosciences, Mississauga, ON, Canada) for plasma and serum derivation, respectively. Whole blood was immediately tested for glucose (Ascensia Contour®, Bayer Inc., Toronto, ON, Canada) and lactate (Accutrend®, Roche Diagnostics, Switzerland) concentrations and heparinised tubes were immediately centrifuged for 10 min at 3000 rpm. Plasma was collected and placed immediately on ice. No additive tubes were left to coagulate at room temperature for ~40 min, centrifuged for 10 min at 3000 rpm. Serum was collected and immediately placed on ice. Plasma and serum were stored at -20°C until later analysis. Serum insulin concentrations were subsequently analyzed via colourimetric immunoassay kit (ALPCO Diagnostics™, Salem, NH, USA) utilising an absorbance microplate reader (ELx808™, BioTek Instruments, Inc., Winooski, VT, USA).

2.2.4.3 Statistics:

All measures were analyzed using a 2-way repeated measures analysis of variance (ANOVA). Significant interaction and main effects were further analyzed using a Tukey HSD post hoc test. All analyses were carried out using statistical analysis software (SigmaStat® 3.1, Systat Software, Inc., Chicago, IL, USA) and values are presented as means $\pm$ standard error of the mean (SEM). Figures were generated utilising GraphPad Prism® 4.0 (GraphPad Software, La Jolla, CA, USA).

2.3 RESULTS

Cardiovascular Data:

There were no differences between conditions with regards to cardiovascular responses to HIE and thus, heart rate data is presented collapsed across condition. AM HIE elicited peak heart rate values of approximately 94±1% peak heart rate ($\%HR_{peak}$) and 91±1% heart rate reserve (%HRR), while PM sessions elicited approximately 96$\%HR_{peak}$ and 94±1%HRR. Each work interval induced an increase in $\%HR_{peak}$ and %HRR relative to its corresponding pre-bout measure ($p<0.001$), with a progressive upward drift such that Pre Bout 4 and Pre Bout 5 %HRRs were greater than the Pre Bout 1 %HRR within each of the respective AM and PM sessions.

Serum Insulin:

HIE induced a progressive reduction in serum insulin concentrations from AM Pre at both the AM Bout 1 and AM Post time points in both conditions, though this reduction only reached statistical significance at AM Post in the HI-LO condition (21±5 to 10±1 μIU/mL, $p<0.05$). However, when collapsed across condition, AM HIE resulted in lessened concentrations of 11±1 μIU/mL at AM Post, irrespective of condition (main effect: time, $p<0.05$). During PM sessions, serum insulin values were significantly elevated in the HI-HI versus HI-LO condition at PM pre (18±3 vs. 6±1 μIU/mL, $p<0.001$), and this difference was maintained through the first bout of HIE (PM Bout 1: 13±2 vs. 6±1 μIU/mL, $p<0.05$). This difference between conditions was abolished by PM Post, in part by a non-significant decrease in serum insulin concentration in the HI-HI condition.

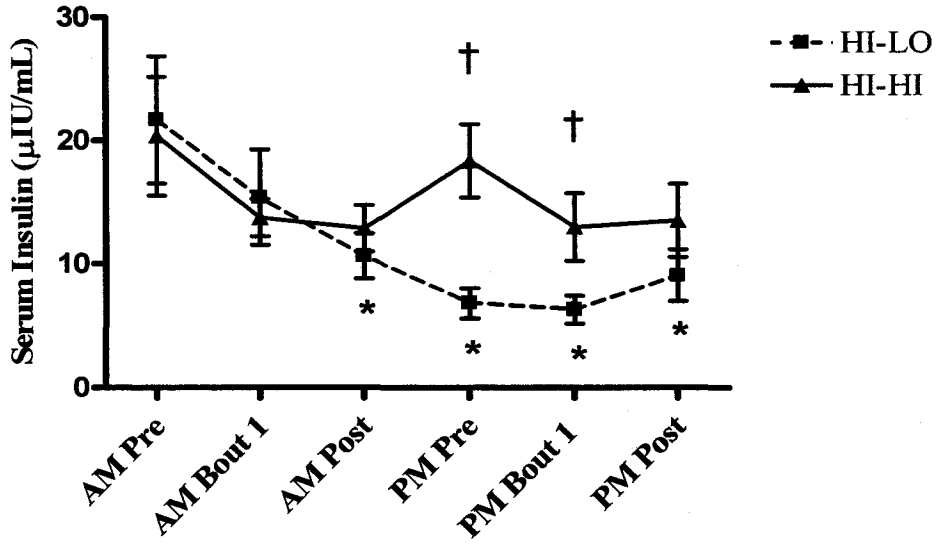


Figure 2.2 – Serum insulin concentrations pre-, following the first work interval, and post-AM and PM HIE sessions with high-CHO or non-energetic placebo ingestion between sessions. *Significantly different from AM Pre time point within condition ($p<0.05$); †Significant difference between conditions at designated time point ($p<0.05$).

Blood Glucose:

Blood glucose showed no change from resting levels throughout either the AM or PM sessions (mean: 4.7±0.1 mmol/L; Figure 2.2). However, a difference between HI-HI and HI-LO conditions did occur at the PM Pre, and PM Bout 1 time points such that the HI-LO condition had greater plasma glucose concentrations, relative to HI-HI (PM Pre: 4.9±0.1 vs. 4.2±0.3 mmol/L, and PM Bout 1: 4.9±0.2 vs. 4.1±0.3 mmol/L, respectively; $p<0.01$). Collapsed across condition, there was an elevation in blood glucose level at AM Post relative to AM Bout 1, indicating an exercise-induced increase in plasma glucose concentration during the latter AM session (5.2±0.2 vs. 4.6±0.1 mmol/L; $p<0.035$).

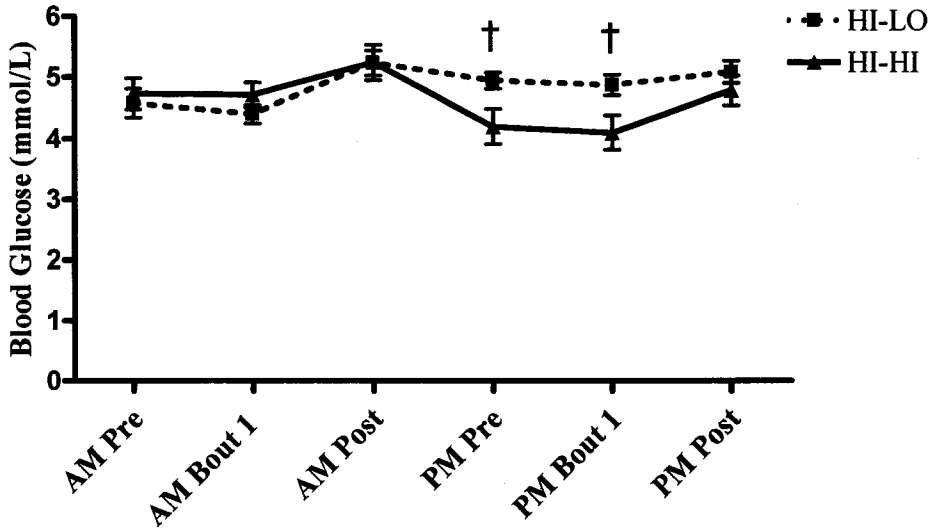


Figure 2.3 – Blood glucose concentrations pre-, following the first work interval, and post-AM and PM HIE sessions with high-CHO or non-energetic placebo ingestion between sessions. †Significant difference between conditions at designated time point ($p<0.01$).

Muscle Glycogen:

Both HIE sessions induced a decrease in muscle glycogen content pre- to post-exercise (Figure 2.4; AM Session: 424±22 to 291±26; PM Session: 305±17 to 203±18 mmol/kg d.w., $p<0.001$). Surprisingly, high CHO delivery between exercise sessions in the HI-HI condition did not result in muscle glycogen resynthesis, and thus, there were no between-condition differences in muscle glycogen content at any point throughout the experimental protocol.

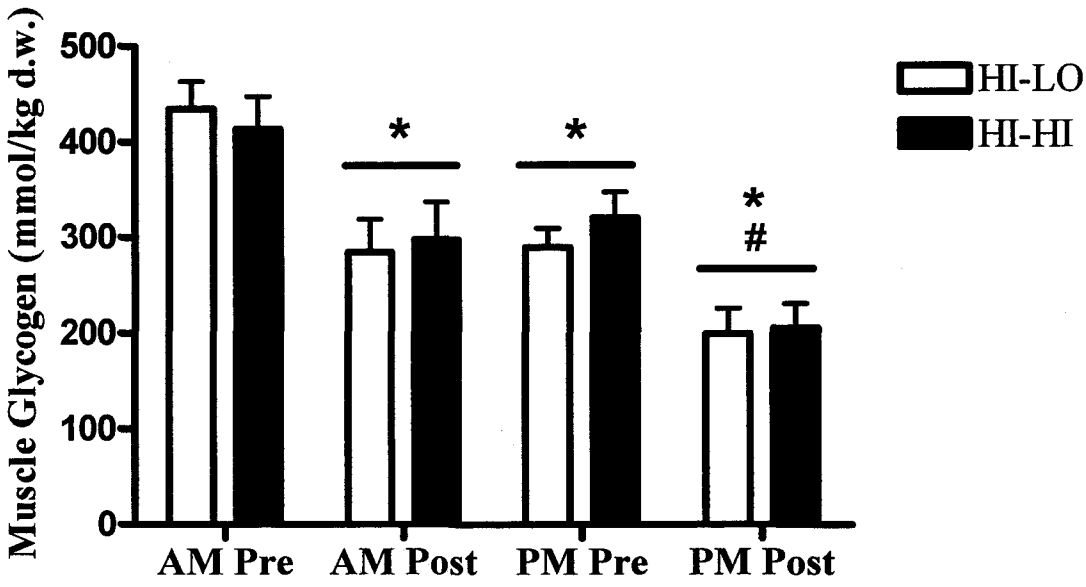


Figure 2.4 – Muscle Glycogen contents pre- and post-AM and PM HIE sessions with high-CHO or non-energetic placebo ingestion between sessions. *Significantly different from AM Pre time point within condition ($p<0.001$); #Significantly different from PM Pre within condition ($p<0.001$).

Energy Status:

With regards to ATP concentrations, there were no exercise-induced differences between AM Pre and AM Post in either the HI-HI, or HI-LO conditions. Interestingly, there was no effects of HIE on PCr concentrations pre to post AM exercise in either HI-HI or HI-LO. However, when taken together, there was a significant main effect for a PCr decrease AM Pre to AM Post (92±6 to 77±4 mmol/kg d.w.; $p=0.018$). Similar to the AM session, HIE induced no changes in either ATP or PCr concentrations in the HI-HI condition pre- to post-exercise. In contrast, the HI-LO condition showed a significant decrease in both ATP and PCr concentrations over the course of PM HIE, resulting in

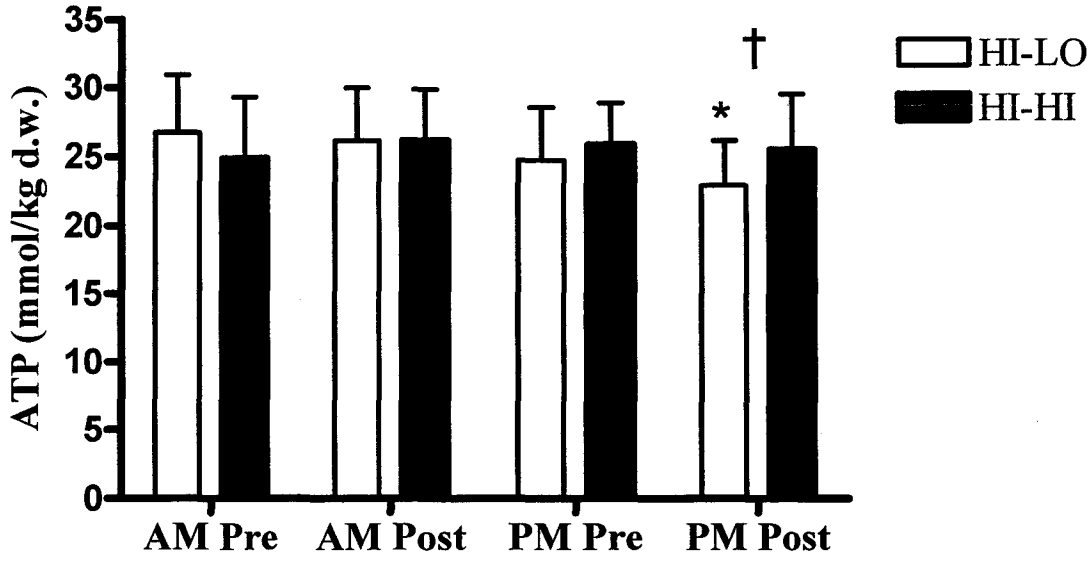
significant differences between conditions at the PM Post time point (Figure 2.5; Table 2.1).

Table 2.1 - Muscle Metabolite Concentrations (mmol/kg d.w.)

	HI-LO				HI-HI			
	AM Pre	**AM Post**	**PM Pre**	**PM Post**	**AM Pre**	**AM Post**	**PM Pre**	**PM Post**
ATP	26±1	26±1	24±1	23±1*†	24±1	26±0	25±1	25±1†
PCr	97±8	83±5†	87±5	61±5*#†	87±5	70±6†	96±4	81±7†

*Significantly different from AM Pre within condition (p<0.001); #Significantly different from PM Pre within condition (p<0.01); †Significant difference between conditions at designated time point (p<0.05)

A:



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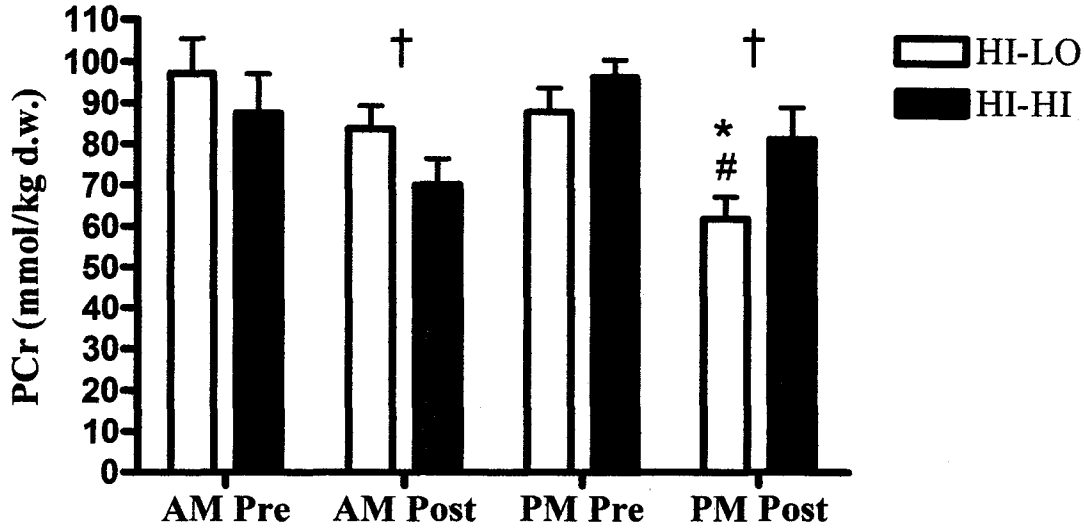

Figure 2.5 – Muscle ATP (A) and PCr (B) concentrations pre- and post-AM and PM HIE sessions with high-CHO or non-energetic placebo ingestion between sessions. *Significantly different from AM Pre time point within condition ($p<0.05$); #Significantly different from PM Pre within condition ($p<0.01$); †Significant difference between conditions at designated time point ($p<0.05$).

AMPK Phosphorylation:

AMPKα subunit phosphorylation showed a HIE-induced increase of greater than 50% AM Pre to AM Post, irrespective of condition (Figure 2.6; $p<0.01$). Surprisingly, while there was a trend for an increase in AMPK phosphorylation during the PM HIE session ($p=0.12$), no statistically significant increase was noted. There were no condition-specific effects to report with respect to AMPK phosphorylation.

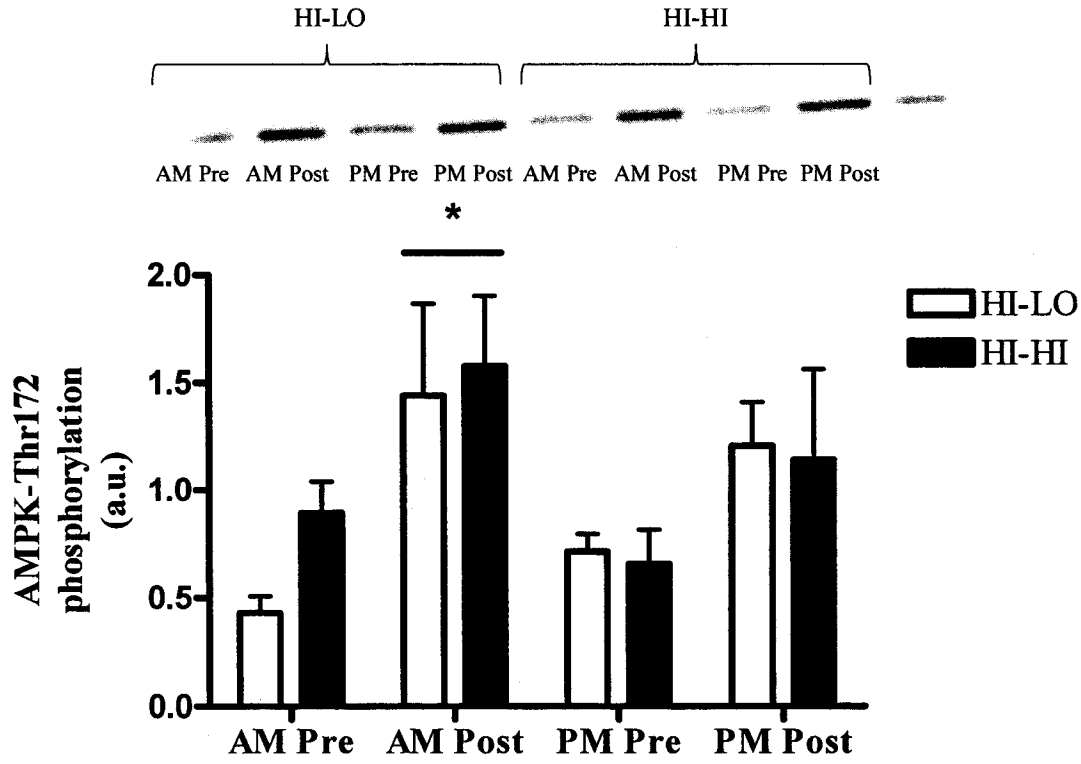

Figure 2.6 – α-AMPK phosphorylation pre- and post-AM and PM HIE sessions with high-CHO or non-energetic placebo ingestion between sessions. *Significantly different from AM Pre time point (main effect, p=0.004).

CaMKII Phosphorylation:

Both AM and PM HIE sessions induced an approximate 40% increase in CaMKII-Thr^{286} phosphorylation when collapsed across condition (AM session: 2.11±0.11 to 2.90±0.19 a.u.; PM session: 2.23±0.10 to 3.15±0.23 a.u.; $p<0.01$). There were no condition-related effects between the HI-HI and HI-LO protocols (Figure 2.7).

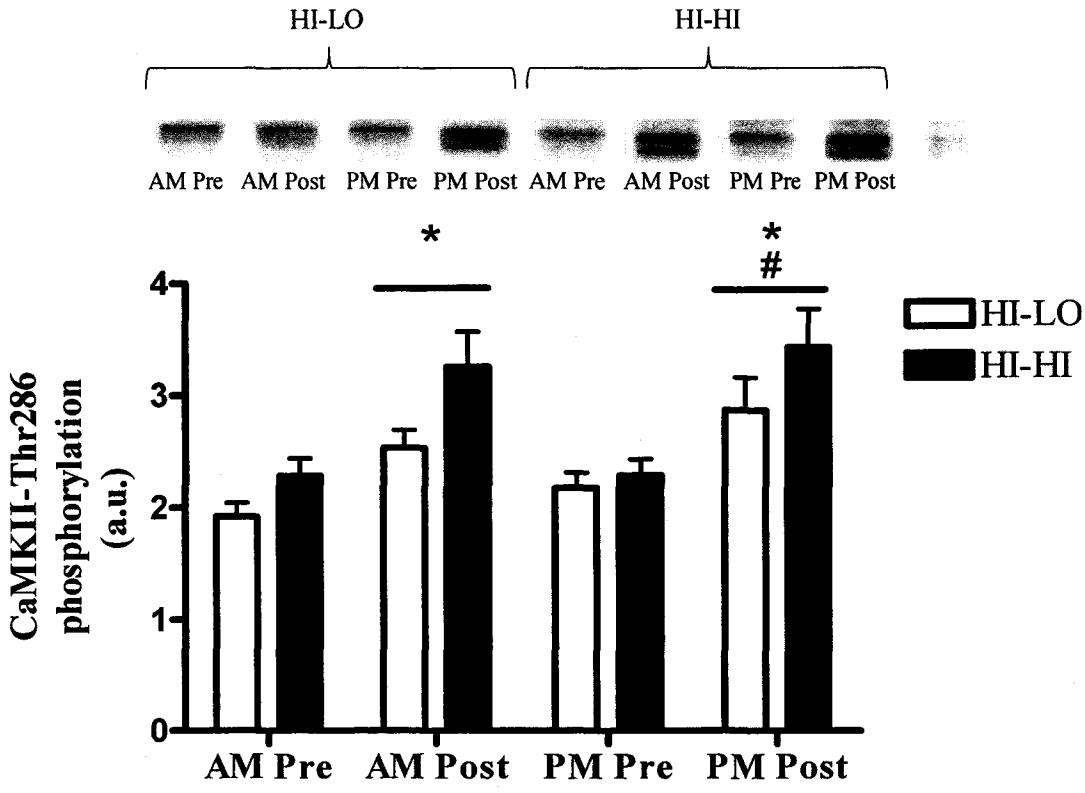

Figure 2.7 – CaMKII phosphorylation pre- and post-AM and PM HIE sessions with high-CHO or non-energetic placebo ingestion between sessions. *Significantly different from AM Pre time point (main effect, $p<0.01$); #Significantly different from PM Pre (main effect, $p<0.01$).

p38 MAPK Phosphorylation:

During AM HIE sessions, p38 phosphorylation status increased approximately 3-fold in each of the HI-LO and HI-HI conditions (HI-LO: 0.64±0.20 to 2.46±0.47 arbitrary units (a.u.); HI-HI: 1.07±0.19 to 3.26±0.65 a.u.; $p<0.001$) before returning to baseline levels during the 3 hour rest period between HIE sessions. During the PM HIE session, there was no exercise-induced increase in p38 phosphorylation when subjects ingested a high amount of CHO in the hours preceding (1.2 g/kg b.w./h). In contrast, the HI-LO

condition showed a 4.5-fold increase in p38 phosphorylation from PM Pre to PM Post (Figure 2.8; $p<0.001$). Thus, HIE-induced p38 phosphorylation was 2.5-fold greater in the HI-LO condition at PM Post versus the HI-HI condition, which remained unchanged from PM Pre.

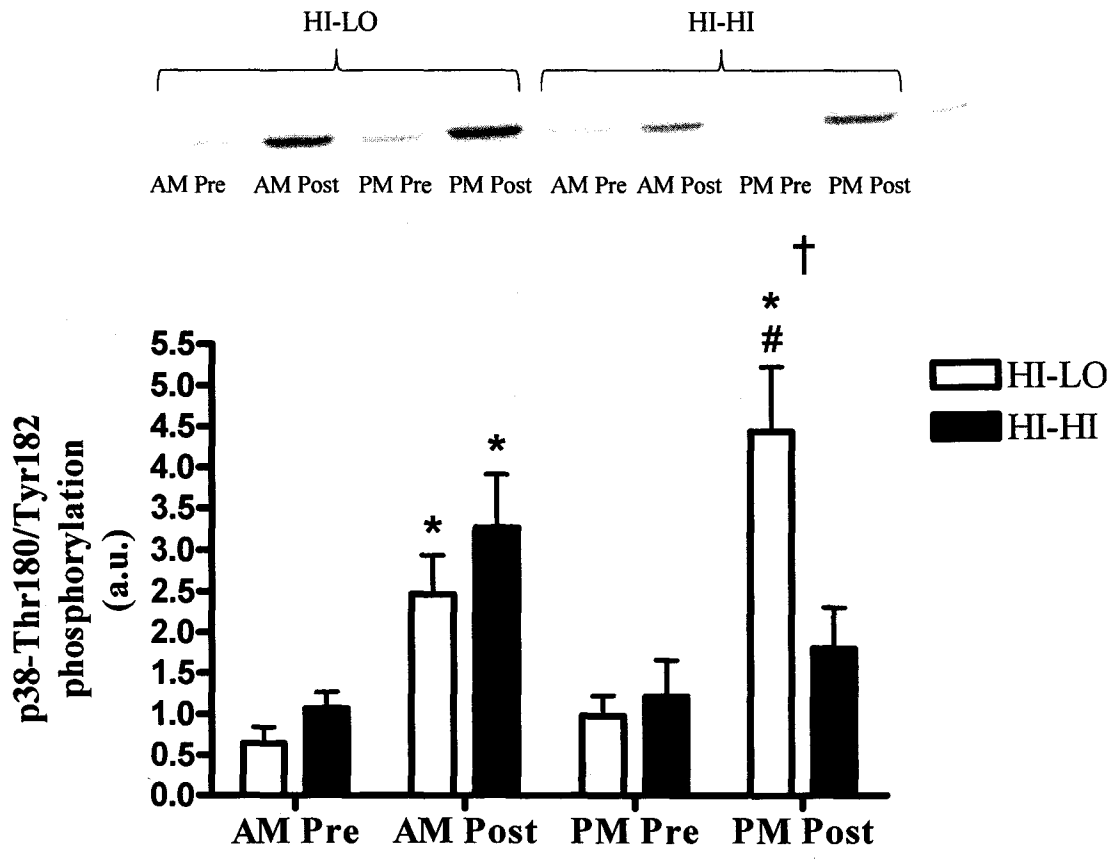

Figure 2.8 – p38 MAPK phosphorylation pre- and post-AM and PM HIE sessions with high-CHO or non-energetic placebo ingestion between sessions. *Significantly different from AM Pre time point within condition ($p<0.001$); #Significantly different from PM Pre within condition ($p<0.001$); †Significant difference between conditions at designated time point ($p<0.001$).

PGC-1α Protein Expression:

There were no exercise-induced changes in PGC-1α protein during either the AM or PM HIE sessions, or throughout the course of the experimental protocol. Interestingly, there was a main effect for condition in which the HI-HI condition showed greater PGC-1α protein at all time points in comparison to the HI-LO condition (Figure 2.8; p=0.05).

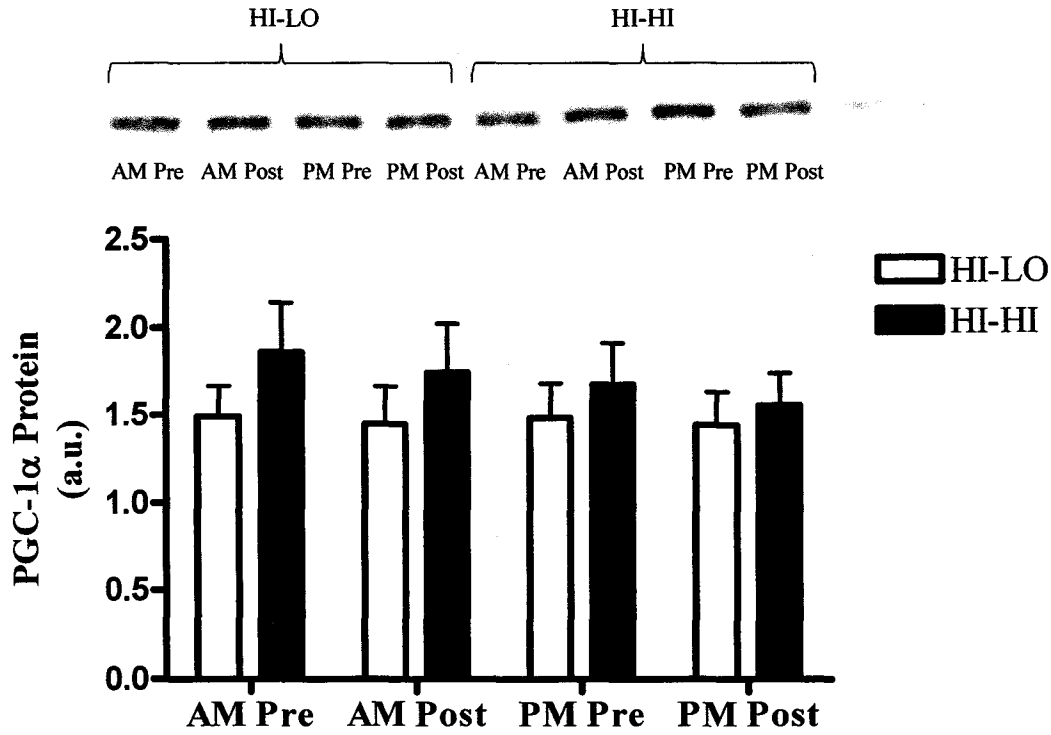

Figure 2.9 – PGC-1α protein expression pre- and post-AM and PM HIE sessions with high-CHO or non-energetic placebo ingestion between sessions.

2.4 DISCUSSION

This study was designed to examine the effects of CHO availability on the human skeletal muscle response to HIE. Specifically, this study was formulated to examine the potential exacerbating effects of reduced CHO availability on signalling proteins

proposed to play a significant role in exercise-induced mitochondrial biogenesis through PGC-1α. This is one of the first studies to examine the effects of HIE on each of the AMPK, CaMK, and p38 MAPK pathways together, and is the first to evaluate the effect of nutrient manipulation on said response. The primary novel finding from this study was that, compared to high CHO ingestion, restricting CHO intake during recovery from HIE exaggerated the exercise-induced p38 activation during a second HIE session performed 3 hours later. These effects occurred in the absence of measurable differences in muscle glycogen, suggesting this effect may have been due to CHO availability *per se*, and not specifically intramuscular glycogen stores. This finding was consistent with our hypothesis that reduced CHO availability during HIE would potentiate the activation of PGC-1α-directed signalling molecules. Furthermore, HIE induced activation of the AMPK, and CaMKII signalling molecules, with no differences between nutritional interventions, indicating muscle glycogen concentration may be more important to these signals than to CHO availability *per* se. Lastly, despite these alterations in protein signalling, there were no effects of either exercise or condition on downstream PGC-1α protein expression over the course of the 5 h protocol.

2.4.1 Effect of CHO Availability on Substrate Metabolism

Serum insulin concentrations at baseline upon subjects' arrival at the laboratory are consistent with values typically reported in the hours following a high CHO meal (30, 39, 103, 129, 132). Similarly, the elevated insulin concentrations seen in the HI-HI condition prior to the PM session resembles previous studies that delivered 1-1.2 g CHO/kg b.w./h in the hours following an exercise bout (18, 57, 60, 113, 122). It is reasonable to assume

that our PM Pre insulin values are reflective of the downslope of the CHO-induced insulin "spike" that reached approximately 45-60 μIU/mL at 2 hours post-exercise, and decreased thereafter to reach its PM Pre level of ~18 μIU/mL. In support of this, Jentjens *et al.*, (60) and van Hall *et al.*, (122) showed that a 1.2 g CHO/kg b.w./h delivery resulted in a progressive increase in insulin values to approximately 50-60 μIU/mL after 2 hours. These researchers continued to feed CHO beyond 2 h and hence direct comparisons at the 3 h post-exercise time point can not be made. However, Tarnopolsky and associates (113) delivered 1 g CHO/kg b.w./h for 2 hours and showed progressive increases in plasma insulin, reaching peaks of 40-45 μIU/mL 2 h post-exercise, before returning to levels of ~20 μIU/mL by the third hour. In contrast to our HI-HI condition, PM Pre insulin values for the HI-LO condition are directly in line with those reported during fasting (30, 38, 39, 132) and did not deviate over the course of the PM session. It may therefore be concluded that we were successful in delivering a significant amount of CHO prior to the afternoon HIE session in the HI-HI condition, whereas no CHO was ingested in the HI-LO condition.

Blood glucose concentration during the AM session of HIE was nearly identical between conditions, each showing a slight, non-significant increase towards the end of the exercise. This result is not surprising, as pre-trial 24-h diets were repeated prior to the beginning of each exercise trial, and subjects abstained from exercise for 24 h or more, presumably resulting in equivalent levels of liver glycogen and exogenous glucose supply during AM exercise. Prior to PM HIE, the HI-HI condition had significantly lower blood glucose concentrations than the HI-LO condition, and this difference was maintained

through the first interval of the PM session before being abolished by the PM Post time point. The lower PM Pre blood glucose in the HI-HI condition is somewhat counterintuitive considering the significant amount of exogenous CHO ingested prior to the PM exercise session (as confirmed by elevated serum insulin concentrations). Previous research has shown however, that the feeding of a large amount of high-glycaemic CHO can result in a lower than pre-feeding circulating glucose concentration approximately 2-3 h following (6, 109, 111, 135). This effect was most likely mediated not only by the high glycaemic index of the CHO supplementation, which often exacerbates the insulin response to feeding (39, 109-111, 129, 135), but also due to the insulin sensitizing effects of the prior bout of exercise (48, 94, 95). Exercise-induced increases in insulin action have also been shown to be mediated in part by muscle glycogen content (40, 56, 143), however, as there were no differences in muscle glycogen following AM HIE, muscle glycogen concentrations did not play a significant role in altering blood glucose concentrations.

High intensity exercise has been shown to induce rapid decreases in muscle glycogen concentration within a very short period of time. In fact, a single bout of 30 s of maximal cycling has been shown to decrease muscle glycogen stores as much as 20-35% (10, 85, 86). High-intensity aerobic interval work has also been shown to result in rapid decreases in muscle glycogen, as 8 x 5 min bouts of HIE eliciting ~85% VO_{2peak} with 1 min rest intervals has been shown to decrease muscle glycogen levels by approximately 50% (108). In the present study, each HIE session resulted in an approximate 30-35% decrement in muscle glycogen, independent of subject condition, and the total reduction

in muscle glycogen over the course of ten, 4 min work bouts (again, independent of condition) was approximately 50%. This is in good agreement with Stepto *et al.* (108) considering the total duration of work intervals was identical (40 min), and the relative intensity was comparable (85 vs. 90% VO_{2peak}). However, it must be remembered that there was a 3 h period during which the subjects in the present study were able to rest and, on one occasion, consume large amounts of CHO.

Rapid muscle glycogen resynthesis following exercise is a high priority for many athletes who train and compete frequently. A surprising finding from the present study was that, following a significant decrease in muscle glycogen concentration during the AM HIE session, delivery of 1.2 g CHO/kg b.w./h for 2 h did not result in significant muscle glycogen resynthesis, with a rate of increase of approximately 7.3 mmol/kg d.w./h. This finding is in contrast to many studies that have fed between 1 and 1.4 g CHO/kg b.w./h for up to 4 h and shown muscle glycogen resynthesis rates of between 25-45 mmol glucosyl units/kg d.w./h (17, 19, 60, 113, 122, 123). For example, Tarnopolsky *et al.* (113) fed 1 g CHO/kg b.w./h immediately and 1 h post-exercise, similar to the current feeding pattern, and showed glycogen resynthesis rates of 40 mmol/kg d.w. over the next 4 hours. However, each of these aforementioned studies delivered CHO following glycogen-depleting exercise (resulting in concentrations $\leq$176 mmol/kg d.w.) whereas our protocol was designed to only lower muscle glycogen concentrations approximately 35-40%. Muscle glycogen resynthesis rates have been inversely associated with the absolute muscle glycogen concentration present in the muscle when CHO feeding begins (92) and evidence suggests that elevated muscle glycogen can inhibit

glycogen synthase activity (82, 141) and that an increased glycogen content can reduce glycogen synthase affinity for glucose (65). Thus, as our muscle glycogen levels remained at approximately 300 mmol glucosyl units/kg d.w. following AM HIE, it is likely that reduced glycogen synthase activity and glucose affinity is the reason we did not see greater resynthesis rates.

One point of interest regarding muscle glycogen resynthesis remains: if a significant amount of CHO was delivered (mean: 93 g CHO/kg b.w./h), as verified by serum insulin increases, and there was significant bodily glucose uptake (presumably evidenced by depressed blood glucose concentrations), but no significant muscle glycogen resynthesis, where did the ingested CHO go? Unfortunately, we cannot determine definitively the physiological destination of the CHO, however, a study by Casey *et al.* (18) may provide some form of insight. Subjects underwent cycling at 70% VO_{2peak} in order to lower muscle and liver glycogen approximately 55-60% (18). Following this, subjects received a single bolus dose of 1 g CHO/kg b.w., and muscle, as well as liver glycogen resynthesis was monitored for the next 4 h (18). Interestingly, during the first hour of recovery, glycogen resynthesis rates were ~40 and 36 mmol/L/h for muscle and liver, respectively (as measured by magnetic resonance spectroscopy), but there was a negative resynthesis rate during the second hour, and by the end of 3 h recovery there was no significant increase in muscle glycogen, but was significant elevation in liver glycogen (18). Thus, it is a possibility that a great deal of CHO disposal in the present study occurred in the liver.

Under the majority of exercise conditions, ATP concentration is defended effectively by the rapid activation of the phosphocreatine, glycolytic and oxidative systems. Under high-intensity exercise situations however, the activation, or rate of activation of these energy-generating pathways is insufficient to meet the demand for ATP, and ATP concentration may subsequently decline (63, 105). The present study indicates that HIE, followed by a high amount of CHO results in the improved maintenance of both ATP and PCr concentrations throughout a subsequent bout of HIE.

Previous research has shown that skeletal muscle ATP concentrations may decrease as much as 40% over the course of a 30 s bout of maximal cycling (58, 72, 105). When these efforts are repeated within 3-4 minutes, these ATP concentrations may be largely repleted, and reduced to a similar extent over the subsequent work bout (72, 104) or may result in a progressive decrease in ATP stores (93). Similarly, ATP decrements are also observed with short, 6-10 s repeated sprints (37), exhaustive high-intensity exercise bouts lasting 2-3 minutes (49, 63), and even during more prolonged high-intensity efforts lasting 10 minutes (126). Unfortunately, there is a paucity in the data regarding aerobic HIE and adenine nucleotides *per se*, however Clark and associates (25) failed to show any significant decrements in ATP concentrations over the course of 8 x 5 min bouts of HIE at approximately 85% VO_{2peak}. Thus, Clark *et al.* (25) support the current study's findings of no changes in ATP concentration during the AM HIE session, but do not support our findings of reduced ATP concentrations during the CHO restricted PM HIE session. However, as Clark and associates (25) did not alter CHO availability prior to, or during HIE exercise, it precludes direct comparison to our afternoon HIE session. With

regards to CHO effects on nucleotide concentrations, it has been shown that CHO can blunt exercise-mediated reductions in ATP during prolonged moderate-intensity exercise to exhaustion (121) though this is not a consistent finding (44, 73). Furthermore, McConell *et al.* (74) showed that CHO ingestion during high-intensity exercise to exhaustion (~83% VO_{2peak}) did not result in better maintenance of ATP concentrations at the point of exhaustion. However, this investigation was performed in highly-trained individuals, which may have prevented any CHO-induced effects due to training-induced improvements in energy homeostasis (74).

Regarding PCr concentrations, the present study's findings support previous research indicating that reduced CHO availability may augment PCr degradation in the first minute of moderate-intensity exercise, and that this difference may be maintained through 10 min (43), 30 min (44), and throughout exercise until exhaustion (44, 120). However other investigators, utilizing very similar exercise models, have shown no such CHO effect on PCr degradation (73, 127). One potential mediator of these discrepancies may be the method of CHO availability alteration as Gibala and colleagues (43) and Green *et al.* (44) each altered muscle glycogen via exercise and low CHO diet, whereas Watt *et al.* (127) and McConell *et al.* (73) altered availability via CHO ingestion pre- and during-exercise. It has been proposed that PCr degradation may be partially mediated by the basal level of pyruvate dehydrogenase (PDH) activity at the onset of exercise, and that increased PDH activity and acetyl group availability may spare PCr during submaximal exercise (51, 118, 119). Interestingly, Watt and associates (127) showed an enhanced basal PDH activity following CHO supplementation, but no reduction in PCr degradation

reduction in PCr degradation at 1 or 10 min of moderate intensity exercise. In contrast, the 2 d low CHO diet utilized by Gibala *et al.* (43) was associated with reduced PDH activity during exercise (43), but did show better PCr maintenance at 1 and 10 minutes of 70% VO_{2peak} cycling. It must be remembered however, that these studies utilized moderate-intensity exercise, and are not directly applicable to the present study's exercise model. Furthermore, the theory of acetyl group availability in the governance of PCr degradation during high-intensity exercise and contraction remains controversial with some showing support (117), while others do not (52, 101). Thus, the unique diet and high-intensity exercise model utilized in the present study to alter CHO availability makes direct comparison to previous studies difficult, but will serve as an intriguing source for future research. Nonetheless, we have shown that CHO availability aids in the defence of PCr degradation during exercise, and extends those findings to include ATP concentrations during high-intensity interval type exercise.

2.4.2 Effect of CHO Availability on Skeletal Muscle Signalling Responses

AMPK has been shown to be activated in an exercise intensity-dependent fashion (21, 41, 131). Similarly, AMPK activity has been linked to reduced CHO availability, specifically in the form of muscle glycogen (33, 106, 130). Thus, we examined the effects of restricted CHO availability on AMPK phosphorylation during high-intensity interval exercise. As expected, 5 x 4 min bouts of HIE significantly increased AMPK phosphorylation over the course of the AM HIE session. These increases in AMPK phosphorylation are likely mediated in part by a HIE-induced increase in AMP, which has been shown to be a critical player in the process of AMPK activation (28) and α-subunit

phosphorylation (100, 102). While the present study did not report any decreases in muscle ATP content over the course of the AM session, numerous studies, including Clark *et al.* (25) have shown significant increases in ADP, AMP, and IMP content during high-intensity exercise both in conjunction and independently of ATP change (53, 75, 85, 93) as has the AMP:ATP ratio (25, 75), reputed to be the most appropriate predictor of AMPK activity (28, 75). Following 3 hours of rest, and ingestion of either placebo or CHO, AMPK-Thr^{172} phosphorylation returned almost to baseline, before being induced to a nearly significant level again during the second HIE session. What is most interesting about these results is that, while there was a trend towards an increase ($p=0.09$) relative AM Pre, AMPK-Thr^{172} phosphorylation was not significantly elevated during the PM HIE session. Furthermore, despite significant differences in ATP and PCr concentration at the end of the PM session, the AMPK response was nearly identical. These findings may be explained in part, again due to the independence of ATP and AMP concentrations, or alternatively due to isoform-specific effects which have been gaining more insight in recent years.

Considerable previous work has examined AMPK regulation in rodents using electrical stimulation models, and these studies showed consistent activation of both the α1 and α2 catalytic subunits (61) as well as α-Thr^{172} phosphorylation being significantly correlated with total AMPK activity (84). Similarly, moderate to high-intensity exercise in humans has been shown to activate both α subunits (21, 25, 125). Thus, we assessed AMPK-αThr^{172} phosphorylation as a marker of total AMPK activity. However, these studies also show that the α2 subunit appears to be more responsive to exercise, and

others have observed activation of solely the α2 subunit in response to exercise (41, 131, 140). It has also recently been suggested that only the α2/β2/γ3 AMPK isoform is responsive to exercise in human skeletal muscle (9). Thus, our results may have been dampened by measuring the α1 subunit. However, as Clark *et al.* (25) did observe α1 activation during high-intensity interval exercise, we felt it was prudent to measure total AMPK phosphorylation as our marker of activity.

The present study showed no effects of CHO availability on basal, or exercise-induced AMPK phosphorylation. This is in contrast to previous studies that have shown both basal, and contraction/exercise-induced AMPK activity may be augmented by reduced CHO in the form of muscle glycogen (33, 106, 130) though not all studies have shown effects (75). These findings may have been partially due to the fact that there was no difference in muscle glycogen concentrations between conditions prior to PM HIE, and that CHO delivery alone, without differences in intramuscular CHO storage has no effect on AMPK phosphorylation (68). In support of this, Akerstrom *et al.* (1) reported that CHO ingestion during 120 minutes of knee-extensor exercise resulted in significantly lessened AMPKα2 response to exercise (1). In contrast, Lee-Young *et al.* (68) reported no differences in AMPK activation in response to CHO ingestion during 120 minutes of cycling exercise. The main differences in the findings between these studies however, was that CHO ingestion resulting in a non-significant muscle glycogen sparing of approximately 100 mmol/kg d.w. ($p=0.08$) in the Akerstrom *et al.* (1), whereas muscle glycogen values appeared nearly identical in the Lee-Young *et al.* (68) study. Thus, muscle glycogen may play a more important role in AMPK activation than CHO alone. It

must be noted however, that exercise-induced AMPK-Thr^{172} phosphorylation was not different between conditions in either study, indicating that AMPK phosphorylation may simply not be a sensitive enough measure to determine subtle differences in activation.

The present study is the first, to our knowledge, to examine the effects of CHO availability and high-intensity interval exercise on CaMKII phosphorylation in human skeletal muscle. Previous research has cited exercise as a potent inducer of CaMKII phosphorylation in human skeletal muscle (96-98). The present data supports and extends these prior findings, suggesting that high-intensity interval exercise is a potent activator of CaMKII in human skeletal muscle, and that this activation may be replicated in response to repeated bouts of exercise, as evidenced by significant CaMKII-Thr^{286} phosphorylation following both HIE sessions.

It was hypothesized that CHO restriction may augment exercise-induced CaMKII phosphorylation based on prior findings that reduced muscle glycogen alters calcium uptake and release (36). However, we found no effects of CHO on the response of CaMKII to HIE. This result may have been influenced by the similar muscle glycogen concentrations seen between conditions prior to the PM HIE session. It may be that CHO availability *per se* does not alter calcium uptake and release, and that muscle glycogen plays a larger role in calcium kinetics than does CHO alone. In support of this, Duhamel and associates (35) recently performed a study in which subjects ingested glucose throughout a cycling trial to exhaustion. Glucose intake (1 g/kg) alone, without differences in muscle glycogen failed to induce any differences from control with regards

to calcium uptake, release, or maximal Ca^{2+}/ATPase maximal activity (35). Thus, it may be muscle glycogen itself that plays a predominant role in calcium handling.

Exercise has repeatedly been associated with increased p38 phosphorylation, most often following exercise of some high intensity, such as marathon running (12, 139), prolonged cycling at approximately 75% VO_{2peak} (76), or high-intensity intervals (140; Gibala *et al.*, submitted). Consistent with these previous findings, the present study shows that 5 x 4 min bouts of HIE eliciting approximately 90% VO_{2peak} results in significant activation of p38 MAPK. Furthermore, this is the first study to show directly that CHO restriction may augment the HIE-induced activation of p38, whereas CHO supplementation may blunt this response. To our knowledge, only one previous study has directly examined the effects of altered CHO availability on p38 phosphorylation (20). In this study, the researchers utilized exercise and either a low- or high-carbohydrate diet to alter pre-exercise muscle glycogen stores prior to undergoing 60 minutes of cycling exercise at approximately 70% VO_{2peak} the following day (20). Results showed that reducing muscle glycogen stores augmented nuclear p38 phosphorylation both at rest, and following exercise, while there was a trend towards an exercise-induced increase in cytosolic p38 phosphorylation only in the low muscle glycogen condition ($p=0.09$). While the present study cannot comment on the nuclear and/or cytoplasmic effects directly, we can support Chan *et al.*'s (20) findings in that reduced CHO availability potentiates the p38 activation. Furthermore, we can extend these findings to show that these effects are mediated by CHO availability *per se* and not directly due to muscle glycogen. Lastly, when CHO is restricted, repeated p38 activation may occur in response

to multiple exercise sessions within a single day. This latter finding that p38 may be phosphorylated, reverse to baseline, and be phosphorylated again in response to exercise all within 5 h is in contrast to previous research citing that exercise-induced p38 phosphorylation is maintained for a period of at least 3 h (116). Interestingly, not only did these researchers find that p38 phosphorylation lasted at least 3 hours, but also that this phosphorylation was associated with enhanced insulin-stimulated glucose uptake during a subsequent euglycaemic-hyperinsulinaemic clamp (116). Thong and associates (116) proposed that p38 may play a mediating role in the enhanced insulin sensitivity seen in the 2-3 h immediately following exercise, though the authors themselves acknowledged the requirement for future research (116). This hypothesis is attractive, as it would provide another potential mediator of the reduced blood glucose concentrations reported in the present study. However, without any direct markers of insulin sensitivity, or further muscle samples to assess p38 phosphorylation during the 3 h rest interval, we cannot attribute any effect on blood glucose to p38, as its phosphorylation was returned to baseline within 3 h of the AM HIE session.

It has recently been proposed that CaMKII may function as an upstream activating kinase to p38, as CaMK inhibition attenuated the p38 activation, and p38-inhibition blocked calcium-mediated increases in PGC-1α expression in vitro (134). The present study shows that, while HIE is capable of activating both CaMKII and p38 in repeated sessions of exercise separated by 3 h, CaMKII could not mediate the CHO-induced effects on p38 phosphorylation due to the finding that CaMKII was increased during the PM HIE session, independent of condition, while p38 phosphorylation was only increased

in the HI-LO condition. These findings do not preclude the possibility that CaMKII modulates p38 activation, however it does suggest that CaMKII is not the sole upstream effector of p38 phosphorylation. It is possible that the CHO-mediated effects on p38 phosphorylation could be carried out by the more traditional p38 mitogen-activated protein kinase kinases MKK3 and/or MKK6. Further research is required to elucidate this possibility.

AMPK has also been proposed to be an upstream kinase to p38 in skeletal muscle, eliciting a variety of effects on glucose transport (69) and fat oxidation (138). However, this proposal has recently been challenged by Ho and colleagues (50) who utilized a number of in vitro and transgenic models to refute the association between AMPK and p38 signalling. The present study further supports this dissociation, as it was shown that while AM HIE activated both AMPK and p38, the PM, CHO-manipulated HIE session showed differing activation patterns. Thus, it appears that again, the CHO-mediated effects on exercise-induced p38 activation are modulated by factors other than AMPK.

PGC-1α has been shown to be an important mediator of muscular phenotype with roles in mitochondrial biogenesis (136), oxidative capacity (124), glucose transport (5, 78), fibre type transition (64, 70), insulin sensitivity (79, 88), and exercise performance (16). Exercise and contraction has been shown to upregulate PGC-1α gene expression acutely both before (55, 114) and following exercise training (91). Furthermore, 6 weeks of traditional endurance training and HIT alike, have been associated with increased PGC-1α protein within human skeletal muscle (13, 99). However, the time-course for exercise-induced increases in PGC-1α protein remains controversial. Previous research

suggests that acute, moderate-intensity cycling for 1 or 3 h does not increase PGC-1α total protein compared to rest (76, 128). However, these findings are in contrast with recent studies which have reported increased total PGC-1α protein content immediately following an exhaustive bout of cycling at 65% VO_{2peak} (71), or 30 minutes following a 50 minute bout of variable-intensity cycling (32). Furthermore, in rodent muscle, PGC-1α protein has been shown to be increased 18 h following two 3 h bouts of swimming (5), and nuclear PGC-1α protein increased immediately following a similar protocol of swimming and running (115). Thus, we assessed whether a single 30 min session of HIE was capable of increasing whole muscle PGC-1α protein immediately, or 3 h post-exercise before the PM HIE session. The present study showed no alterations in total PGC-1α protein content in response to either bout of HIE, or at any time over the course of a 5 h exercise protocol, supporting the findings of McGee & Hargreaves (76) and Watt and colleagues (128). One explanation for the discrepancies between the current study and DeFilippis *et al.* (32) and Mathai *et al.* (71) could be the fact that we utilized a HIE protocol eliciting approximately 90% VO_{2peak}, while the aforementioned studies utilized more continuous lower-intensity protocols. The significant activation of AMPK during AM HIE could also partially explain the lack of protein increase at AM Post, as AMPK activation during times of increased energy demand have been reported to play a role in protein synthesis inhibition (11, 34). Other possibilities include exercise duration, as subjects only performed 20 minutes of exercise during work bouts compared to a minimum of 50 minutes of lower intensity exercise in all of the previous studies. However, while we may be able to hypothesize that the higher intensity and AMPK

activation within the current study contribute to the lack of PGC-1α protein production over the course of our 5 h protocol, the reasons behind the discrepancies between Watt *et al.* (128) and McGee & Hargreaves (76), and those of De Filippis *et al.* (32) and Mathai *et al.* (71) are difficult to explain. Thus, further investigation into the effect of acute exercise and the time-course for PGC-1α protein production is required.

2.4.3 Limitations

The present study was designed to assess the effects of reduced CHO availability on the acute response of human skeletal muscle to high-intensity interval exercise with regards to signalling proteins associated with enhanced PGC-1α expression. While we have shown here that restricting CHO during the intervening 3 h between two HIE sessions may augment p38 phosphorylation and that these effects are independent of muscle glycogen status, a number of limitations to the present study remain. First, we are limited by the number of muscle and blood samples that we have taken. Further muscle and blood sampling may have allowed us to better characterize the effects of our high CHO intervention, and monitor serum insulin and blood glucose concentrations more thoroughly. We are unable to precisely account exactly for the disposal of the high amount of CHO that our subjects ingested in the HI-HI condition. While we have hypothesized that some of this CHO may have been taken up by the liver, liver biopsies are impractical for these research purposes and thus, we may not say for certain. Isotope tracer methodology could have provided us with a better account of glucose disposal, and would provide more insight into this question. Further muscle biopsies after the PM session may have also allowed us better opportunity to examine the PGC-1α protein

response to acute HIE with CHO manipulation. Secondly, while we have connected these various exercise-induced signalling pathways with the PGC-1α protein and gene expression, we have not measured PGC-1α mRNA directly. This is the next stage of our analysis, to examine whether the enhanced p38 MAPK activation translates into greater PGC-1α expression. We also have not measured the individual fibre-type responses within our human muscle samples, nor the specific muscle glycogen "populations" that occur within these muscle fibres to assess potential differences in response to HIE and CHO ingestion. It has been shown previously that high-CHO feeding prior to and during exercise may result in fibre-specific muscle glycogen sparing (31), and thus, the fed condition and high insulin values during the AM HIE session may have somewhat hindered our ability to detect muscle glycogen in our mixed fibre samples.

2.4.4 Future Directions

The present study raises a number of questions which could be investigated in future projects. First and foremost, considering the CHO-mediated effects on p38 phosphorylation in response to acute exercise, what are the long-term effects if this exercise protocol is repeated regularly? We are currently examining this question within our laboratory by conducting a 2 week training study utilizing the present protocol. We have also provided an interesting question as to what is the upstream mediator of the CHO effects on p38. We have provided some insight with regards to this question indicating that neither AMPK nor CaMKII are likely to perform this function, but future research should examine potential mediators such as MKK3/MKK6, catecholamines, reactive oxygen species, and other mediators that have been connected with CHO

availability such as interleukin-6. Furthermore, it would be interesting to further examine the relationship between muscle glycogen and the AMPK and CaMKII signalling molecules, as we have shown that CHO delivery alone, without alteration in muscle glycogen has no effect on their subsequent response to HIE.

2.5 CONCLUSIONS

This is the first study to show that CHO availability, and not necessarily changes in muscle glycogen availability, may affect exercise-induced activation of p38. These findings hold meaningful implications for athletics (16), obesity and disease states such as insulin resistance (7, 79, 88), as recent research has tied p38 MAPK phosphorylation directly to enhanced expression of the putative master regulator of mitochondrial biogenesis, PGC-1α (2, 3, 54, 134). Furthermore, we have shown that CHO supplementation may reduce HIE-induced cellular stress as reflected by ATP and PCr concentrations. We are the first to show that CaMKII may be activated repeatedly in response to high-intensity interval exercise, independent of CHO availability, when pre-exercise muscle glycogen levels remain above 300 mmol/kg d.w. Lastly, we have shown that high-intensity aerobic interval type exercise is a potent activator of three of the major protein signalling pathways linked to PGC-1α gene and protein expressions. Though we cannot make further hypotheses without directly measuring PGC-1α expression, an enhanced p38-mediated PGC-1α expression may enhance the potential for protein production, and lead to greater metabolic adaptation in response to exercise. Nonetheless, we have provided a fertile footing for future research to investigate the methods by which

may be augmented via nutrient manipulation, and have provided a number of new research questions regarding the upstream mediators of p38.

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APPENDIX I

SUBJECT SCREENING AND CONSENT FORMS

Hamilton
Health Sciences

RESEARCH ETHICS BOARD

McMaster University

REB Office, 1057 Main St. W., Hamilton, ON L8S 1B7
Telephone: 905-521-2100, Ext. 42013
Fax: 905-577-8379

August 1, 2007

PROJECT NUMBER: **07-219**

PROJECT TITLE: Effect of glycogen availability on the acute response of human skeletal muscle to high-intensity interval training

PRINCIPAL INVESTIGATOR: **Dr. M. Gibala**

This will acknowledge receipt of your letter dated July 9, 2007, which enclosed the revised REB application, a copy of the Par-Q Test and the revised Participant Information Sheet and Consent Form, version #2 dated July 4, 2007 for the above-named study. These issues were raised by the Research Ethics Board at their meeting held on June 19, 2007. Based on this additional information, we wish to advise your study has been given ***final*** approval from the full REB. The research proposal, including the Participant Information Sheet and Consent Form, version #2 dated July 4, 2007 and the recruitment poster was found to be acceptable on both ethical and scientific grounds. ***Please note*** attached you will find the Information Sheet/Consent forms with the REB approval affixed; all consent forms and recruitment materials used in this study must be copies of the attached materials.

We are pleased to issue final approval for the above-named study for a period of 12 months from the date of the REB meeting on June 19, 2007. Continuation beyond that date will require further review and renewal of REB approval. Any changes or amendments to the protocol or information sheet must be approved by the Research Ethics Board.

The Hamilton Health Sciences/McMaster Health Sciences Research Ethics Board operates in compliance with the ICH Guidelines Good Clinical Practice Guidelines, the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans and Division 5 Health Canada Food and Drug Regulations.

Investigators in the Project should be aware that they are responsible for ensuring that a complete consent form is inserted in the patient's health record. In the case of invasive or otherwise risky research, the investigator might consider the advisability of keeping personal copies.

A condition of approval is that the physician most responsible for the care of the patient is informed that the patient has agreed to enter the study.

PLEASE QUOTE THE ABOVE-REFERENCE PROJECT NUMBER ON ALL FUTURE CORRESTPONDENCE

Sincerely,

J. Holland /sm

Jack Holland, MD, FRCP, FRCP (C)
Chair, Research Ethics Board

McMaster University

PARTICIPANT INFORMATION SHEET

Title of Study

Effects of glycogen availability on the acute response of skeletal muscle to high-intensity interval exercise

Locally Responsible Investigator and Principal Investigator

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Sponsors

Canadian Olympic Committee
Natural Sciences and Engineering Research Council (NSERC) of Canada

OVERVIEW

You are being invited to participate in this research study because you are a healthy, regularly active person between the ages of 18 and 35. In order to decide whether or not you want to be part of this research study, you should understand what is involved and the potential risks and benefits. This form gives you detailed information about the research study, which will be discussed with you. Once you understand the study, you will be asked to sign this form if you wish to participate. Please take your time to make your decision. Feel free to discuss it with your friends and family or your family physician.

WHY IS THIS RESEARCH BEING DONE?

We are generally interested in factors that regulate skeletal muscle energy metabolism and exercise performance. We use specific exercise or nutritional interventions to "perturb" metabolism, and then we examine the response by measuring changes in biochemical markers in

We are generally interested in factors that regulate skeletal muscle energy metabolism and exercise performance. We use specific exercise or nutritional interventions to "perturb" metabolism, and then we examine the response by measuring changes in biochemical markers in skeletal muscle. It is well known that carbohydrate is the main source of fuel for repeated high-intensity sprint exercise. Traditionally, it is thought that having a high level of carbohydrate stored in your body (in a form called glycogen) during training is the most effective way to train hard and induce adaptations that improve performance. Recently, it was suggested that training without a lot of carbohydrate stored within your muscle might be more effective for stimulating muscle adaptations and improving performance. However, the limited studies of this topic to date have used training interventions and performance tasks (single leg kicking) that do not resemble normal athletic training competition. It is our purpose to examine what happens within the muscle cells when an acute session of "real life" exercise is performed with either a normal or low amount of muscle glycogen. We will measure changes in key energy metabolites and proteins in skeletal muscle in order to detect changes between before and after exercise when it is performed with and without carbohydrate. The results will provide basic science information regarding the regulation of skeletal muscle metabolism and may have practical applications for people who are interested in exercise and fitness.

WHAT IS THE PURPOSE OF THE STUDY?

Our main purpose is to examine changes in the skeletal muscle when high intensity interval exercise is performed with either low or high amounts of carbohydrate. We will test our hypothesis that certain signal proteins will be found in larger concentrations and certain metabolites will undergo greater change when high intensity sprint exercise is performed with a low amount of stored carbohydrate in comparison to a high amount of stored carbohydrate.

WHAT ARE MY RESPONSIBILITIES IF I PARTICIPATE IN THE STUDY?

You will first be asked to complete two questionnaires that are designed to identify potential medical or other reason that might preclude your participation in the study. These forms will be administered by one of the investigators listed above. Following successful completion of the forms and entry into the study, you will make four visits to the laboratory over the course of approximately three weeks. During the first two visits, we will test your baseline fitness level and allow you to become familiar with exercise testing devices. The final two visits will be the actual experimental trial days. Total time spent in the lab will be approximately 1 hour for each of the first two visits, and approximately five hours for each of the experimental trials. Thus the total time commitment for the entire study is approximately 12 hours.

VISIT #1: VO_{2peak} Test. This test involves cycling on a stationary bike (cycle ergometer) at progressively higher workloads while the amount of oxygen taken up by

your body is determined from a mouthpiece connected to a gas analyzer. The test will last ~30 min.

VISIT #2: Interval Familiarization. This test will consist of cycling for five x four-minute intervals on a cycle ergometer at a fixed workload equivalent to 90% of your VO_{2peak}. You will have two minutes of recovery between each interval during which you can rest or pedal lightly. We will use the mouthpiece connected to the gas analyzer to make sure that you are working at the proper intensity and also monitor your heart rate from a strap placed around your chest.

VISITS #3 and #4: Experiment Trials. Each experiment trial will consist of two exercise sessions performed over a period of approximately five hours. For each trial, after you arrive at the lab, a catheter will be inserted into an arm vein and one of your legs will be prepared for muscle biopsy sampling. A resting blood sample and muscle biopsy sample will be obtained. The details of the blood sampling and biopsy procedures and associated risks are thoroughly described on the attached forms entitled "Description of Medical Procedures." You will then perform the first exercise bout, which will consist of a bout of interval exercise similar to that performed during the familiarization session. Specifically, you will perform five x four-minute intervals at 90% of your VO_{2peak} with 2 minutes of rest between each interval. A second muscle biopsy sample will be obtained after the last interval. You will then rest for 3 hours while eating either a high or low carbohydrate meal that we will provide for you. After the recovery period, a third muscle biopsy sample will be obtained, and then you will repeat the interval exercise session. A fourth and final biopsy will be obtained after the last interval. In addition, blood samples will be obtained each time a muscle biopsy is taken (i.e. immediately before and after each exercise session). The entire experimental trial will be performed on two occasions separated by approximately one week, such that on one occasion you receive a low carbohydrate meal during the recovery period and on the other occasion you receive a high carbohydrate meal. We will also ask that you try and keep you normal diet as similar as possible for 24 hours before each trial, and refrain from any exercise aside from normal activities of daily living.

DESCRIPTION OF POTENTIAL RISKS AND DISCOMFORTS

Exercise testing. The potential risks and discomforts associated with the exercise testing procedures are similar to those associated with any form of strenuous physical activity. These include fatigue, fainting, abnormal blood pressure, irregular heart rhythm, and in very rare instances, heart attack, stroke or death. Every effort will be made to minimize these potential risks by evaluation of preliminary information relating to your health and fitness and by careful observations during testing.

Venous blood and muscle biopsy sampling. Please refer to the attached form entitled "Description of Medical Procedures" for a complete description of the invasive medical

procedures to be performed during the study and the potential risks and discomforts associated with these procedures.

HOW MANY PEOPLE WILL BE IN THIS STUDY?

We plan to recruit and test 10 subjects.

WHAT ARE THE POSSIBLE BENEFITS FOR ME AND/OR FOR SOCIETY?

This is a basic science study into the regulation of skeletal muscle energy metabolism and training. You will gain some insight into your exercise capacity and perhaps some information regarding nutritional strategies to improve performance.

WHAT INFORMATION WILL BE KEPT PRIVATE?

All data obtained in connection with this study will remain confidential. Appropriate measures, consistent with Research Ethics Board guidelines, will be taken to ensure privacy. The results from this study will be used for educational purposes and shared with the scientific community. However, all personal information will be removed from the data and subjects will only be identified by a code number. If the results of the study are published, your name will not be used and no information that discloses your identity will be released. Upon completion of the study, you will have access to your own data and the group data for your own interest.

CAN PARTICIPATION IN THE STUDY END EARLY?

If you volunteer to be in this study, you may withdraw at any time. You also have the option of removing your data from the study. You may also refuse to answer any questions you don't want to answer and still remain in the study. The investigators may also withdraw you from the study if circumstances arise which warrant doing so.

WILL I BE PAID TO PARTICIPATE IN THE STUDY?

If you agree to take part, you will receive an honorarium of $250.00 in order to compensate you for your time and effort. In the event that you do not complete the study, you will receive a pro-rated amount based on the proportion of the study completed.

WILL THERE BE ANY COSTS?

Your participation in the study will not involve any costs to you.

WHAT HAPPENS IF I HAVE A RESEARCH-RELATED INJURY?

If you are injured as a direct result of taking part in this study, all necessary medical treatment will be made available to you at no cost. Financial compensation for such things as lost wages, disability or discomfort due to this type of injury is not routinely available. However, if you sign this consent form it does not mean that you waive any legal rights you may have under the law, nor does it mean that you are releasing the investigator(s), institution(s) and/or sponsor(s) from their legal and professional responsibilities.

IF I HAVE ANY QUESTIONS OR PROBLEMS, WHOM CAN I CALL?

If you have questions about this research project now or later, you may contact any one of the investigators listed on first page of this document.

If you have questions regarding your rights as a research participant, you may contact the Office of the REB Chair at 905-521-2100, ext. 42012.

The REB Project Number for this study is 07-219.

CONSENT STATEMENT

SIGNATURE OF RESEARCH PARTICIPANT

I have read the preceding information thoroughly. I have had the opportunity to ask questions, and all of my questions have been answered to my satisfaction. I agree to participate in this study. I understand that I will receive a signed copy of this form.

_____
Name of Participant

_____ _____
Signature of Participant Date

Consent form administered and explained in person by:

_____
Name and title

_____ _____
Signature Date

SIGNATURE OF INVESTIGATOR

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

_____ _____
Signature of Investigator Date

McMaster University

DESCRIPTION OF MEDICAL PROCEDURES

Venous blood sampling. A small Teflon catheter will be inserted into a forearm vein with the assistance of a small needle, which is subsequently removed. The discomfort of this procedure is transient and is very similar to having an injection by a needle, or when donating blood. Once the needle is removed there should be no sensation from the catheter. During the course of the experiment, blood will be drawn periodically from the catheter. In any one experiment the total blood loss is typically less than 100 ml, which is approximately 1/6 of the blood removed during a donation to a blood bank. In this specific experiment, each blood sample will remove approximately 6 ml of blood. This means that a total of 24 ml will be extracted each of the last 2 visits to the lab. It is not enough of a blood loss to affect your physical performance in any way. After each blood sample has been taken, the catheter is "flushed" with a sterile saline solution in order to prevent blood from clotting in the catheter. This is a salt solution that is very similar in composition to your own blood and it will not affect you. Following removal of the catheter, pressure will be placed on the site in order to minimize bleeding and facilitate healing.

Muscle biopsy sampling. This procedure involves the removal of a small piece of muscle tissue using a sterile hollow needle. The area over the muscle to be sampled will be cleaned and a small amount of local anesthetic ("freezing") will be injected into and under the skin over the vastus lateralis (quadriceps) muscle. A small incision (~4 mm) in the skin will then be made in order to create an opening through which to put the biopsy needle into the muscle. There is a small amount of bleeding from the incision, but this is minimal. The incision will be covered with sterile gauze and surgical tape. At those times during the experiment when a biopsy is required, the bandage will be removed and the biopsy needle will be inserted into your thigh through the incision. A small piece of muscle (~50-100 mg; about the size of the eraser on the end of a pencil) will quickly be obtained and then the needle will be removed. During the time that the sample is being taken (~5 sec), you may feel the sensation of deep pressure in the muscle and on some occasions this is moderately painful. However, the discomfort very quickly passes and you are quite capable of performing exercise. Occasionally, a biopsy sample yields little or no tissue sample and in this instance, it may be necessary to make a second attempt through the same incision site. If a biopsy sample is required during an exercise trial, the exercise bout is briefly interrupted in order to obtain the sample, and the muscle may feel a little "tight" during the first few seconds as you begin to exercise again. Following the exercise bout, the biopsy sites will be closed with sterile bandage strips or a suture (stitch)

and wrapped with a tensor bandage. You should refrain from excessive muscle use for the remainder of the day. Once the anesthetic wears off, your leg may feel tight and often there is the sensation of a deep bruise or "Charlie Horse". You should not take any aspirin based medicine for 24 hours following the experiment as this can promote bleeding in the muscle. However, other analgesics such as Ibuprofen or Tylenol are acceptable alternatives. It is also beneficial to keep your limb elevated when you are sitting, and the periodic application of an ice pack will help to reduce any swelling and residual soreness. The following day your muscle may feel uncomfortable upon movement, e.g., going down stairs. The tightness in the muscle usually disappears within 1-2 days, and subjects routinely begin exercising normally within 2-3 days. In order to allow the incisions to heal properly and minimize any risk of infection, you should avoid prolonged submersion in water for 2-3 days. Daily showers are acceptable, but baths, swimming, saunas, etc. should be avoided.

WHAT ARE THE POSSIBLE RISKS AND DISCOMFORTS?

Venous blood sampling procedure. The insertion of a venous catheter for blood sampling is a common medical practice and involves minimal risk provided proper precautions are taken. The catheter is inserted under completely sterile conditions, however there is a theoretical risk of infection. There is also chance of bleeding if adequate pressure is not maintained upon removal of the catheter. This may cause some minor discomfort and could result in bruising/skin discoloration that could last up to a few weeks. There is also the remote risk that trauma to the vessel wall could result in the formation of a small blood clot, which could travel through the bloodstream and become lodged in a smaller vessel. However, we have never experienced such a complication in our laboratory after several thousand venous catheter placements.

Muscle biopsy procedure. The biopsy technique is routinely used in physiological research, and complications are rare provided that proper precautions are taken. However, there is a risk of internal bleeding at the site of the biopsy, which can result in bruising and temporary discolouration of the skin. On occasion a small lump of fibrous tissue may form under the site of the incision, but this normally disappears within 2-3 months. As with any incision there is also a slight risk of infection, however this risk is virtually eliminated through proper cleansing of the area and daily changing of wound coverings. If the incision does not heal within a few days or you are in any way concerned about inflammation or infection, please contact us immediately. In very rare occasions there can be damage to a superficial sensory nerve that will result in temporary numbness in the area. There is also an extremely remote chance (1 in ~1,000,000) that you will be allergic to the local anesthetic. It is the collective experience of members in our laboratory that, in healthy young subjects, 1 in ~2,500 have experienced a local skin infection; 1 in ~1,000 have experienced a small lump at the site of the biopsy (in all cases this disappeared within approximately one week using gentle massage over the area of the lump); 1 in ~2,000 have experienced a temporary loss of sensation in the skin at the site of incision (an area of numbness about the size of a quarter that lasted up to 4 months),

and 1 in ~100 have experienced mild bruising around the site of incision that lasted for ~4-5 days. There is also a theoretical but extremely small risk of damage to a small motor nerve branch leading to the muscle that is being sampled but we have never experienced this complication.

McMaster University

SUBJECT SCREENING QUESTIONNAIRE

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

Name: _____ Date: _____

1. Have you ever been told that you have a heart problem?

YES NO

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

YES NO

3. Have you ever been told that you have kidney problems?

YES NO

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

YES NO

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

YES NO

6. Have you ever had any allergies to medication?

YES NO

7. Have you ever had any allergies to food or environmental factors?

YES NO

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

YES NO

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

YES NO

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

YES NO

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

YES NO

12. Are you currently taking ANY medication or have you taken any medication in the last two days? If yes, please indicate medication(s).

YES NO

13 Have you ever taken part is a study that involved needle biopsy or blood sampling? If yes, please provide details in space below

YES NO

Please use the space below or on the back of this page to transmit any additional comments or concerns you may have.