EXERCISE-INDUCED CELL SIGNALLING RESPONSES OF HUMAN SKELETAL MUSCLE: THE EFFECTS OF REDUCED CARBOHYDRATE AVAILABILITY

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ABSTRACT

It is well documented that regular endurance exercise induces skeletal muscle mitochondrial biogenesis. However, the optimal training stimulus and nutritional intervention for which to maximize mitochondrial adaptations to endurance exercise is not well known. Developments in molecular techniques now permit the examination of the cell signalling responses to acute exercise therefore increasing our understanding of how manipulation of the training protocol and nutrient availability may enhance the training stimulus to a given bout of exercise. The primary aim of this thesis is to therefore characterise the skeletal muscle cell signalling responses thought to regulate mitochondrial biogenesis following an acute bout of high-intensity interval exercise and moderate-intensity continuous exercise. A secondary aim is to subsequently examine how manipulation of carbohydrate (CHO) availability may enhance the activation of key regulatory cell signalling pathways.

The aim of the first study (Chapter 4) was to develop two exercise protocols of varied activity profile, which induced comparable total oxygen consumption and energy expenditure after being matched for average intensity, duration and distance ran. In a repeated measures and randomised design, eight active males performed an acute bout of high-intensity interval (HIT) running (6 x 3 min at 90 % $\dot{V}O_{2max}$ interspersed with 6 x 3 min at 50 % $\dot{V}O_{2max}$ also performed with a 7-min warm up and cool down at 70 % $\dot{V}O_{2max}$) and an acute bout of moderate-intensity continuous (CONT) running (50-min continuous running at 70 % $\dot{V}O_{2max}$). As a result of average intensity (70 % $\dot{V}O_{2max}$) duration (50-min) and distance ran (9843 ± 176) being equal between protocols, total oxygen consumption (HIT; 162 ± 6, CONT; 166 ± 10 L) and energy expenditure (HIT; 811 ± 30, CONT; 832 ± 48 kcal) were matched between protocols ($P > 0.05$). Despite higher ratings of perceived exertion in HIT compared with CONT (HIT; 14 ± 0.5, CONT; 13 ± 0.4 AU, $P < 0.05$), subjects reported greater ratings of perceived enjoyment in the HIT protocol (HIT; 87 ± 2, CONT; 61 ± 4 AU, $P < 0.05$) according to the Physical Activity Enjoyment scale. By matching these two protocols for work done, these data therefore provided an appropriate framework for which to examine the molecular signalling responses of human skeletal muscle to acute HIT and CONT.

The aim of the second study (Chapter 5) was to characterise the skeletal muscle cell signalling responses associated with the regulation mitochondrial biogenesis following HIT and CONT. In a repeated measures and randomised design, muscle biopsies (vastus lateralis) were obtained pre-, post- and 3 h post-exercise from ten active males who performed the HIT and CONT protocols developed in Chapter 4. Despite the obvious difference in activity profiles between protocols, muscle glycogen (HIT; 116 ± 11, CONT; 111 ± 17 mmol/kg dry wt) decreased similarly between protocols ($P < 0.05$), and phosphorylation (P-) of AMPK (HIT; 1.5 ± 0.3, CONT; 1.5 ± 0.1) and p38MAPK (HIT; 1.9 ± 0.1, CONT; 1.5 ± 0.2) increased immediately post-exercise before returning to baseline 3 h post exercise. P-p53 (HIT; 2.7 ± 0.8, CONT; 2.1 ± 0.8) and expression of PGC-1α mRNA (HIT; 4.2 ± 1.7, CONT; 4.5 ± 0.9) increased 3 h post-exercise in both HIT and CONT though there were no difference between protocols ($P > 0.05$). Data therefore demonstrate comparable

cell signalling responses between HIT and CONT when matched for work done, average intensity, duration and distance ran. Furthermore, this is the first time exercise is shown to up-regulate p53 phosphorylation in human skeletal muscle therefore highlighting an additional pathway by which exercise may regulate mitochondrial biogenesis.

Progressing from the role of the exercise stimulus in initiating mitochondrial biogenesis, the aim of the third study (Chapter 6) was to examine the effects of reduced CHO availability on modulating the exercise-induced activation of the cell signalling pathways as characterised in Chapter 5. Although HIT and CONT protocols resulted in comparable signalling in Chapter 5, we chose HIT as our chosen exercise model given that it is perceived as more enjoyable than CONT, has application for improving both human health and performance and also because of its relevance as a training modality for elite athletes in team and endurance sports. In a repeated measures and randomised design, muscle biopsies (vastus lateralis) were obtained from eight active males pre-, post and 3 h after performing an acute bout of high-intensity interval running with either high (HIGH) or low CHO availability (LOW). In LOW, subjects performed a bout of glycogen depleting exercise the night before and reported to the laboratory on the subsequent morning in a fasted state as well as restricting CHO before, during and after exercise. Subjects in HIGH CHO loaded for 24 h before reporting to the laboratory to perform HIT with CHO consumed before, during and after exercise. Resting muscle glycogen (HIGH, 467 ± 19; LOW, 103 ± 9 mmol.kg^{-1} dw) and utilisation (HIGH, 142 ± 34; LOW, and 30 ± 12) was greater in HIGH compared with LOW ($P < 0.05$). Phosphorylation (P-) of ACC^{Ser79} (HIGH, 1.4 ± 0.4; LOW, 2.9 ± 0.9), a marker for AMPK activity, and p53^{Ser15} (HIGH, 0.9 ± 0.4; LOW, 2.6 ± 0.8) was higher in LOW immediately post- and 3 h post-exercise, respectively ($P < 0.05$). Before and 3 h post-exercise, mRNA content of PDK4, Tfam, COXIV and PGC-1α were greater in LOW compared with HIGH ($P < 0.05$) whereas CPT1 showed trend towards significance ($P = 0.09$). However, only PGC-1α expression was increased by exercise ($P < 0.05$) where 3-fold increases occurred independent of CHO availability. Data demonstrate that low CHO availability enhances p53 phosphorylation in a manner that may be related to upstream signalling through AMPK. Given the emergence of p53 as a potential molecular regulator of mitochondrial biogenesis, such nutritional modulation of contraction-induced p53 activation may have implications for both athletic and clinical populations.

In summary, the work undertaken from the studies in this thesis provides novel information in relation to the regulation of exercise-induced cell signalling responses associated with mitochondrial biogenesis. Specifically, this is first report to examine cell-signalling responses to running exercise where comparable signalling between HIT and CONT was observed when protocols are matched for average intensity and duration. Furthermore, these data provide the first report of an exercise-induced increase in p53 phosphorylation in which data demonstrate low CHO availability augments the exercise-induced increase in p53 signalling which may be related to upstream signalling through AMPK. Further studies would now benefit from addressing the nuclear and mitochondrial abundance of p53 in response to an acute exercise challenge as well as comprehensively examining how training status, exercise intensity and CHO availability affects p53 regulation and downstream target genes.

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LIST OF ABBREVIATIONS

ACC, acetyl-CoA carboxylase; ADP, Adenosine diphosphate; AMP, Adenosine monophosphate; AMPK, AMP-activated protein kinase; ATF-2, Activating transcription factor-2; ATP, Adenosine triphosphate; β-Had, β-Hydroxacyl-CoA dehydrogenase; Ca^{2+}, Calcium; CD36, Cluster of Differentiation 36; CONT, Moderate-intensity continuous exercise; CS, Citrate synthase; COX, Cytochrome c oxidase; COXII, Cytochrome c oxidase subunit II; COXIV, cytochrome *c* oxidase subunit IV; CPT-1, carnitine palmitoyltransferase 1; FABPm, Fatty acid binding protein; FAT/CD36, Fatty acid transporter; FFA, Free fatty acids; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GLUT4, Glucose Transport Type 4; GPT, Glutamate pyruvate transaminase; GS, Glycogen Phosphorylase; HIT, High-intensity interval exercise; HK, Hexokinase II; H_2O_2, Hydrogen Peroxide; HSP72, Heat Shock Protein 72; IMP, Inosine monophosphate; LDH, Lactate dehydrogenase; MAPK, mitogen-activated protein kinase; MEF2, Myocyte enhancer factor 2; PK, Pyruvate kinase; PDK-4, pyruvate dehydrogenase-4; PFK, Phosphofructokinase; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; Pi, Inorganic phosphate; ROS, Reactive Oxygen Species; PRC, PGC-1 related coactivator; RT-PCR, Real time polymerase chain reaction; TFAM, mitochondrial transcription factor A; SCO2, synthesis of cytochrome c oxidase 2; SDH, Succinate dehydrogenase; SIRT1, Sirtuin1; SR, Sarcoplasmic reticulum; TCA; Tri-carboxylic acid cycle; UCP3, Uncoupling Protein 3

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CHAPTER 1

General Introduction

1.1. BACKGROUND

It is well established that regular bouts of endurance exercise (i.e. endurance training) is beneficial for human health and performance, as evidenced by improved cardiovascular (Blomqvist & Saltin 1983) and metabolic function (Holloszy & Coyle 1984). At the 'whole body level', adaptations to endurance training are perhaps most widely recognised functionally by an increase in the individual's maximal oxygen uptake ($\dot{V}O_{2max}$). This improvement in $\dot{V}O_{2max}$ is mediated by physiological adjustments to various links of the oxygen transport chain, which ultimately result in improved oxygen delivery, and extraction and utilisation by the exercising muscle. For example, regular endurance exercise promotes left ventricular hypertrophy (Longhurst et al. 1981), an increased maximal cardiac output (Blomqvist & Saltin 1983), an improved capillary to muscle fibre ratio (Andersen & Henriksson 1977) as well as increasing the mitochondrial content and activity of skeletal muscle (Holloszy 1967; Holloszy & Coyle 1984). The training-induced increase in mitochondrial content of skeletal muscle (i.e. mitochondrial biogenesis) is particularly important for mediating improved endurance performance by permitting an improvement in energy homeostasis and conferring increased resistance against muscle fatigue (Hawley & Holloszy 2009).

Although it is well documented that regular physical activity induces favourable adaptations for improving both health and performance, it is less clear what is the most beneficial exercise prescription (i.e. intensity, duration, frequency etc) to yield the desired training stimulus. Public health guidelines typically advocate that training should occur 3-5 days per week and be performed at a fixed absolute workload at an exercise intensity of 40-80% $\dot{V}O_{2max}$ for durations of 20-60 minutes (Donnelly et al. 2009) However, in recent years a growing body of literature now demonstrates that aerobic high-intensity interval training (exercise consisting of periods of high-intensity activity interspersed with periods of lower-intensity activity) induces greater improvements in $\dot{V}O_{2max}$ compared with traditional models of moderate-intensity continuous training that is matched for total work done (Dubach et al. 1997; Rognmo et al. 2004; Wisløff et al. 2007; Helgerud et al. 2007). Furthermore, at the cellular level of skeletal muscle, data also demonstrates that brief periods of high-intensity interval exercise induce similar improvements in oxidative capacity of skeletal muscle when compared with continuous exercise of longer duration (Gibala et al. 2006; Burgomaster et al. 2008). Remarkably, these similar mitochondrial adaptations were achieved despite a distinct difference in the total training volume between training interventions where approximately 3 times more work was performed in the continuous training approaches. Taken together, these data suggest that high-intensity interval training may be a more time efficient training method than conventional continuous training approaches.

Although the physiological and biochemical adaptations to endurance training are well documented, the molecular mechanisms underpinning mitochondrial biogenesis remain to be precisely elucidated. An increased understanding of the molecular mechanism underpinning skeletal muscle adaptation is particularly important as it may help to aid training protocol development so as to maximise training adaptations (Coffey & Hawley 2007). At a molecular level, adaptation to training

following repeated exercise bouts can be viewed as transient changes in gene expression leading to increased steady state levels of specific proteins (Coffey & Hawley 2007). In considering possible 'signals' responsible for exercise-induced changes in transcriptional activity, increases in intracellular calcium (Chin 2005), lactate (Hashimoto et al. 2007), reactive oxygen species (Pattwell & Jackson 2004), hypoxia (Flück 2003), mechanical stretch (Hornberger et al. 2005) and substrate availability / utilisation (Hawley et al. 2007) have been identified as possible factors. These signals (acting alone or likely in combination with each other) can activate a multitude of signal transduction pathways (i.e. phosphorylation networks), which can result in transcription factor activation and subsequent binding to the promoter region of those genes involved in adaptation to exercise. In general, the time-course of these events to endurance type exercise protocols is transcription factor activation and mRNA accumulation during or within several hours after exercise (Pilegaard et al. 2005; Pilegaard et al. 2000) which may then induce subsequent translation of the protein in the hours to days following the exercise challenge (Perry et al. 2010). Despite recent advances in understanding the molecular responses of human skeletal muscle to continuous exercise protocols, no studies have directly compared the molecular responses and signalling pathways activated by continuous exercise versus high-intensity interval exercise. Such research is particularly important as a host of signals activated by muscle contraction, (e.g. redox balance, hypoxia, energy depletion, and lactate production) are likely to be exacerbated during the brief periods of high-intensity exhaustive efforts occurring during interval exercise protocols.

In addition to the nature of the training stimulus, the energy status/substrate availability of the cell is now also emerging as one of the most potent regulators of exercise-gene interactions (Hawley et al. 2011). In this regard, current sport nutrition guidelines (ACSM et al. 2009) typically advocate that carbohydrate (CHO) should be consumed before (Sherman et al. 1991; Achten et al. 2004), during (Costill et al. 1973; Coyle et al. 1986; Nicholas et al. 1995; Sugiura & Kobayashi 1998) and after (Ivy et al. 1988; van Loon et al. 2000; Tarnopolsky et al. 1997) exercise so as to maximise endogenous/exogenous CHO availability during exercise as well as promote muscle glycogen resynthesis. However, in recent years several researchers observed that deliberately commencing exercise with reduced levels of CHO availability augment improvements in skeletal muscle oxidative capacity compared to the typical nutritional guidelines (Hansen et al. 2005; Yeo et al. 2008; Morton et al. 2009; Van Proeyen et al. 2011). This model of nutritional periodization has been termed as the *train low : compete high* model surmising that a portion of training sessions be deliberately commenced with reduced CHO availability yet competition always be performed with high CHO availability (Burke 2010). Taken together, these data demonstrate that carbohydrate restriction before, during and after exercise may enhance the training response though the molecular mechanisms underpinning these adaptations remain unknown.

1.2. AIMS, OBJECTIVES AND STRUCTURE OF THESIS

The aim of this thesis is to characterise the skeletal muscle cell signalling responses in human skeletal muscle following an acute bout of high-intensity interval exercise versus moderate-intensity continuous exercise. Having established which mode of exercise provides the greatest training stimulus in terms of activation of acute cell signalling pathways, a further aim is to investigate the role of carbohydrate availability in modulating these responses. As such, it is hoped that the studies contained in this thesis will help inform training prescription guidelines so as to potentially maximise training adaptations.

The aims of this thesis will be achieved by:

a) The development of moderate-intensity continuous and high-intensity interval exercise protocols that are matched for total oxygen consumption, average intensity, duration, energy expenditure and distance ran (Study 1).
b) Characterising the acute skeletal muscle cell signalling responses (associated with the regulation of mitochondrial biogenesis) of human skeletal muscle to an acute bout of high-intensity interval exercise and moderate-intensity continuous exercise that is previously matched for energy expenditure and total oxygen consumption (Study 2).
c) Examining the effects of carbohydrate availability on exercise-induced cell signalling responses of human skeletal muscle (Study 3).

CHAPTER 2

Literature Review

2.0 INTRODUCTION

Skeletal muscle is a malleable tissue that has the ability to undergo major adaptations in response to exercise training. The most prominent adaptation to endurance exercise training is that of *mitochondrial biogenesis*, usually defined as an increase in mitochondrial number and content per gram of tissue (Hood, 2001). Since the pioneering work of Holloszy (1967) first documenting this response in rodents and the introduction of the muscle biopsy technique to the exercise sciences in the late 1960s, the field of investigation examining mitochondrial adaptations to endurance training has grown considerably over the last 40 years (Bylund et al. 1977; Andersen & Henriksson 1977; Ingjer 1979; Salmons & Henriksson 1981; Schantz et al. 1983; Wibom et al. 1992; Coggan et al. 1992; Phillips et al. 1996; MacDougall et al. 1998; Dubouchaud et al. 2000; Perry et al. 2010). In addition to increased mitochondrial size and number, fundamental compositional adaptations of mitochondria include increased enzyme protein content and activity of those enzymes of the TCA cycle as well as those involved in substrate transport (Holloszy et al. 1970; Oscai & Holloszy, 1971; Holloszy et al. 1973; Gollnick et al. 1972; 1973; Winder et al. 1974). The functional relevance of these adaptations is that exercise in the trained state is associated with smaller disturbances in cellular homeostasis (i.e. less accumulation of ADP, AMP, Pi etc) such that trained individuals have increased reliance on lipid metabolism (with a down-regulation of CHO metabolism) for a given exercise intensity compared with untrained individuals (Christensen 1939; Hermansen et al. 1967; Holloszy, 1967; Mole et al. 1971; Baldwin et al. 1972; Gollnick et al. 1977; Henriksson 1977; Hurley et al. 1986; Constable et al. 1987; Kiens et al. 1993). Such training-induced improvements in metabolic control and alterations in substrate utilisation are considered as one of the major contributing factors by which regular endurance training confers increased fatigue resistance to an acute exercise challenge.

In addition to these biochemical adaptations, the last decade of research has seen a significant expansion in studies examining the potential molecular mechanisms underpinning mitochondrial adaptations to endurance training. Such research has immediate translational potential to the exercising human considering that identifying important molecular pathways may allow for the development of highly tailored training protocols to induce optimal adaptation. In this chapter, the reader is initially presented with an overview of some of the key signalling pathways currently thought to mediate mitochondrial biogenesis followed by a critical review of the role of the training and nutritional stimulus in enhancing the training stimulus. As such, this chapter aims to provide the reader with an extended introduction and rationale as to the nature of the studies undertaken in this thesis. Data are predominantly reviewed from human studies though where appropriate rodent and cell culture data are also reviewed so as to substantiate discussion points.

2.1. MOLECULAR MECHANISMS REGULATING SKELETAL MUSCLE MITOCHONDRIAL BIOGENESIS

Exercise-induced mitochondrial adaptations in skeletal muscle are thought to be due to the result of cumulative effects of repeated bouts of exercise (Widegren et al. 2001; Perry et al. 2010). At a molecular level, the initiation of mitochondrial biogenesis in muscle commences with the activation of putative signals brought about by muscle contractions (Hood 2001). The activation of early signals, known as primary messengers, is transduced into signalling cascade pathways that subsequently amplify and interact with other pathways that end in activation of one or more transcription factors. Recent advances in technology and development of analytical techniques such as western blotting, and real time polymerase chain reaction (RT-PCR) have allowed exercise physiologists to examine the molecular and cellular responses of human skeletal muscle to various training and nutritional interventions. These new and exciting findings have provided scientists, coaches and practitioners with an insight into specific skeletal muscle adaptation and potential new clues into future and novel training methodologies. In the following sections, the author reviews some of the most well studied signalling pathways currently thought to regulate exercise-induced mitochondrial biogenesis.

2.1.1. Regulation of Cellular Adaptation

Skeletal muscle adaptations to endurance exercise training are regulated by a complex series of partially redundant signalling molecules (Drummond et al. 2009). Upon the onset of contraction, a variety of primary signalling molecules and messengers (e.g. intracellular Ca^{2+}, reactive oxygen species, changes in ATP/ADP ratio, reduced energy availability etc) activate downstream transcription and co-transcriptional factors to induce a co-ordinated up-regulation of gene expression (Mu et al. 2001; Chin et al., 2004; Akimoto et al. 2005; Hardie & Sakamoto, 2006; Jäger et al. 2007; Wright et al. 2007; Powers et al. 2010; Philp et al. 2012). Although various animal and cell culture models have examined the potential signalling pathways responsible for metabolic adaptations to endurance exercise, it is noteworthy that despite certain subunits of proteins or whole proteins being knocked out in a range of cell lines and animal models (Jorgensen et al. 2005), mitochondrial adaptations still take place thus emphasising the complexity of the signalling transduction network that exists. Nevertheless, the following sub-sections examine the regulation and downstream effects of two well characterised signalling kinases activated during endurance exercise and two downstream transcription factors that are thought to be required for exercise-induced mitochondrial biogenesis.

2.1.1.1. AMP-Dependent Protein Kinase (AMPK)

The AMPK was first discovered in the 1980's (Carling et al. 1987) in the regulation of enzymes associated with fatty acid and cholesterol synthesis. It was termed AMPK after its activator, nucleotide 5'-AMP (Carling & Hardie 1989). Due to the downstream effects AMPK possesses, it is recognised as the primary energy sensor of the cell and has several targets, (e.g. peroxisome proliferator-activated receptor γ coactivator 1α, PGC-1α) in the signalling transduction network that

is associated with mitochondrial biogenesis. The AMPK is a heterotrimeric Ser/Thr kinase comprised of catalytic α- and regulatory ß- and γ- subunits (Hardie 2007). In humans AMPK is a highly conserved system comprising of multi-subunit complexes. For example, there are two genes encoding alternate isoforms of the α subunits (α1 and α2), two encoding ß subunits (ß1 and ß2) and three encoding γ subunits (γ1, γ2, and γ3). The α subunits are the catalytic components of the protein and contain the Thr^{172} residue, whose phosphorylation is required for full enzymatic activity (Hawley et al. 1996). The ß subunit contains a carbohydrate-binding domain that allows the association of AMPK with glycogen (Hudson et al. 2003; Polekhina et al. 2003) and the γ subunits appear to be involved in binding of the activator, AMP (Cheung et al. 2000). All different combinations of the α, ß and γ subunits appear able to form a complex with up to twelve combinations possible. A schematic of AMPK can be seen in Figure 2.1. Any metabolic stress that causes a drop in the ADP : ATP ratio results in the production of AMP. This rise in AMP results in an increase in the AMP : ATP ratio and signals that the cell is metabolically stressed and therefore, causes an instant 10-fold activation of AMPK activity (Suter et al. 2006) and phosphorylation of $AMPK^{Thr172}$. In addition to the role of AMP in activating AMPK, ADP also appears to be important if not the main activating component contributing to AMPK phosphorylation (Bland & Birnhaum, 2011; Oakhill et al. 2011). This opinion is derived from the fact that the intracellular ADP concentrations generally exceed that of AMP thus suggesting ADP as the physiological determinant of the state of AMPK phosphorylation. Furthermore, it is reported that ADP possesses the ability to protect AMPK from dephosphorylation thus enhancing and prolonging its increased activity. In the absence of AMP and ADP, AMPK can also be activated by increases in intracellular Ca^{+} (Hawley et al. 2005; Woods et al. 2005) although data on this remains controversial.

Figure 2. 1 Two views of a crystal structure of a partial heterotrimeric complex of mammalian AMPK (Hardie et al. 2012)

Since the discovery that AMPK responds to changes in AMP concentration, muscle contraction has become recognised as a potent stimulus for AMPK phosphorylation at site Thr^{172} (Aschenbach, Sakamoto, & Goodyear, 2004). The AMPK was first shown to become active in rodent skeletal muscle following exercise (Winder & Hardie, 1996) and these findings have since been confirmed in human skeletal muscle studies following knee extensor (Frosig, Jorgensen, Hardie, Richter, & Wojtaszewski, 2004) and cycling exercise (Chen et al. 2000; Chen et al. 2003; Coffey et al. 2006; Gibala et al. 2009; Egan et al. 2010). The catalytic α subunits are only responsive to increasing exercise intensity (Chen et al. 2003) demonstrating 80 % $\dot{V}O_{2max}$ to be a more potent stimulus for increased α2 activity than 40 % $\dot{V}O_{2max}$ or 60 % $\dot{V}O_{2max}$. This is further supported by recent evidence from Egan et al. where $AMPK^{Thr172}$ phosphorylation was significantly greater following 36 min cycling at 80 % $\dot{V}O_{2max}$ compared to 70 min cycling at 40 % $\dot{V}O_{2max}$ (Egan et al. 2010). In addition to the exercise-induced phosphorylation of $AMPK^{Thr172}$ following an acute exercise bout, cumulative bouts of exercise within a training period result in increased protein content and basal activity of several AMPK subunits (Frosig et al. 2004). In contrast, it is also apparent that cumulative bouts of exercise fail to increase total protein content of AMPK (Benziane et al. 2008). Furthermore, $AMPK^{Thr172}$ phosphorylation also fails to activate to similar levels during a criterion exercise bout following a period of training as to pre-training suggesting trained individuals have to work at a higher intensity in order to maintain an adequate training stimulus. Despite these data on knee extensor and cycling models, no data exist on running as the chosen exercise mode therefore highlighting a need for further work characterising the AMPK response following running.

In regard to the regulation of the ß subunits on the AMPK trimer, glycogen loading is shown to suppress AMPK signalling in response to exercise suggesting glycogen is an AMPK negative controller (Wojtaszewski et al. 2002). If AMPK is suppressed by high glycogen then it is tempting to suggest that during aerobic exercise when glucose levels are low or the cell is in a state of low energy (due to nutritional manipulation) then AMPK would be activated further. Support for this hypothesis stems from Hardie et al. (1998) where AMPK in budding yeast triggers the switch from anaerobic metabolism to oxidative phosphorylation when glucose in the medium becomes depleted. Furthermore, both Wojtaszewski et al. (2003) and Yeo et al. (2010) demonstrated AMPK activation is greater in skeletal muscle with low muscle glycogen than high muscle glycogen following a bout of acute endurance exercise (a detailed overview of carbohydrate availability on AMPK can be seen in section 2.4). The AMPK is also shown to facilitate glucose uptake for glucose oxidation since glucose uptake appears to be dependent upon GLUT4 and AMPK activation is shown to correlate with translocation of GLUT4 to the plasma membrane (Kurth-Kraczek et al. 1999; Mu et al. 2001). Moreover, AMPK also regulates fatty acid uptake by phosphorylating and deactivating acetyl CoA carboxylase (ACC), thereby lowering malonyl CoA levels (Vavvas et al. 1997). Malonyl CoA is an inhibitor of the FFA transporter, carnitine palmitoyltransferase I (CPT-I), thus increasing the potential for mitochondrial fatty acid uptake. Taken together, it is suggested that both muscle

contraction and low energy status of the cell can activate AMPK, which has downstream effects on glucose uptake and fatty acid oxidation.

Whilst a focus of AMPK has centred on what regulates this signalling protein kinase, less is known regarding the effects AMPK has on downstream proteins in the signalling transduction network. In response to muscle contraction, AMPK can directly phosphorylate PGC-1α, at Thr^{177} and Ser^{538}, a co-transcription factor that is regarded as the 'master regulator of mitochondrial biogenesis' (see section 2.3.2.4) (Jäger et al. 2007). This phosphorylation of AMPK on PGC-1α is thought to be required to initiate many of the gene regulating functions that AMPK possesses in skeletal muscle. In support of these gene-regulating functions, in mice knocked out for AMPKα2, mitochondrial proteins are expressed to lower levels than compared to their wildtype counterparts. In contrast, overexpression of AMPKγ3 results in greater AMPK activity and thus higher levels of mitochondrial proteins (Nilsson et al. 2006). Taken together, these data highlight the importance of AMPK for inducing mitochondrial adaptations following exercise.

2.1.1.2. p38 Mitogen Antigen Protein Kinase (MAPK) signalling

The p38MAPK is a mechanical stress related protein and is shown to have vital roles within the signalling network of mitochondrial biogenesis. One role associated with p38MAPK is transcriptional regulation of the redox status in skeletal muscle. Immediately following exercise there is a rapid induction of reactive oxygen species (ROS) that accompany the adaptive mechanisms to metabolic stress. Hydrogen peroxide (H_2O_2) is shown to activate p38MAPK in an intensity-dependent manner (Kefaloyianni et al. 2006) and triggers phosphorylation of p38MAPK and glucose transport (Kim et al. 2006). Furthermore, inflammatory cytokines, which are associated with the immune system, are known to activate the p38MAPK signalling pathway, which subsequently exerts its effects on PGC-1α (Akimoto et al. 2005). Similar to AMPK, p38 MAPK consists of various isoforms (p38α, p38β, p38δ, and p38γ) of which have different roles. The isoform of particular note is p38γ as this is the isoform found exclusively in skeletal muscle (Li et al. 1996) and is activated in response to acute running (marathon) exercise (Boppart et al. 2000). Further support for the importance of this isoform in skeletal muscle comes from Pogozelski et al. (2009) where they demonstrated in p38γ knockout mice, reduced mitochondrial adaptations following endurance exercise. Furthermore, the purported role that p38MAPK plays in directly phosphorylating PGC-1α (via activating transcription factor-2 (ATF-2)) has been demonstrated in mice and cell culture (Puigserver et al. 2001; Akimoto et al. 2005) adding to the importance this signalling protein kinase plays in exercise-induced mitochondrial adaptations.

There is a growing body of evidence showing phosphorylation of p38MAPK in rodents after treadmill exercise (Goodyear et al. 1996; Nader & Esser 2001), cycling in humans (Widegren et al. 1998; Gibala et al. 2009; Cochran et al. 2010; Egan et al. 2010; Yeo et al. 2010) and marathon running in humans (Boppart et al. 2000; Yu et al 2001). Recently, Gibala et al. (2009) displayed a significant increase in p38MAPK in human skeletal muscle following four short term (30 seconds) but extremely high intensity bouts of all out interval cycling which subsequently resulted in a

significant increase in PGC-1α mRNA expression. Taken with recent evidence from Wright et al. (2007) whereby p38MAPK directly phosphorylates PGC-1α, it appears p38MAPK is a vital component of the signalling process for mitochondrial biogenesis. Whilst evidence supports p38MAPK in playing a role signalling for mitochondrial biogenesis in both forms of endurance exercise (i.e. cycling and running), marathon running may not be deemed a practical model for the recreational population, thus highlighting a need for further work on characterising the p38MAPK response following more practical models of running exercise.

2.1.1.3. Peroxisome Proliferator-Activated Receptor γ coactivator 1α (PGC-1α)

Since its discovery in 1998, where it was identified as a coactivator of PPARγ in brown adipose tissue and a key player in adaptive thermogenesis (Puigserver et al. 1998), PGC-1α is considered the 'master regulator' of mitochondrial biogenesis given its function as a transcriptional co-activator that binds to and activates transcription factors NRF-1, NRF-2, Tfam, TFB1m, TFB2M, ERRα, and MEF-2 resulting in an increased expression of their target genes. The importance of PGC-1α in regulating mitochondrial content and function is evident from rodent studies demonstrating that overexpression increases oxidative enzyme activity (Lin et al. 2002), improves insulin sensitivity (Benton et al. 2008), protects against sarcopenia (Wenz et al. 2009), and also improves exercise capacity (Calvo et al. 2008).

It is now being recognised that PGC-1α can be activated in a number of ways via muscle contraction. For example, AMPK is shown to phosphorylate PGC-1α at Thr177 and Ser536 (Jäger et al. 2007), which is required for the PGC-1α-dependent induction of mitochondrial biogenesis and p38MAPK is known to directly converge upon PGC-1α (Akimoto et al. 2005; Wright et al. 2007) and increase its expression. In addition, deacetylation through the AMPK-SIRT1 pathway (Gerhart-Hines et al. 2007; Rodgers et al. 2005) is also recognised as another important regulatory pathway. With this in mind PGC-1α has now become one of the most widely studied proteins involved in exercise-induced mitochondrial biogenesis (Lin et al. 2002). During basal conditions, PGC-1α is located in the cytosol, however, upon activation, PGC-1α relocates itself to the nucleus (Wright et al. 2007; Little, Safdar, Cermak, et al. 2010a; Little, Safdar, et al. 2011b) whereby it can auto-activate its expression leading to a second phase of adaptation where there is an increase in PGC-1α expression and protein content.

The first convincing evidence that demonstrated PGC-1α as a critical mediator of mitochondrial biogenesis was performed in cell culture (Wu et al. 1999) and mouse skeletal muscle (Lin et al. 2002). In accordance, overexpression of PGC-1α in mice skeletal muscle resulted in increased proportions of Type I fibres and improved endurance capacity (Lin et al. 2002). Furthermore, both whole-body knockout and muscle-specific knockout of PGC-1α in rodents display a reduced mRNA and/or protein content of mitochondrial proteins and ATP synthase (Leone et al. 2005). In humans, a single bout of knee extensor exercise was shown to significantly elevate PGC-1α mRNA (Pilegaard et al. 2003) which led to the examination of various modes of exercise, intensities and durations (Gibala et al. 2009; Harber et al. 2009; Leick et al. 2010; Nordsborg et al. 2010; Perry et

al. 2010) on increasing PGC-1α mRNA. An elegant study by Perry et al. (2010) characterised the time course response of PGC-1α responses at both the mRNA and protein level following two weeks of high-intensity interval cycling. Indeed, 4 h after an acute bout of high-intensity interval cycling PGC-1α mRNA increased > 10 fold but returned to baseline 24 h after recovery. This saw-tooth effect was repeated for the two-week training duration, however, the magnitude of the 4 h increase progressively decreased over the two weeks. Concomitantly, PGC-1α at the protein level increased but unlike the mRNA responses there was a gradual increase, reaching a plateau in the final training bouts. These data suggest that a continual modification of exercise intensity and frequency is required to maintain exercise-induced PGC-1α expression. Whilst these data demonstrate the importance of PGC-1α it appears maximising its transcriptional response may be integral to enhanced mitochondrial adaptations.

Based on the above data, a schematic overview of a potential signalling pathway regulating mitochondrial adaptations to endurance training is shown in Figure 2.1. The studies contained in this thesis will be designed to test the presence of some of these signalling pathways in human skeletal muscle following differing modes of exercise and nutrient status.

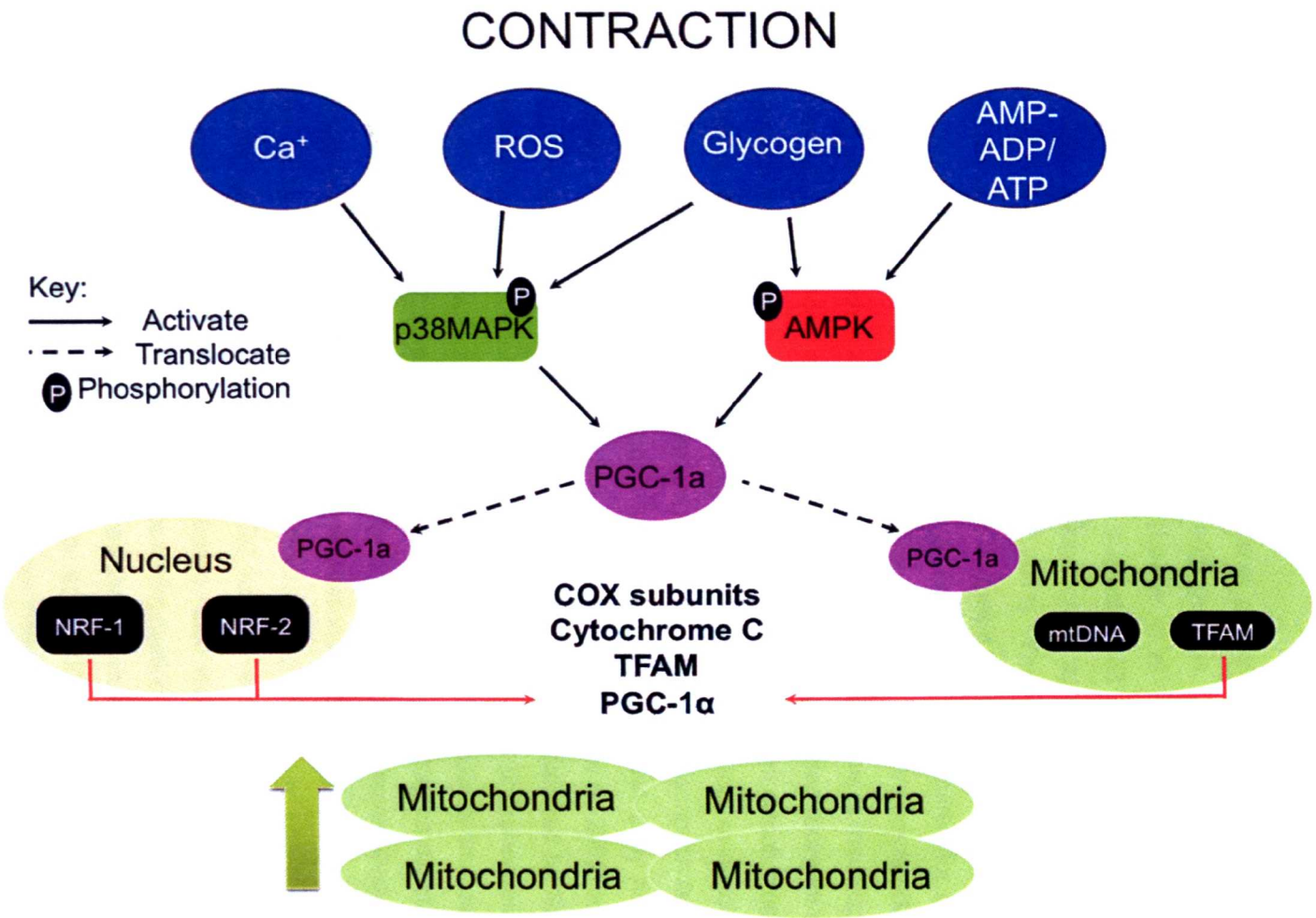

Figure 2. 2 PGC-1α mediated mitochondrial biogenesis. Contraction induces primary signalling of calcium, ROS, glycogen and AMP-ADP/ATP. These signals result in phosphorylation of p38MAPK and AMPK, which converge on PGC-1α, that subsequently translocates to the nucleus and mitochondria where it co-activates and interacts with transcription factors NRF-1/2 and Tfam. The result is an increase in protein content of COX subunits, cytochrome c, Tfam and PGC-1α, which culminates in an increase in mitochondrial content.

2.1.1.4. p53

The role p53 plays as a tumor suppressor protein and the effects it has on cell cycle arrest, apoptosis, pro- and antioxidant activity, angiogenesis, DNA repair, differentiation, fertilisation, aging and senescence is well documented (Levine et al. 2006). Whilst these were recognised as primary functions, emerging evidence suggests p53 is also involved in regulating pathways of energy metabolism and mitochondrial biogenesis (Lago et al. 2011; Matoba et al. 2006; Park et al. 2009; Saleem et al. 2009; Saleem et al. 2011). The most conclusive evidence in support of p53 regulating pathways for mitochondrial biogenesis derives from the ability of p53 to regulate the synthesis of cytochrome *c* oxidase 2 (SCO2), a protein required for the assembly of mtDNA-encoded cytochrome c oxidase (COX) II subunit in the COX IV complex (Matoba et al. 2006). The COX complex, which is located in the mitochondrial membrane, is made up of 13 subunits of which three are mitochondrial encoded whilst ten are nuclear-encoded (Schmidt et al. 2001). COX plays a pivotal role in aerobic respiration by accepting electrons from cytochrome *c* and transferring them to an oxygen molecule, thereby reducing it to H_20. In addition, evidence in animal and cell line models suggests p53 directly interacts with mitochondrial transcription factor A (Tfam) (Park et al. 2009) in the mitochondria, which is known to regulate mtDNA copy number and transcriptional activity (Yoshida et al. 2003). Interestingly, AMPK (Jones et al. 2005) and p38MAPK (She et al. 2001) directly phosphorylate p53 at Ser^{15} that is further supported by Saleem et al. (2009) who demonstrated an acute bout of exercise results in temporal phosphorylation of AMPK, p38MAPK and p53. Furthermore, knockout of p53 impairs aerobic capacity and exercise performance and results in greater fatigue leading to the possibility that p53 may be directly implicated in exercise-induced mitochondrial adaptations (Saleem et al. 2009). Moreover, evidence supporting a role for p53 in exercise-induced mitochondrial adaptations is illustrated by the discovery of a p53 binding site on the PGC-1α promoter (Irrcher et al. 2008), which may also suggest p53 plays a role in regulating the steady-state level of PGC-1α. As well as the effect that exercise has on p53 activation, nutrient deprivation, i.e. glucose, also leads to $p53^{Ser15}$ phosphorylation via AMPK (Jones et al. 2005). This action of glucose deprivation on p53 is particularly intriguing as potential mechanisms responsible for the enhanced skeletal muscle oxidative capacity following training with reduced carbohydrate availability (Hansen et al. 2005; Yeo et al. 2008; Morton et al. 2009) (see section 2.5). Whilst the understanding of exercise-induced p53 regulation has improved in the last decade, it is still unknown as to the actions of p53 in human skeletal muscle and thus further work investigating the p53 exercise-induced pathway may provide additional clues as to the regulation of exercise-induced mitochondrial adaptations.

2.2. OPTIMAL TRAINING STIMULUS TO INDUCE SKELETAL MUSCLE MITOCHONDIRAL BIOGENESIS

Whilst traditionally endurance training has been performed as a fixed steady state of moderate-intensity, a wealth of emerging evidence demonstrates that high-intensity interval training (exercise

models of which consist of both supra-maximal activity lasting 6-30 s and sub-maximal activity lasting 1-4 min) is a more potent mode of exercise for inducing similar or enhanced skeletal muscle adaptations when compared with high-volume endurance training. In this regard, coaches, practitioners and exercise physiologists are now becoming concerned with the optimal training stimulus to induce the greatest training response. The following section reviews recent data on the 30 s Wingate model before moving onto the more practical 1-min on/1-min off model followed by data characterising the efficacy of the 3-4 min high-intensity model. An overview of studies that have characterised the acute and longitudinal effects of high-intensity interval exercise can be seen in Tables 2.2 and 2.3.

The Wingate model was first introduced in 1974 (Nelson & Morehouse 1974) and is typically used as a test of an individual's anaerobic capacity. The test requires all out supra-maximal effort for 30 s with periods of active or passive recovery. The Wingate test has recently been used as a training model whereby 4-6 Wingate tests are interspersed by 4-min recovery. Remarkably, this mode of training induced comparable performance and skeletal muscle oxidative adaptations, i.e. cytochrome *c* oxidase (COX), COXII and COXIV content when compared against traditional continuous approaches whereby subjects cycled for 90-120 min at 65 % VO_{2peak} (Gibala et al. 2006). Furthermore, these adaptations took place despite total training time commitment being 2.5 h for the high-intensity group and 10.5 h for the endurance-training group. These data are further supported from the same laboratory (Burgomaster et al. 2008) in that 6 weeks of training that consisted of 4-6 Wingate tests interspersed by 4-min recovery 3 times a week induced similar performance and skeletal muscle oxidative adaptations (PGC-1α protein content) compared with 40-60 min cycling at 65 % VO_{2peak} 5 times a week. Again, these adaptations occurred despite the total exercise commitment being considerably less (90 % in this case) in the Wingate group compared with the continuous group. Taken together, the authors commented that this model of training, i.e. sprint interval training, is a time-efficient stimulus for inducing rapid skeletal muscle oxidative and metabolic alterations that are comparable to high-volume continuous training. Whilst these data are intriguing the mechanisms responsible are unclear. In order to gain an insight into the potential mechanisms that are responsible for the improvement in skeletal muscle oxidative capacity, Gibala et al. (2009) characterised an acute bout of Wingate training and demonstrated that following the 4th Wingate, phosphorylation of AMPK and p38MAPK increased concomitant with a 2-fold increase in expression of PGC-1α. The authors therefore postulated this acute response as a mechanism contributing to improved skeletal muscle oxidative capacity.

As a lack of time is regularly cited as the reason for lack of exercise adherence, it was suggested that the Wingate model of HIT exercise might be applicable to those at risk of diminished health (Babraj et al. 2009; Richards et al. 2010; Whyte et al. 2010). In this regard, Whyte et al. (2010) employed the Wingate model of training and prescribed 2-weeks of training to a group of sedentary/obese individuals. They observed that this model of training is indeed capable of increasing insulin sensitivity and fat oxidation and decreasing systolic blood pressure and carbohydrate oxidation in the fasted state. Furthermore, performance and hip and waist

circumference improved following training, therefore, demonstrating this model of training as a time-efficient mode of exercise for improving vascular and metabolic parameters in sedentary/obese individuals. Collectively, these data demonstrate that this model of training is beneficial for improving aspects of human health and performance and that similar adaptations occur compared with traditional continuous approaches despite a reduction of up to 90 % of total exercise time commitment.

Whilst the Wingate model appears beneficial from a time perspective, this mode of exercise also contains several limitations. For example, in order to carry out the exercise a specialist piece of equipment (a mechanically braked cycle ergometer) is required. Furthermore, there are reports of light-headedness and feelings of nausea (Richards et al. 2010) which may also make this Wingate type of exercise an impractical model for some individuals. As such, Little et al. (2010b) developed a practical high-intensity interval model that required subjects to perform six sessions over 2-weeks of 8-12, 60 second bouts at 100 % of peak power interspersed by 75 s of rest. Similar to previous findings, exercise performance and maximal activity of citrate synthase and COX increased as well as total protein content of citrate synthase, COXII, COXIV and mitochondrial transcription factor A (Tfam). These findings are similar to those observed following Wingate training although the difference between protocols is a decreased intensity, longer duration and a shorter recovery period. Nevertheless, total exercise time remained < 30 min thus resulting in a time-efficient stimulus for improved mitochondrial adaptations. Whilst these changes occurred in recreationally active men, the same authors (Little et al. 2011a) subsequently evaluated the efficacy of HIT in type II diabetic subjects. Despite a slight difference in protocol (exercise bouts performed at 90 % HRmax and recovery time reduced to 60 seconds), average 24 h blood glucose concentration decreased and 3 h post-prandial areas under the curve for breakfast, lunch and dinner had all reduced. From a skeletal muscle perspective, training increased citrate synthase activity and protein content of complex II, complex III, subunit IV of complex IV, mitofusin and GLUT4. Taken together, these data demonstrate low-volume HIT is capable of rapidly improving glucose control as well as skeletal muscle markers of metabolic control in type II diabetic patients. In addition, Hood et al. (2011) demonstrated an improved insulin sensitivity (35 %) and muscle oxidative capacity as shown by increases in protein content of citrate synthase (35 %), cytochrome c oxidase subunit IV (35 %), PGC-1α (56 %) and GLUT4 (260 %) in aged adults. Furthermore, Gillen et al. (2012) reported a 65 % improvement in time spent in hyperglycaemia following an acute bout of HIT (the same used in previous studies) demonstrating an improved glycaemic control in type II diabetic patients following HIT. All together, these data demonstrate that despite altering the exercise bout to a more practical model (which includes lower intensity but longer duration), HIT is still a time-efficient stimulus for inducing beneficial metabolic and skeletal muscle adaptations that manifest in improvements in human health.

Although the benefits of the 1-min on/1-min off model is well documented, this short duration bout of exercise may not be applicable to elite athletes where durations of intervals typically last up to 4-min (Stone & Kilding 2009). In this regard, Perry et al. (2008) examined the responses of a 4-min

model whereby subjects were required to perform 6-weeks of 10 x 4-min bouts at 90 % of VO_{2peak} interspersed by 2-min recovery three times a week. Despite this model being an hour in duration, exercise intensities are still as high as the 1-min model previously described. Concomitant with improvements in power output and maximal oxygen uptake, there were improvements at rest in cytochrome c oxidase IV (18 %), and maximal activities of citrate synthase (26 %), β-hydroxacyl-CoA dehydrogenase (29 %), aspartate amino transferase (26 %) and pyruvate dehydrogenase (21 %), fatty acid (FAT/CD36, FABPpm) and lactate (MCT1 and MCT 4) transporters and increased muscle glycogen (59 %). During exercise, adaptations also occurred whereby 6-weeks HIT induced a reduction in glycogenolysis, lactate accumulation and substrate utilisation and increased fat oxidation.

In an attempt to provide a time-course of skeletal muscle molecular adaptations the same authors (Perry et al. 2010) utilised the same exercise protocol, however, reduced the training period to 2-weeks. In addition, muscle biopsies were taken at regular intervals during the course of the 2-weeks so as to characterise the early (4 h) transcriptional response and longer-term (24 h) protein changes to training. Expression of PGC-1α mRNA increased 10-fold following the first bout of exercise and returned to baseline 24 h post exercise. This 'saw-tooth' effect continued until the cessation of the 2-week period, however, with smaller increases following each bout. In line with this observation, citrate synthase maximal activity progressively increased over the course of the 2-weeks along with increases in PGC-1α protein. This time-course of temporal skeletal muscle molecular adaptation can be seen in Figure 2.2.

Figure 2. 3 Temporal responses of PGC-1α mRNA and PGC-1α protein and CS maximal activity following 2-weeks high-intensity interval training (Perry et al. 2010).

Collectively, these data conclude that from a short-term perspective high-intensity interval training is both a time-efficient means and powerful mode of exercise for inducing improvements in performance and mitochondrial function and content, which have implications for both health and athletic populations. Subtle differences in protocols between studies suggest how alterations in the intensity and duration of the exercise protocol underpin the muscle specific changes that occur. Whilst these data are highly informative, the molecular mechanisms underpinning the potency of HIT remain unclear and indeed it is still unknown whether high-intensity interval exercise induces superior cell signalling responses compared with moderate-intensity continuous exercise when matched for work done, duration, average intensity and duration.

Table 2. 1 High-intensity interval longitudinal training studies showing improvements in mitochondrial function and content and performance

Author	Subjects	Exercise Protocol	Findings
Gibala et al. (2006)	Sixteen healthy males	2 weeks cycling, 3 times a week 1) 4-6 30 second Wingate's interspersed with 4.5 min recovery (SIT) 2) 90-120 min at 65 % $\dot{V}O_{2peak}$ (ET)	• 50 KJ (ET; 3.5 %, SIT; 4.1 %) and 750 KJ (ET; 7.5 %, SIT; 10.1 %) time trial performance improved • COX, COXII and COXIV increased similarly in both groups • Muscle buffering capacity increased (ET; 4.2 %, SIT; 7.6 %) • Resting muscle glycogen (ET; 17 %, SIT; 28 %) increased similarly in both groups
Helgerud et al. (2007)	Fifty-five healthy males	8 weeks running, 3 times a week 1) 45-min at 70 % HR_{max} (LSD) 2) 24.25 min at 85 % HR_{max} (anaerobic threshold) (LT) 3) 15 s on at 95 % HR_{max} /15 s off at 70 % HR_{max} x 47 reps 4) 4 x 4 min intervals at 90-95 % HR_{max} interspersed with 3-min recovery bouts at 70 % HR_{max}	• VO_{2max} increased greater in 15/15 and 4x4 than LDS and LT • Running economy improved in all groups (7.5-11.7 %) • Stroke volume increased greater in 15/15 and 4x4 than LDS and LT • Velocity at lactate threshold (vLT) improved in all groups (9.6 %)
Mohr et al. (2007)	Thirteen healthy males	8 weeks running, 3-6 times a week 1) 15 x 6 s runs at 95 % max speed interspersed with 1-min recovery (ST) 2) 8 x 30 s runs at 130 % $\dot{V}O_{2max}$ interspersed with 1.5 min recovery (SET)	• Na^+ /H^+ exchanger isoform 1 (31%) and Na^+-K^+-ATPase isoform α2 (68%) increased in SET only • Na^+-K^+-ATPase β 1-isoform and monocarboxylate transporter 1 (MCT1) increased in both groups • ST improved sprint performance, however, SET improved performance in exhaustive continuous treadmill running • Improvement in Yo-Yo IRT2 was greater in SET

			(29 %) than ST (10 %)
Burgomaster et al. (2008)	Twenty (5 male and 5 female per group)	6 weeks cycling 1) 4-6 30 second Wingate's interspersed with 4.5 min recovery (SIT) (3 x week) (SET) 2) 40-60 min at 65 % $\dot{V}O_{2peak}$ (5 x week) (ET)	• Peak power output increased 17 % in SIT and 7 % in ET whereas mean power output increased (7 %) in SET only • Maximal activities of CS and β-Had and protein content of PDH increased similarly between groups • Muscle glycogen was increased at 60-min of exercise post-training and glycogenolysis reduced similarly in both groups
Perry et al. (2008)	Eight healthy (3 males, 5 females)	6 weeks cycling, 3 times a week 10 x 4 min at 90 % $\dot{V}O_{2peak}$ interspersed with 2 min rest	• Power output increased 21 % and time to exhaustion at pre-training 90 % VO_{2peak} improved 111 % • CS maximal activity increased 26 % and COXIV protein increased 18 % • β-Had (29 %) maximal activity and protein content of FAT/CD36 (16 %), FABPpm (30 %) all increased • Amino aspartate transferase (26 %) and PDH (21 %) increased and muscle glycogen increased 56 % • GLUT4 (21 %), MCT1 (14 %) and MCT4 (16 %) protein all increased
Babraj et al. (2009)	Sixteen health males	2 weeks cycling, 3 times a week 4-6 30 second Wingate's interspersed with 4.5 min recovery (SIT)	• Area under the glucose (12 %), insulin (37 %) and NEFA (26 %) curves all reduced • Insulin sensitivity improved 23 %
Iaia et al. (2009)	Seventeen endurance trained males	4 weeks running, 3.4-4 times a week 1) 8-12 x 30 s at 93 % max speed interspersed by 3-min recovery (SET) 2) 52-min of moderate-intensity continuous running (Con)	• Running economy improved in SET only • UCP3 tended to be higher in SET only • CS and β-Had remained unchanged in both groups • Maximal oxygen uptake and 10km time trial unchanged

Perry et al. (2010)	Nine healthy males	2 weeks cycling, 3 times a week 10 x 4 min at 90 % $\dot{V}O_{2peak}$ interspersed with 2 min rest	• mtDNA increased 118 %, Tfam mRNA increased 54 % and Tfam protein was unchanged. NRF-2 protein increased 50 % • CS mRNA (48 %) and β-Had mRNA (17 %) increased and maximal activities of CS (17 %) and β-Had (8 %) increased • COXIV mRNA (29 %) and COXIV protein (20 %) increased • PGC-1α mRNA increased 10-fold with a gradual decrease during training. PGC-1α protein increased 42 % • PGC-1β increased and remained 61-87 % higher that rest during training. PGC-1β protein increased following session 5 but retuned to pre-levels by post-training • PPARβ/δ mRNA increased 50 % and decreased to resting following each session. PPARβ/δ protein increased 31 %. PPARα protein increased 60 %.
Little et al. (2010)	Seven healthy males	2 weeks cycling, 3 times a week 8-12 x 60 s intervals at 100 % PPO interspersed with 75 s recovery	• 50 KJ (11 %) and 750 KJ (9 %) time trial performance improved • Maximal activity of COX increased 29 %, and protein content of COX II (35 %) and COXIV (38 %) increased • CS maximal activity (16 %) and protein content (20 %) increased • Nuclear PGC-1α increased 24 % and SIRT1 protein (56 %) and Tfam (37 %) protein increased • GLUT4 protein (119 %) and muscle glycogen (17 %) increased
Richards et al. (2010)	Five males and	2 weeks cycling, 3 times a week	• Insulin sensitivity increased

	seven females	4-6 30 second Wingate's interspersed with 4.5 min recovery (SIT)	
Whyte et al. (2010)	Ten overweight/obese sedentary males	2 weeks cycling, 3 times a week 4-6 30 second Wingate's interspersed with 4.5 min recovery (SIT)	• Maximal oxygen uptake and PPO improved • Insulin sensitivity increased • Fat oxidation and CHO oxidation increased and decreased, respectively, as rest • Systolic blood pressure decreased • Hip and waist circumference decreased
Hood et al. (2011)	Seven sedentary healthy males	2 weeks cycling, 3 times a week 8-12 x 60 s intervals at 60 % PPO interspersed with 60 s recovery	• Protein content of CS (35 %), cytochrome c subunit IV 3(5 %) and PGC-1α increased 56 % • GLUT protein (260 %) and Insulin sensitivity (35 %) improved
Little et al. (2011)	Eight diabetic males	2 weeks cycling, 3 times a week 8-12 x 60 s intervals at 90 % HR_{max} interspersed with 60 s recovery	• Average 24 h blood glucose reduced from 7.6 ± 1.0 to 6.6 ± 0.7 $mmol.L^{-1}$ • CS maximal activity increased 20 % • Protein content of complex 2 70 kDa subunit (37 %), complex 3 core 2 (51 %) and complex IV subunit IV (68 %) • Mitofusin 2 (71 %) and GLUT4 (369 %) both increased
Serpiello et al. (2011)	Ten healthy males	4 weeks running, 3 times a week 5 x 4 second sprints interspersed with 20-sec between sprints and 4.5 min between sets	• AMPK protein (18 %), AMPK phosphorylation (38 %) and ACC protein (46 %) increased • PGC-1α protein (33 %) and NRF-1 mRNA (33 %) increased • Tfam mRNA (18 %) and COXIV protein (14 %) increased

Table 2. 2 High-intensity interval studies characterising the acute mitochondrial responses

Author	Subjects	Exercise Protocol	Findings
Gibala et al. (2009)	Six healthy males	4 x 30 second Wingate's interspersed with 4.5 min recovery	• P-AMPK and P-p38MAPK increased immediately post 4th interval bout • PGC-1α mRNA increased 2-fold 3 h post exercise
Wang et al. (2009)	Nine sedentary males	1) 90 min of 12 s at 120 % $\dot{V}O_{2max}$ interspersed with 18 s 20 % VO_{2max} (IE) 2) 90 min continuous cycling at 67 % VO_{2max} (CE)	• Muscle glycogen reduced 35-40 % in both conditions • PGC-1α mRNA increased 10-fold and 13-fold following CE and IE, respectively and PRC increased 6-fold in both conditions • AMPKα1 mRNA increased following CE only • NRF-2 and Tfam mRNA increased 1.7 and 2.1-fold, respectively, in CE only • TFB1M and TFB2M mRNA increased 1.7 and 2.8-fold, respectively, in CE only • PDK4 mRNA increased following both conditions and LPL mRNA increased 2-fold in CE only
Wang et al. (2011)	Six male and 1 female healthy but sedentary	1) 90 min of 12 s at 120 % $\dot{V}O_{2max}$ interspersed with 18 s 20 % $\dot{V}O_{2max}$ (IE) 2) 90 min continuous cycling at 67 % $\dot{V}O_{2max}$ (CE)	• Muscle glycogen reduced 40 % in both conditions • PGC-1α and PDK4 mRNA increased 5-8-fold in both type I and II fibres following both CE and IE
Gillen et al. (2012)	Seven type II diabetic individuals	8-12 x 60 s intervals at 90 % HR_{max} interspersed with 60 s recovery	• Hyperglycaemia as measured as time above 10 $mmol.L^{-1}$ reduced • Post-prandial hyperglycaemia, as measured by area under the glucose curve was reduced after exercise

2.3. NUTRITIONAL MODULATIONS OF TRAINING ADAPTATION

In addition to modification of the training stimulus through manipulation of the training protocol (i.e. varying intensity and duration), the energy status of the cell is now emerging as a key regulator in modulating training-induced adaptations. Since the early work of Bergstrom and colleagues (1967), it has become well documented that CHO feeding prior to and during prolonged sub-maximal exercise enhances performance and delays time to fatigue (Bergström et al. 1967; Coyle et al. 1986). Recreational and elite athletes have therefore consistently consumed high CHO diets in order to increase muscle glycogen stores to subsequently train harder and longer with the aim of achieving enhanced training adaptations. However, feeding CHO prior to training now appears to attenuate the acute signalling response and alters fuel selection and utilisation during subsequent exercise (Cluberton et al. 2005; Pilegaard et al. 2005), therefore, affecting the rate of transcription and translation of specific proteins associated with mitochondrial biogenesis. Such data led to the development of the 'train low' approach to training whereby deliberately commencing exercise with reduced CHO availability may enhance the training response and skeletal muscle adaptations. The following section reviews recent evidence of CHO availability manipulation through 1) reduced muscle glycogen, 2) fasted exercise and 3) restricting CHO intake in the recovery period, before examining the molecular mechanisms that may underpin these training-induced adaptations.

2.3.1. Carbohydrate availability and the training response/adaptation

The first study to examine the acute response to endurance exercise with reduced muscle glycogen was that by Pilegaard et al. (2002). In this study, subjects underwent glycogen-depleting exercise the prior afternoon followed by a diet high or low in CHO. The authors reported that commencing exercise with reduced muscle glycogen enhanced the transcriptional activation of metabolic genes (PDK4, UCP3, HKII, LPL), therefore, surmising that transcriptional activation of metabolic genes may be sensitive to muscle glycogen and/or FFA availability. Whilst these findings demonstrate commencing exercise in a reduced muscle glycogen state enhances the acute transcriptional response it was unknown as to what the effect is of cumulative bouts of reduced muscle glycogen training. In this regard, Hansen et al. (2005) subjected seven untrained males to knee extensor exercise 5 d/wk for 10 wk. Subjects trained both legs but according to a different schedule. Subjects trained one leg once every day (HIGH) and the other leg twice a day every other day (AM and PM) meaning every second training session in the twice a day group was commenced with low muscle glycogen (LOW). Muscle biopsies were taken from the vastus lateralis pre-training, at 5 wk and 10 wk and performance tests were undertaken to examine exercise capacity. Surprisingly, resting muscle glycogen content in LOW increased post-training compared to no change in HIGH, there were superior increases in citrate synthase (CS) activity following LOW, and exercise performance (time to exhaustion) increased in LOW only, suggesting that altering substrate availability (i.e. reduced muscle glycogen) during training enhances training adaptation and exercise performance.

Whilst these initial data proved insightful, the exercise protocol employed by Hansen and colleagues can be critiqued in that it is not representative of real world exercise and the subjects studied were untrained. Therefore, Yeo et al. (2008) studied the effect of cycling exercise with low muscle glycogen availability in highly trained endurance athletes. Subjects were split into a control (HIGH) group and experimental (LOW) group. The HIGH group trained 6 days/wk for 3 wk alternating between 100-min steady state cycling at 70 % VO_{2peak} (AT) and high-intensity interval cycling (HIT; 8 x 5 min bouts at maximal effort with 1-min recovery in between bouts). The LOW group trained twice per day, every second day, performing AT in the morning followed by HIT 1-2 h later. An experimental trial was performed prior to and after the training period where subject's rode for 60-min steady state at 70 % VO_{2peak} (60SS) followed by a 60-min time trial. Muscle biopsies from the vastus lateralis were obtained immediately before and after the 60SS and expired gas was collected to examine substrate oxidation. Results demonstrated that resting muscle glycogen stores increased in LOW compared to HIGH after training, maximal enzyme activity of CS and β-HAD increased in LOW compared to HIGH and COX subunit IV increased in LOW compared to HIGH after training. In addition, rates of fat oxidation were enhanced to a greater extent in LOW compared to HIGH. However, exercise performance was similar between LOW and HIGH after training despite the superior increases in mitochondrial adaptations and resting muscle glycogen in LOW. These data therefore confirm that even in well trained subjects, a 'cycling' of muscle substrate stores can further enhance the training stimulus. Using a similar training design to Yeo et al, Hulston and colleagues (Hulston et al. 2010) also reported similar time-trial performance with both conditions, and an enhanced ability to oxidise lipids in the LOW group only. Taken together, these data demonstrate that commencing exercise whereby a portion of sessions is commenced with reduced muscle glycogen enhances the adaptive training response.

Another potential approach to commencing exercise with reduced CHO availability is that of fasted exercise. Data from two independent laboratories (Civitarese et al. 2005; Cluberton et al. 2005) demonstrate that commencing an acute bout of endurance exercise following an overnight fast enhances the transcriptional response of exercise-induced metabolic genes compared to feeding breakfast and/or CHO before and during exercise, which is shown to attenuate the transcriptional response. Whilst these data are intriguing from an acute perspective it unknown if these changes also occur following accumulated bouts of exercise. In an attempt to elucidate whether fasted training enhances training adaptation, Van Proeyen et al. (2011) subjected twenty males to a 6 week block of training whereby they either commenced exercise in the fasted state and received breakfast after training or commenced exercise following breakfast. The authors reported increased citrate synthase and β-HAD maximal activities following training in the fasted state compared to no change when exercising in the fed state. Despite these improvements in oxidative capacity both the fasted and the fed group showed similar improvements in VO_{2max} and performance. Collectively, these findings confirm and extend previous studies in that restricting CHO availability enhances oxidative enzyme adaptations. An overview of studies examining this 'train low' phenomenon can be seen in Table 2.4.

In addition to commencing exercise with reduced muscle glycogen or in the fasted state, another potential approach is to restrict CHO in the hours following exercise (recovery stage). Indeed, Pilegaard et al. (2005) subjected nine males to 75 minutes of cycling at 75 % VO_{2max} followed by a 24 h diet that was either high or low in CHO. Whilst muscle glycogen was restored to baseline by 5 h in the high group, it remained depressed throughout the 24 h recovery period. Plasma free fatty acid levels was 3-7 fold higher in the LOW group during the 24 h recovery period whilst leg glucose uptake was 5-15 fold higher in the HIGH group. Although exercise itself induced a transcriptional response in a number of genes, i.e. PDK4, UCP3, LPL, CPT1, HKII, PGC-1α and PPARα, feeding a high CHO diet reversed this transcriptional response of PDK4, UCP3, LPL and CPT1 5-8 h post-exercise compared to sustained/enhanced activation up until 24 h in the LOW CHO group. These data suggest that feeding CHO in the recovery period following exercise may attenuate the acute transcriptional response initiated by endurance exercise. Whilst restricting CHO in the 24 h post-exercise may not be a practical approach with the athletic population, one way of achieving this 'low CHO availability' approach may be to train in the evening and then sleep whilst low in muscle glycogen (sleep low). In theory, this would then ensure the transcriptional response induced by the exercise bout is maintained for as long as possible in a time in which exercise and feeding is not undertaken. Taking into account the findings of Van Proeyen and colleagues (2011), exercise may also be commenced in the fasted state in the morning, therefore, providing the individual with a 'double hit' of reduced CHO availability training. This mode of dietary and exercise manipulation is yet to be tested and therefore warrants investigation.

Table 2. 3. Skeletal muscle adaptations to a period of training with normal/high carbohydrate availability v low carbohydrate availability

Reference	Subjects	Duration	Exercise Protocol	Findings
Hansen et al. (2005)	7 untrained men	5 x week for 10 weeks	Knee extensor exercise with one leg trained in a low glycogen (LOW) protocol and the other leg trained in a high glycogen (HIGH) protocol.	• CS increased greater in LOW • β-Had increased in LOW only
Mortensen et al. (2007)	7 untrained men	5 x week for 10 weeks	Knee extensor exercise with one leg trained in a low glycogen (LOW) protocol and the other leg trained in a high glycogen (HIGH) protocol.	• No effect of condition on PGC-1α mRNA expression following an acute bout of 3 h knee extensor exercise, i.e. similar fold change before and after training
De Bock et al. (2008)	20 active men	3 x week for 6 weeks	1-2 h cycling at 75 % $\dot{V}O_{2max}$ with one group training in the fasted state (LOW) and the other with CHO feeding before (HIGH).	• SDH increased similarly between groups • GLUT4 increased similarly between groups • HK II increased similarly between groups • FABPm increased in LOW only
Yeo et al. (2008)	14 trained cyclists / tri-athletes	4 x week for 3 weeks	100-min steady state cycling @ 70 % $\dot{V}O_{2max}$ followed either 2 h or 24 h later by 8 x 5-min @ maximal pace for 3 weeks	• CS increased in LOW only • β-Had increased in LOW only • COXIV increased in LOW only • PGC-1α showed no change
Morton et al. (2009)	30 active men	4 x week for 6 weeks Low+Glu and Low+Pla 2 x session every second day and NORM once a day	5 x 3 min @ 90 % $\dot{V}O_{2max}$ interspersed with 6 x 3 min @ 50 % $\dot{V}O_{2max}$. Low+Glu ingested CHO before and during exercise, Low+Pla ingested placebo before and during and NORM ingested water	• PGC-1α increased similarly in all conditions • HSP70 increased similarly in all conditions • HSP60 increased similarly in all conditions • αB Crystallin increased similarly in all conditions • SDH increased greater in Low+Pla compared to Low+Glu and Norm

Hulston et al. (2010)	14 trained cyclists	6 x week for 3 weeks	HIGH performed alternating days of steady state (AT) (90 min @ 70 % $\dot{V}O_{2max}$) and HIT (8x5 min efforts). LOW performed AT and HIT in the same day every other day	• β-Had increased in LOW only • FAT/CD36 tended to increase greater in LOW than HIGH • GLUT-4 increased greater in HIGH than LOW
Van Proeyen et al. (2010a)	20 active men	4 x week for 6 weeks	1-1.5 h cycling at 70 % $\dot{V}O_{2max}$ with one group training in the fasted state (LOW) and the other with CHO feeding before (HIGH).	• CS increased in LOW only • β-Had increased in LOW only
Van Proeyen et al. (2010b)	20 active men	3 x week for 6 weeks	1-2 h at 75 % $\dot{V}O_{2max}$ with one group training in the fasted state (LOW) and the other with CHO feeding before and during exercise (HIGH).	• Phosphorylation of AMPK^{Thr172} abolished post training in both conditions • Phosphorylation of ACC^{Ser229} abolished post training in both conditions • Phosphorylation of p38MAPK$^{Thr180/Tyr182}$ abolished post training in both conditions
Van Proeyen et al. (2010c)	20 active men	4 x week for 6 weeks	60-90 min at 70-85 % $\dot{V}O_{2max}$ with one group training in the fasted state (LOW) and the other with CHO feeding before and during exercise (HIGH).	• GLUT4 protein increased in LOW only • Phosphorylation of AMPKα increased in LOW only • FAT/CD36 mRNA post training increased in LOW only • CPT-1 mRNA post training increased in LOW only • CS increased similarly between conditions

2.3.2. Mechanisms underpinning changes in carbohydrate modulated training adaptation

An overview of studies examining the acute responses to carbohydrate modulated training adaptation can be found in Table 2.5. The first study to characterise the acute skeletal muscle signalling responses when in a state of low muscle glycogen was by Wojtaszewski et al. (2003). Muscle glycogen was manipulated to two extremes whereby the HIGH group had starting muscle glycogen of approximately 900 mmol/kg dry wt and in the LOW group approximately 160 mmol/kg dry wt. The authors reported phosphorylation of AMPK before exercise began in the LOW group only and this difference persisted up until 1 h post exercise. Chan et al. (2004) subsequently reported that endurance exercise commenced with reduced muscle glycogen (163 ± 27 mmol glucosyl U/kg dry mass) resulted in greater nuclear abundance of phosphorylation of p38MAPK both before and after exercise. It wasn't till after the host of reduced muscle glycogen training studies (see section 2.4.1) that there were further developments in our understanding of the mechanisms that govern mitochondrial adaptations when training with reduced muscle glycogen. In response to their training study in 2008, Yeo and colleagues (2010) set out to try and determine the mechanisms responsible for the changes they observed previously. In this study, the authors utilised a similar model to their previous study (Yeo et al. 2008), where endurance trained cyclists performed an acute bout of high-intensity interval exercise (HIT) (8 x 5-min at a self selected intensity) either 1-2 h following aerobic training (AT) (100-min at 70 % $\dot{V}O_{2max}$) (LOW) or 24 h following AT (HIGH). The data show a significant increase in P-AMPK following HIT in both LOW and HIGH though the magnitude of increase was greater in LOW than HIGH. In contrast, there was no activation of p38MAPK either before or after HIT training in both the HIGH and LOW conditions.

In a differently designed train-low study, Cochran et al. (2010) recruited 10 healthy active men that were not specifically trained and performed 5 x 4-min interval bouts (HIE) at 90 – 95 % heart rate reserve (HRR), once in the morning (AM) and then 3 h after (PM). During the 3 h recovery period, subjects in the carbohydrate fed (HI-HI) group consumed 1.2 g · kg body wt^{-1}· h^{-1} for the first 2 h and in the non-carbohydrate fed group (HI-LO) subjects consumed an isovolumetric taste-matched placebo. Skeletal muscle biopsies were taken from the vastus lateralis pre-, post- and 3 h post the AM session and immediately post- PM session. In contrast to the findings of Wojtaszewski et al. (2003) and Yeo et al. (2010), P-AMPK was not different between the fed and unfed group, however, consistent with findings from Chan and colleagues, P-p38MAPK was 50 % higher in LOW following the PM session compared with HIGH. Downstream of these signalling events, PGC-1α mRNA content significantly increased 3 h after AM with no difference regardless of feeding and stayed elevated following PM with no difference between conditions. Furthermore, COXIV mRNA showed similar increases 3 h following the AM sessions with no difference between groups or after the PM session. Taken together these data suggest both p38MAPK and AMPK may be two nutrient sensitive signalling molecules regulating the enhanced oxidative adaptations associated with training with reduced CHO availability.

Whilst the majority of studies have analysed the effect of reduced muscle glycogen on signalling responses others have also examined the effect of commencing exercise in the fasted state or following CHO feeding. In this regard, Akerstrom et al. (2006) reported following 2 h knee extensor exercise that phosphorylation of AMPKα2 was attenuated with CHO feeding compared with phosphorylation in the fasted state. In contrast, Lee-Young and colleagues (2006) failed to observe any difference in phosphorylation of AMPKα2 when fed CHO or placebo (Lee-Young et al. 2006). Despite the differences observed for AMPK signalling, one common observation is that CHO has no effect on PGC-1α mRNA expression. For example, Russell et al. (2005) failed to observe any difference in PGC-1α mRNA expression after glucose or water ingestion following 2 h cycling at 50 % W_{max} and Cluberton et al. (2005) also demonstrated glucose ingestion before 60-min cycling at 75 % $\dot{V}O_{2max}$ had no attenuating effects on PGC-1α mRNA expression compared with placebo.

Despite PGC-1α being purported as the master regulator of mitochondrial biogenesis it is interesting that carbohydrate has no apparent effect on this transcriptional co-activator at least in terms of its transcriptional regulation. It is therefore possible that there is an alternative signalling pathway that may be contributing to the training responses during differing energy states. In keeping with this hypothesis, one potential candidate that has received little attention is p53 (see section 2.3.2.3 for review). Indeed, Jones et al. (2005) reported an AMPK-p53 signalling relationship when in a nutrient deprived state and given that both AMPK and p38MAPK respond to differing levels of energy status and that p53 activation increases in a temporal fashion following AMPK and p38MAPK, it is tempting to speculate that this pathway may have a potential role to play in the regulation of train low induced skeletal muscle oxidative adaptations. This hypothesis, however, has yet to be tested and therefore warrants further investigation.

Table 2. 4 Acute signalling and metabolic responses to exercise with normal/high carbohydrate availability vs. low carbohydrate availability

Reference	Subjects	Exercise Protocol	Findings
Pilegaard et al. (2002)	Study a) 6 active males	1-legged muscle glycogen depleting exercise followed by 2-legged cycling the next day	• PDK4, LPL and HKII elevated pre-exercise in reduced muscle glycogen leg only • PDK4 and UCP3 elevated in response to exercise in the reduced muscle glycogen leg only
	Study b) 6 active males	3 h of 2-legged knee extensor exercise with normal of low muscle glycogen	• PDK4 and UCP3 elevated in response to exercise in the reduced muscle glycogen leg only
Wojtaszewski et al. (2003)	8 trained men	60 min cycling at 70 % $\dot{V}O_{2max}$; 1 group with reduced pre-exercise muscle glycogen (LOW) and 1 group with loaded muscle glycogen (HIGH).	• AMPKα2 activated in LOW only • ACC phosphorylation greater in LOW compared with HIGH
Russell et al. (2005)	7 untrained men	2 h cycling at 50 % Wmax with HIGH condition fed CHO before, during and after exercise. LOW condition restricted CHO before, during and after exercise.	• PGC-1α increased similarly between conditions (12-fold) • PRC increased similarly between conditions (3.3-fold)
Civitarese et al. (2005)	7 untrained men	2 h cycling at 50 % PPO with HIGH condition fed CHO before, during and after exercise. LOW condition restricted CHO before, during and after exercise.	• GLUT-4 increased in LOW with no change in HIGH • PDK-4 increased in LOW with no change in HIGH • HIGH resulted in suppression of AMPK, CD36, CPT-1 and UCP3 • No change in ACC with exercise or condition
Cluberton et al. (2005)	6 active men	60 min cycling at 75 % $\dot{V}O_{2max}$ with HIGH condition fed CHO before, during and after exercise. LOW condition restricted CHO before, during and after exercise	• PGC-1α increased similarly between conditions (3-4-fold) • PDK-4 increased 23-fold in LOW with no change in HIGH • GLUT-4 increased 2-fold in LOW with no change in HIGH • UCP3 increased 7-fold in LOW with no change in HIGH
Pilegaard et al.	9 active men	75 min cycling at 75 % $\dot{V}O_{2max}$ with HIGH	• PDK4 returned to baseline 8 h post-exercise in HIGH

(2005)		condition fed high CHO diet in 24 h post-exercise. LOW condition consumed low CHO diet in 24 h post-exercise	• UCP3 returned to baseline 8 h post-exercise in HIGH • LPL returned to baseline 8 h post-exercise in HIGH • CPT1 returned to baseline 8 h post-exercise in HIGH • All these genes remained elevated for 24 h in LOW
Akerstrom et al. (2006)	9 active men	2 h one-legged knee extensor exercise with LOW condition unfed and HIGH condition fed carbohydrate before exercise.	• Activation of AMPKα2 attenuated in HIGH condition • No effect of CHO supplementation on AMPKα1 activity
Lee Young et al. (2006)	9 active men	120 min cycling at 65 % $\dot{V}O_{2max}$ with either no CHO feeding (LOW) or CHO feeding (NORM).	• Phosphorylation of AMPKα2 was similar in both groups • Phosphorylation of ACC was similar in both groups
Steinberg et al. (2006)	7 active men	60 min cycling at 70 % $\dot{V}O_{2max}$ with either reduced pre-exercise muscle glycogen (LOW) or normal muscle glycogen (NORM).	• Activation of AMPKα2 was enhanced in LOW • Activation of ACC was enhanced in LOW
Yeo et al. (2010)	12 trained cyclists / tri-athletes	100-min steady state cycling at 70 % $\dot{V}O_{2max}$ followed either 2 h or 24 h later by 8 x 5-min at maximal effort	• AMPK increased 2-fold in LOW and 0.6 HIGH • p38 showed no change between groups or with training
Cochran et al. (2010)	10 active men	Two bouts of 5 x 4 min cycling at 90–95% of heart rate reserve separated by 3 h of recovery during which subjects ingested a high-CHO drink (HIGH) or placebo (LOW) before PM exercise.	• AMPK increased similarly between conditions post PM • p38MAPK increased greater in LOW post PM than HIGH • PGC-1α increased similarly between conditions post PM exercise • COXIV increased similarly between conditions post AM exercise

2.4. Summary

Mitochondrial biogenesis has long been one of the most well studied adaptations of skeletal muscle to endurance training. The introduction of molecular biology to the exercise sciences has allowed exercise physiologists to examine the molecular signalling pathways regulating this hallmark adaptation. It is currently thought that mitochondrial adaptations to endurance training are due to the cumulative effects of transient changes in gene expression in response to each training session and that key signalling proteins and transcription factors are involved in a co-ordinated regulation of both the nuclear and mitochondrial genomes. An increased understanding of these pathways would allow for the development of highly tailored training programs to maximise the training stimulus. Furthermore, the role of the specific training stimulus and nutrient availability is increasingly being recognised as key regulators determining the extent of skeletal muscle adaptation to a given work load. The studies contained in this thesis are designed to further examine how manipulation of the exercise protocol (e.g. HIT v CONT) and nutrient availability (e.g. LOW v HIGH CHO provision) can regulate the acute cell signalling pathways thought to regulate mitochondrial adaptations to exercise.

CHAPTER 3

General Methodology

3.1. GENERAL METHODOLOGY

3.1.1. Location of testing and ethical approval

All of the exercise testing and biochemical analysis was carried out in the physiology and biochemistry laboratories at the Research Institute for Sport and Exercise Science. The ethical committee of Liverpool John Moores University approved all experimental protocols and exercise procedures.

3.1.2. Subjects

All of the subjects who participated in each study were young healthy and recreationally active males. A comparison of the subjects' physical and physiological characteristics is shown in Table 3.1. All participants gave written informed consent to participate after details of the study had been fully explained. Participants refrained from additional exercise outside of the study requirements and from alcohol and caffeine intake for at least 24 h prior to any of the testing sessions. All participants had no history of neurological disease or musculoskeletal abnormality and none were under any pharmacological treatment during the study.

Table 3. 1 Subjects physical and physiological characteristics

	Study 1	Study 2	Study 3
Age (years)	25 ± 1.9	20 ± 0.5	25 ± 1.5
Height (m)	1.74 ± 0.01	1.77 ± 0.01	1.77 ± 0.01
Body mass (kg)	73 ± 1.9	73 ± 2.5	78 ± 2.9
$\dot{V}O_{2max}$ *(ml.kg^{-1}.min^{-1})*	57 ± 1.4	52 ± 2.1	55 ± 1.9

3.1.3. Anthropometry

Subject's height and body mass was assessed on their first visit to the laboratories that coincided with their assessment of physiological fitness (see section 3.3). The subject's height (SECA, Birmingham, UK) and nude weight (SECA, Birmingham, UK) was assessed and was kept consistent for every subject.

3.2. CARDIO-RESPIRATORY MEASUREMENTS

3.2.1. Heart rate

Subjects were fitted with a short-range radio telemetry system for the measurement of heart rate (Polar S610i, Kempele, Finland) in all exercise related experiments.

3.2.2. Assessment of respiratory gases during exercise

Subjects were required to wear a facemask for measurement of respiratory gases during exercise. Expired oxygen and carbon dioxide were analysed during breath-to-breath measurement via a CPX Ultima series online gas analysis system (Medgraphics, Minnesota, US).

3.2.3. Assessment of energy expenditure

Energy expenditure during exercise was estimated in Study 1 (see Chapter 4) via indirect calorimetry (Medgraphics, Minnesota, US) according to the calculations of Zuntz (1901) assuming a non-protein respiratory exchange ratio.

Table 3. 2 Table of Zuntz; analysis of the oxidation of mixtures of carbohydrate and fat (Zuntz, 1901).

R.Q	Percentage of total oxygen consumed by:		Percentage of total heat produced by:		Calories per litre O_2	
	Carbohydrate	Fat	Carbohydrate	Fat	Number	Logarithm
0.707	0	100	0	100	4.686	0.67080
0.71	1.02	99	1.10	98.9	4.690	0.67114
0.72	4.44	95.6	4.76	95.2	4.702	0.67228
0.73	7.85	92.2	8.40	91.6	4.714	0.67342
0.74	11.3	88.7	12.0	88.0	4.727	0.67456
0.75	14.7	85.3	15.6	84.4	4.739	0.67569
0.76	18.1	81.9	19.2	80.8	4.751	0.67682
0.77	21.5	78.5	22.8	77.2	4.764	0.67794
0.78	24.9	75.1	26.3	73.7	4.776	0.67906
0.79	28.3	71.7	29.9	70.1	4.788	0.68018
0.80	31.7	68.3	33.4	66.6	4.801	0.68129
0.81	35.2	64.8	36.9	63.1	4.813	0.68241
0.82	38.6	61.4	40.3	59.7	4.825	0.68352
0.83	42.0	58.0	43.8	56.2	4.838	0.68463
0.84	45.4	54.6	47.2	52.8	4.850	0.68573
0.85	48.8	51.2	50.7	49.3	4.862	0.68683
0.86	52.2	47.8	54.1	45.9	4.875	0.68793
0.87	55.6	44.4	57.5	42.5	4.887	0.68903
0.88	59.0	41.0	60.8	39.2	4.899	0.69012
0.89	62.5	37.5	64.2	35.8	4.911	0.69121
0.90	65.9	34.1	67.5	32.5	4.924	0.69230
0.91	69.3	30.7	70.8	29.2	4.936	0.69339
0.92	72.7	27.3	74.1	25.9	4.948	0.69447
0.93	79.1	23.9	77.4	22.6	4.961	0.69555
0.94	79.5	20.5	80.7	19.3	4.973	0.69663
0.95	82.9	17.1	84.0	16.0	4.985	0.69770
0.96	86.3	13.7	87.2	12.8	4.998	0.69877
0.97	89.8	10.2	90.4	9.58	5.010	0.6984
0.98	93.2	6.83	93.6	6.37	5.022	0.70091
0.99	96.6	3.41	96.8	3.18	5.035	0.70197
1.00	100.0	0	100.0	0	5.047	0.70303

3.2.4. Assessment of carbohydrate and fat oxidation rates

Carbohydrate and fat oxidation rates were estimated during exercise in Study 3 (see Chapter 6) via indirect calorimetry (Medgraphics, Minnesota, US), as calculated according to the calculation of Peronnet and Massicotte (1991).

$$\text{Carbohydrate oxidation} = 4.585 \bullet \dot{V}CO_2 - 3.226 \bullet \dot{V}O_2$$

$$\text{Fat oxidation} = 1.695 \bullet \dot{V}O_2 - 1.701 \bullet \dot{V}CO_2$$

3.3. ASSESSMENT OF MAXIMAL OXYGEN UPTAKE ($\dot{V}O_{2max}$)

All subjects were assessed for $\dot{V}O_{2max}$ using an incremental exercise test performed on a motorised treadmill (HP Cosmos). Oxygen uptake ($\dot{V}O_2$) was measured during exercise by breath-by-breath measurement using a CPX Ultima series online gas analysis system (Medgraphics, Minnesota, US) after being calibrated with known reference gases. The test began with a 3-min stage at a treadmill speed of 10 km.h^{-1} followed by 3-min stages at 12 km.h^{-1}, 14 km.h^{-1} and 16 km.h^{-1}. Upon completion of the 16 km.h^{-1} stage the treadmill inclined by 2 % every 3-min thereafter until termination of the test. Termination of the test occurred upon volitional exhaustion despite strong verbal encouragement. The $\dot{V}O_{2max}$ was taken as the highest $\dot{V}O_2$ value obtained in any 10-sec period and was stated as being achieved by the following end-point criteria: 1) heart rate within 10 beats.min^{-1} of age-predicted maximum, 2) respiratory exchange ratio > 1.1, and 3) plateau of oxygen consumption despite increased workload. In an attempt to provide a clearer profile of the participants O_2 uptake and to accurately match the participants running velocities with their respective $\dot{V}O_2$ values in Study 1, a further physiological assessment of running economy was made. The exercise protocol commenced at a treadmill speed of 8 km.h^{-1} and increased by 1 km.h^{-1} thereafter every 3 min until at least 95 % of the participant's $\dot{V}O_{2max}$ had been reached (taken from previous $\dot{V}O_{2max}$ test).

3.4. MEASUREMENT OF PSYCHO-PHYSIOLOGICAL VARIABLES

3.4.1. Ratings of perceived exertion

Subjects reported ratings of perceived exertion during exercise according to a 15-point Borg Scale (Borg 1970). Table 3.2 displays the category ratio scale that was used.

Table 3. 3 Borg scale used for subjects ratings of perceived exertion during exercise (Borg, 1970).

Rating	Description
6	No Exertion At All
7	Extremely Light
8	
9	Very Light
10	
11	
12	
13	Somewhat Hard
14	
15	Hard
16	
17	Very Hard
18	
19	Extremely Hard
20	Maximal Exertion

3.4.2. Ratings of perceived enjoyment

Subjects reported ratings of perceived enjoyment immediately after exercise in Study 1 according to the Physical Activity Enjoyment Scale (PACES) questionnaire validated by Kendzierski and DeCarlo (1991). According to the scale, the higher the score the more enjoyable the subject found

the exercise session. An illustration of the PACES enjoyment questionnaire can be seen in Table 3.3.

Table 3. 4 Illustration of Physical Activity Enjoyment Scale validated by Kendzierski and DeCarlo (1991).

*	I enjoy it	1	2	3	4	5	6	7	I hate it
	I feel bored	1	2	3	4	5	6	7	I feel interested
	I dislike it	1	2	3	4	5	6	7	I like it
*	I find it pleasurable	1	2	3	4	5	6	7	I find it unpleasurable
*	I am very absorbed in this activity	1	2	3	4	5	6	7	I am not at all absorbed in this activity
	It's no fun at all	1	2	3	4	5	6	7	It's a lot of fun
*	I find it energising	1	2	3	4	5	6	7	I find it tiring
	It makes me depressed	1	2	3	4	5	6	7	It makes me happy
*	It's very pleasant	1	2	3	4	5	6	7	It's very unpleasant
*	I feel good physically while doing it	1	2	3	4	5	6	7	I feel bad physically while doing it
*	It's very invigorating	1	2	3	4	5	6	7	It's not at all invigorating
	I am very frustrated by it	1	2	3	4	5	6	7	I am not at all frustrated by it
*	It's very gratifying	1	2	3	4	5	6	7	It's not at all gratifying
*	It's very exhilarating	1	2	3	4	5	6	7	It's not at all exhilarating
	It's not at all stimulating	1	2	3	4	5	6	7	It's very stimulating
*	It gives me a strong sense of accomplishment	1	2	3	4	5	6	7	It doesn't give me a strong sense of accomplishment
*	It's very refreshing	1	2	3	4	5	6	7	It's not at all refreshing
	I felt as though I would rather be doing something else	1	2	3	4	5	6	7	I felt as though there is nothing else I would rather be doing

* Reverse when totalling scores

3.5. PROCUREMENT, STORAGE AND ANALYSIS OF BLOOD SAMPLES

Blood samples were drawn from a superficial vein in the anticubital crease of the forearm using either standard venepuncture techniques or via a cannula (NHS supply chain, Alfreton, UK) (Figure 3.1). Samples were collected into vacutainers (Vacutainers Systems, Becton, Dickinson) containing EDTA, lithium heparin or serum and stored on ice or at room temperature for 1 h (serum samples) until centrifugation at 1500 g for 15 min at 4°C. Following centrifugation, aliquots of plasma and serum were stored at -80°C for later analysis. Plasma glucose, lactate, fatty acids and glycerol samples were analysed using commercially available kits (Randox Laboratories, Antrim, UK) and insulin (Cobas, Roche Diagnostics, Indianapolis, USA) concentration was determined via an electrochemiluminescence immunoassay (ECLIA). The % CV for plasma glucose, lactate, NEFA and glycerol in our lab is < 5 %.

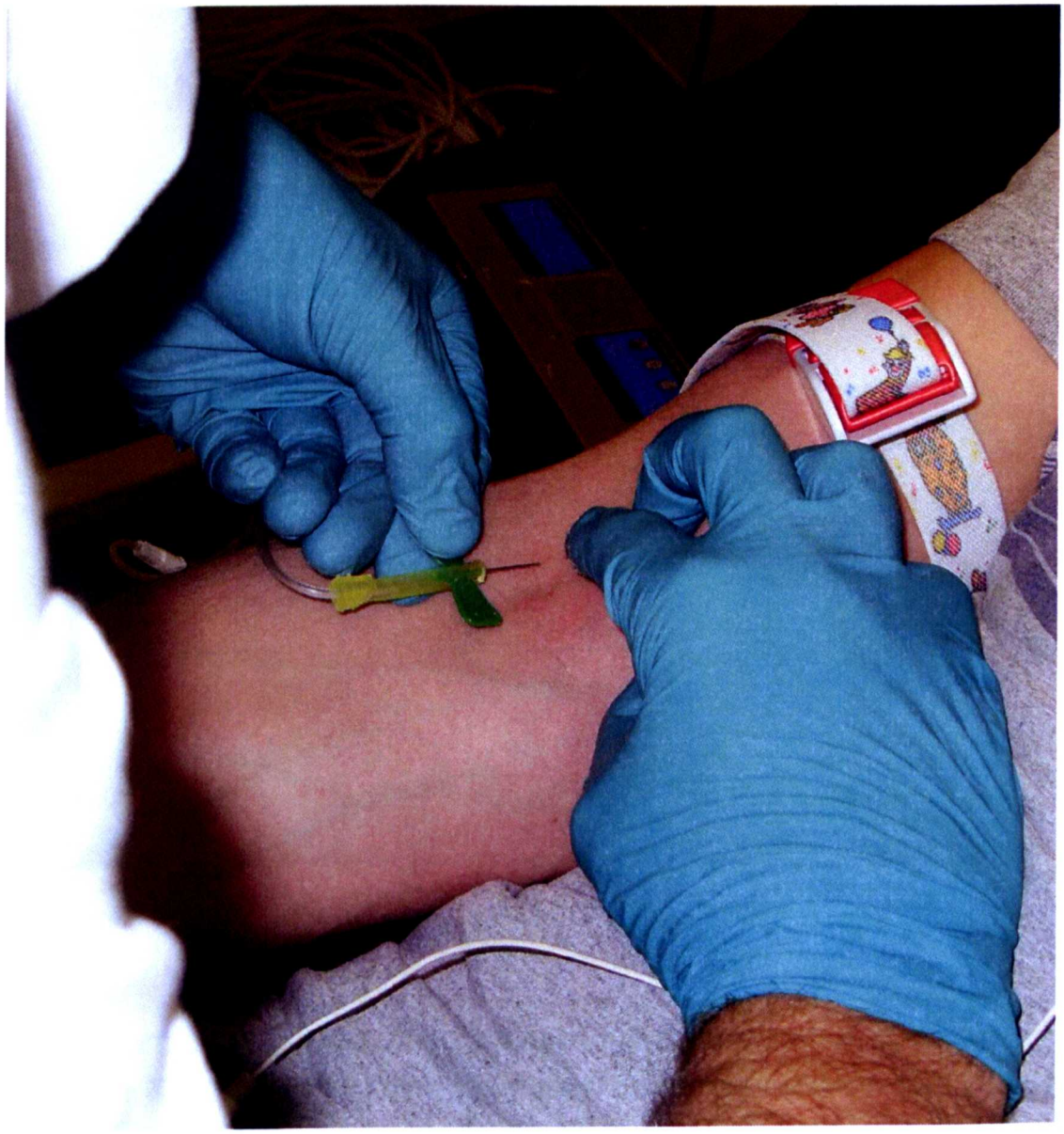

Figure 3. 1 Venous blood sample taken from the anticubital crease of the forearm

3.6. MUSCLE BIOPSIES

Muscle biopsies were taken from the vastus lateralis (Figure 3.2). Samples were obtained under local anaesthesia (0.5 % marcaine) using a Pro-Mag 2.2 biopsy gun (MD-TECH, Manan Medical Products, Northbrook, IL). Once the biopsy needle is inserted through the fascia, the 'firing' of the biopsy gun operates with a feed-forward of up to 2.5-3.5 cm, depending on the angle of insertion of the needle. Once obtained the samples (~60mgs) were frozen in liquid nitrogen and stored at -80°C for later analysis.

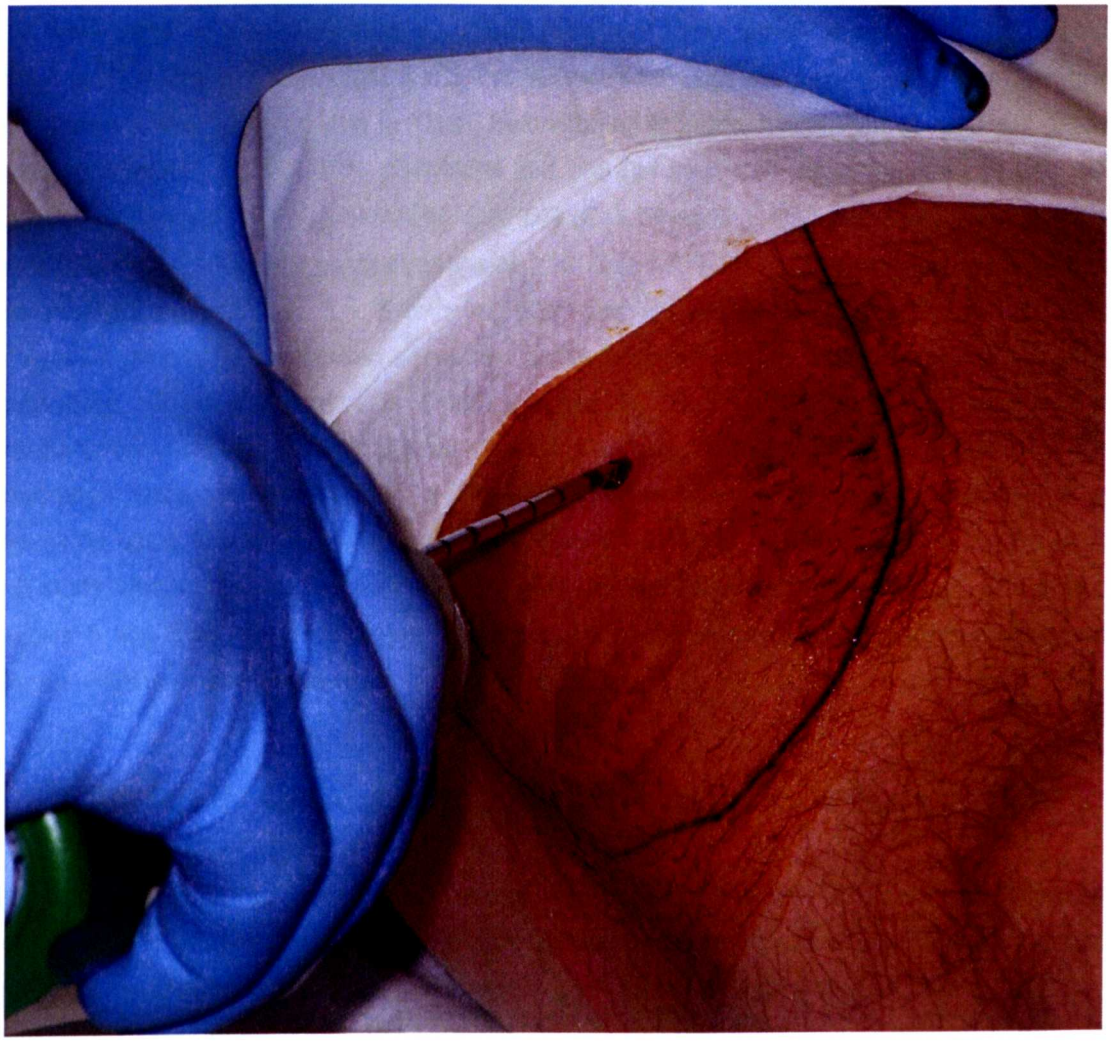

Figure 3. 2 Typical muscle biopsy of the vastus lateralis muscle

3.7. SKELETAL MUSCLE ANALYSIS USING SDS-PAGE AND WESTERN BLOTTING

Approximately 20-30 mg of frozen muscle was ground to powder and homogenised in 120 µl of ice cold lysis buffer (25 mM Tris/HCl [pH 7.4], 50 mM NaF, 100 mM NaCl, 5 mM EGTA, 1 mM EDTA, 10 mM Na-Pyrophosphatase, 1 mM Na_3VO_4, 0.27 M sucrose, 1 % Triton X-100, 0.1 % 2-

mercaptoethanol) and supplemented with a protease inhibitor tablet (Complete mini, Roche Applied Science, West Sussex, UK). Homogenates were centrifuged at 14,000 g for 10 min at 4°C and the supernatant was collected. The protein content of the supernatant was determined using a bicinchoninic acid assay (Sigma, UK). Each sample was diluted with an equal volume of 2X Laemmli buffer (National Diagnostics, USA) and boiled for 5 min at 100 °C. For each blot, a standard and internal control was loaded along with 50-100 µg of protein from each sample and then separated in Tris-glycine running buffer (10 X Tris/Glycine, Geneflow Ltd, Staffordshire, UK) using self-cast 4% stacking and 10 % separating gels (National Diagnostics, USA). Gels were transferred semi-dry onto nitrocellulose membrane (Geneflow Ltd, Staffordshire, UK) for 2 h at 200 V and 45 mA per gel in transfer buffers (anode 1; 0.3 M Tris, 20 % methanol, pH 10.4; anode 2; 0.25 M Tris, 20 % methanol, pH 10.4; cathode; 0.4 M 6-amino hexanoic acid, 20 % methanol, pH 7.6). After transfer, membranes were blocked for 1 h at room temperature in Tris-buffered saline (TBST: 0.19 M Tris pH 7.6, 1.3 M NaCl, 0.1 %Tween-20) with 5% non-fat milk. The membranes were then washed for 3 x 5 min in TBST before being incubated overnight at 4 °C with phosho-specific antibodies; ACC, AMPK, p38MAPK and p53 (all from Cell Signalling, UK) as well as the total protein content; AMPK, p38MAPK, GAPDH (Cell Signalling, UK) and PGC-1α (Calbiochem, Merck Chemicals, UK) all at concentrations of 1:1000 in 1 X TBST. The next morning, membranes were washed for a further 3 x 5 min in TBST and subsequently incubated with anti-species horseradish peroxidise-conjugated secondary antibody (Bio-Rad, UK or Dako, UK) for 1 h at room temperature. After a further 3 x 5 min washes in TBST, membranes were exposed in a chemiluminescence liquid (SuperSignal, Thermo Fisher Scientific, Rockford, IL, USA) for 5-min. Membranes were visualised using a Bio-Rad Chemi-doc system, and band densities were determined using ImageLab image-analysis software. In order to ensure the antibodies used in each study was specific to the protein of interest, a secondary control was run on every gel. In chapters 5 and 6 all raw densitometry data were used for statistical analysis purposes so as to compare within-subject responses.

3.8. ANALYSIS OF mRNA CONTENTS OF SKELETAL MUSCLE USING QUANITITATIVE REAL TIME PCR

Total RNA was isolated from muscle biopsies (20-30 mg) using Trizol reagent (Invitrogen), according to the manufacturer's protocol. RNA quality and quantity were determined using Implen Nanophotometer (Implen, Munchen, Germany) and the RNA was stored at -80 °C. cDNA was synthesised using random hexamers (Applied Biosystems) and Superscript III enzyme (Invitrogen), using manufacturer's protocol. Gene specific expression data was obtained using probes selected from Human Universal Probe Library (Roche Diagnostics) with compatible oligonucleotide primers (MWG Eurofins). One microliter of each sample was analysed in triplicate with negative controls using AB 7500 Real-Time Quantitative PCR instrument (Applied Biosystems) and Agilent Brilliant II qPCR Master Mix with Low ROX (Agilent Technologies). One microliter of cDNA, 500 nM of primer and 200 nM of probe were used for each 20-µl reaction (Table 1). The following cycling parameters were used: 50 °C for 2 minutes, initial denaturation at 95 °C for 10 minutes, followed by 40 cycles

of denaturation at 95°C for 15 s and annealing/elongation at 60°C for 1 minute. Data was collected and analysed using AB SDS 1.43 Software (Applied Biosystems, Foster City, USA). Changes in mRNA content were calculated according to the 2-ΔΔCt method where GAPDH was used as the housekeeping gene (Heid et al. 1996). In order determine the optimum housekeeping gene(s) GAPDH, β2M and β-actin pre-tests were run on all samples to ensure no variability between time points. The use of GAPDH as a single reference gene was used due to variability in other housekeeping genes (β2M and β-actin).

Table 3. 5 Primer and probe sequences used for real-time PCR during chapter 5 and 6

Gene	Forward primer	Reverse primer	Probe
GAPDH	AGCCACATGGCTCAGACAC	GCCCAATACGACCAAATCC	60
PDK4	CTGGTATATACAGAGCCTGATGGA	TGTCTATTTCGAACTTTGATGAGTG	31
CPT-1	GACAATACCTCGGAGCCTCA	AATAGGCCTGACGACACCTG	-
PGC-1α	TGAGAGGGCCAAGCAAAG	ATAAATCACACGGCGCTCTT	13
SCO2	GCAGCAAAAGCGAACAGAA	GTGATCCAGCAGGTGGAAGT	67
COXIV	CCATGTCAAGCACCTGTC	CAGCAAAGCTCTCCTTGA	-
HSP72	TTCCAGATCTCGTCCATGC	CAGGTCAAAGATGAGCACGTT	70
MnSOD	CTGGACAAACCTCAGCCCTA	TGATGGCTTCCAGCAACTC	22
TFAM	TGGCAAGTTGTCCAAAGAAACCTGT	GTTCCCTCCAACGCTGGGCA	-

3.9. ANALYSIS OF MUSCLE GLYCOGEN CONCENTRATION OF SKELETAL MUSCLE

Muscle glycogen concentration was determined according to the method described by (van Loon et al. 2000). Approximately 3-6 mg of freeze dried sample was powdered, dissected free of all visible non-muscle tissue and subsequently hydrolyzed by incubation in 500 µl of 1 M HCl for 3-4 hours at 100°C. After cooling to room temperature, samples were neutralized by the addition of 250 µl 0.12 mol. L^{-1} Tris/2.1 mol. L^{-1} KOH saturated with KCl. Following centrifugation, 150 µl of the supernatant was analysed in duplicate for glucose concentration according to the hexokinase method using a commercially available kit (GLUC-HK, Randox Laboratories, Antrim, UK). Glycogen concentration is expressed as mmol.kg^{-1} dry weight (dw) and intra-assay coefficients of variation was <5%.

3.10. STATISTICAL ANALYSIS

All statistical analyses were performed using the statistical package for social sciences (SPSS; version 17, IBM, USA). All data in text, figures and tables are presented as means ± SEM with P values ≤ 0.05 indicating statistical significance.

CHAPTER 4

THE DEVELOPMENT OF LABORATORY BASED HIGH-INTENSITY INTERVAL AND MODERATE-INTENSITY CONTINUOUS RUNNING PROTOCOLS MATCHED FOR ENERGY EXPENDITURE

The aim of this study was to develop two different exercise protocols in terms of activity profiles (i.e. HIT and CONT) that were matched for total oxygen consumption and energy expenditure after being matched for average intensity, duration and distance ran. Having initially matched the protocols for all of the aforementioned indices, these data provide an appropriate framework for future chapters to subsequently characterise the cell signalling responses of human skeletal muscle to acute HIT and CONT exercise. This study was presented as a poster at the 2nd International Sport Science and Sport Medicine Conference (ISSSMC) in Newcastle, UK 2010 and published as an abstract in the British Journal of Sports Medicine (see Appendix 1) and also published as a full manuscript in the Journal of Sport Sciences (see Appendix 2).

4.1. INTRODUCTION

Recent data from cycling-based protocols demonstrate that high-intensity interval training is a more time efficient mode of training compared with moderate-intensity continuous training exercise (CONT) (Gibala et al. 2006; Daussin et al. 2007; Daussin et al. 2008; Burgomaster et al. 2008). In addition to cycling interventions, Helgerud et al. (2007) also observed that HIT (4x4 min intervals at a running velocity corresponding to 90 % HR_{max} interspersed with 3-min recovery periods at 70 % HR_{max}) is a more time efficient mode of training than CONT (45-min continuous running at a running velocity corresponding to 70 % HR_{max}), as evidenced by superior training-induced increases in $\dot{V}O_{2max}$, stroke volume, and running economy. Despite the obvious advantage of HIT in inducing beneficial training adaptations these studies did not match their exercise protocols for average intensity or duration therefore it is unknown what the overriding factor is in driving the enhanced adaptation. Furthermore, given the methodological difficulties in measuring power output on motorised treadmills, these researchers also quantified and prescribed exercise intensity on the basis of % HR_{max}. Whilst this approach is beneficial in the field setting it is not without limitations as measurement of heart rate per se may not always reflect energy production, owing to problems of cardiovascular drift and the effect of environmental and psychological influences (Gilman, 1996). Alternatively, Morton (2007) suggested that a more accurate method to quantify, prescribe and match exercise intensity during HIT and CONT running protocols performed in the laboratory is to match both exercise protocols for the average velocity corresponding to a particular % $\dot{V}O_{2max}$ (Morton 2007). In this way, both protocols can therefore be matched for average intensity and exercise duration as well as distance ran despite the obvious difference in activity profiles between protocols.

The aim of the present study was to therefore develop laboratory based HIT and CONT running exercise protocols that are matched for all of the aforementioned indices and moreover, total oxygen consumption and energy expenditure. These protocols are to be then used in Chapter 5 (Study 2) in order to characterise the skeletal muscle cell signalling events following HIT and CONT running in human skeletal muscle.

4.2. METHODS

4.2.1. Subjects

Eight recreationally active men volunteered to participate in the study (mean ± SEM: age, 25 ± 1.9 yr; weight, 73 ± 1.9 kg; height, 1.74 ± 0.01 m; maximum oxygen uptake ($\dot{V}O_{2max}$), 57 ± 1.4 ml. kg^{-1}. min^{-1}). The study was approved by the Ethics Committee of Liverpool John Moores University.

4.2.2. Design

At least 5 days after having initially been assessed for $\dot{V}O_{2max}$ and running economy (RE) (see section 3.3) participants completed two running exercise protocols consisting of high-intensity interval exercise and moderate-intensity continuous exercise in a fully randomised and crossover design (Altman, 1991). Subjects were required to run a high-intensity interval (HIT) protocol and a moderate-intensity continuous (CONT) protocol. Each protocol consisted of running exercise and was performed on the same motorised treadmill (HP Cosmos, Germany). The HIT running protocol commenced with a 7-min warm up at a running velocity corresponding to 70 % $\dot{V}O_{2max}$ followed by six 3-min bouts at a running velocity corresponding to 90 % $\dot{V}O_{2max}$. The high-intensity intervals were separated by 3-min active recovery periods at a running velocity corresponding to 50 % $\dot{V}O_{2max}$. Following the interval and recovery periods, participants then performed a 7-min cool down at a running velocity corresponding to 70 % $\dot{V}O_{2max}$. The exercise protocol therefore gave a total of 18-min of interval exercise and 18-min of active recovery time, thus giving a total interval exercise time of 36 min. When including the warm-up and cool-down times, the total duration of the exercise protocol was 50-min therefore ensuring the protocols were matched for duration. The CONT protocol consisted of 50-min continuous running at a running velocity corresponding to 70 % $\dot{V}O_{2max}$. The average intensity during the HIT protocol and the CONT protocol, when quantified according to average running velocity, equated to 70 % $\dot{V}O_{2max}$. A graphical illustration of the exercise protocols can be seen in Figure 4.1.

The exercise protocols were performed 7 days apart after an overnight fast and at the same time of day to rule out any circadian effects on exercise performance (Reilly & Brooks 1986). Oxygen uptake (see section 3.2.2), heart rate (see section 3.2.1) and RPE (see section 3.5.1) were recorded throughout exercise and participants also reported perceived enjoyment upon completion of each protocol (see section 3.5.2). Energy expenditure was calculated (see section 3.2.3) so as to match the energy expenditure between the HIT and CONT protocols. A comparison of running velocities corresponding to specific % of $\dot{V}O_2$ for each subject is shown in Table 4.1. In addition a methodological breakdown of participants' typical running velocities and total distance ran during both protocols are shown in Table 4.2.

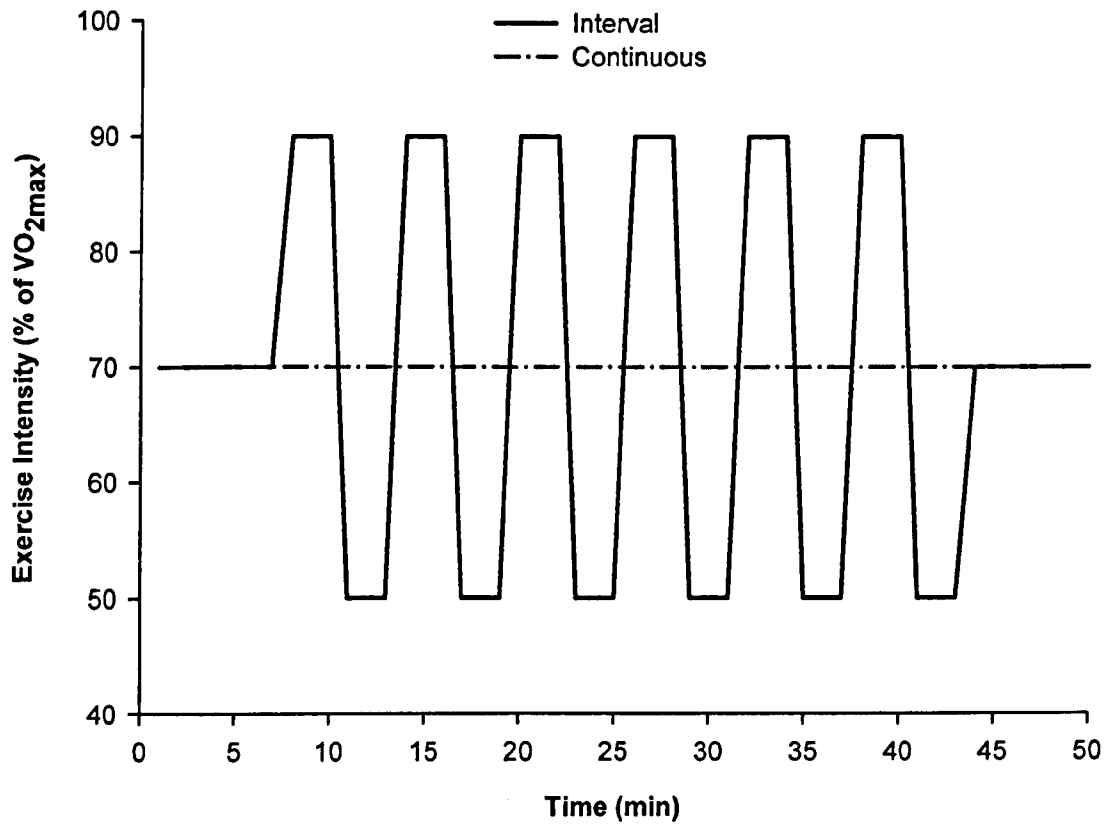

Figure 4. 1 Schematic illustration of the exercise intensity profile during the HIT and CONT running protocols

Table 4. 1 Subjects exercise intensity and respective running velocities for the HIT and CONT protocols

Subject	HIT (Intensity and Speed km.h^{-1})			CONT
	50%	*70%*	*90%*	*70%*
1	8	11	14	11
2	9.5	12.5	15.5	12.5
3	9	12	15	12
4	9	12	15	12
5	9	12	15	12
6	8	11	14	11
7	9.5	12.5	15.5	12.5
8	9.5	11.5	13.5	11.5

HIT: High-intensity interval exercise, CONT: Moderate-intensity continuous exercise

Table 4. 2 A methodological breakdown of a typical subject's running velocities and total distance ran during the HIT and CONT protocols.

Protocol	Time (min)	Speed ($km.h^{-1}$)	Intensity (% $\dot{V}O_{2max}$)	Distance Ran ($m.min^{-1}$)	Total Distance Ran (m) (time * distance)
CONT	50	12.5	70	208.3	10415
HIT					
Warm Up	7	12.5	70	208.3	1458.1
Bout 1	3	15.5	90	258.3	774.9
Recovery 1	3	9.5	50	158.3	474.9
Bout 2	3	15.5	90	258.3	774.9
Recovery 2	3	9.5	50	158.3	474.9
Bout 3	3	15.5	90	258.3	774.9
Recovery 3	3	9.5	50	158.3	474.9
Bout 4	3	15.5	90	258.3	774.9
Recovery 4	3	9.5	50	158.3	474.9
Bout 5	3	15.5	90	258.3	774.9
Recovery 5	3	9.5	50	158.3	474.9
Bout 6	3	15.5	90	258.3	774.9
Recovery 6	3	9.5	50	158.3	474.9
Cool Down	7	12.5	70	208.3	1458.1
Total	**50**	**n/a**	**n/a**	**n/a**	**10415**

HIT: High-intensity interval exercise, CONT: Moderate-intensity continuous exercise

4.2.3. Statistical Analysis

Statistical analysis was conducted using the Statistical Package for Social Sciences software program (version 17). All data were initially analysed for normality according to the Shapiro-Wilks test. Once verified, a comparison of physiological responses between exercise protocols was assessed using student's t-tests for paired samples and a two way general linear model was used to determine whether there was an interaction or any significant differences over time and between conditions for heart rate and RPE. All data in text, figures, and tables are presented as means ±SEM with P values of ≤0.05 indicating statistical significance.

4.3. RESULTS

4.3.1. Physiological responses to the HIT and CONT protocols

Despite there being a significant interaction and main effect for time for heart rate between protocols throughout the 50-min of exercise ($P < 0.001$) (Figure 4.2) there was no significant difference in average heart rate ($P = 0.547$) or average % HR_{max} ($P = 0.528$) between HIT and CONT (Figure 4.3). Similarly, there was no difference in average $\dot{V}O_2$ ($P = 0.389$) and % $\dot{V}O_{2max}$ ($P = 0.305$) between HIT and CONT (Figure 4.4). Furthermore, total $\dot{V}O_2$ ($P = 0.358$) and energy expenditure ($P = 0.383$) was similar between the HIT and CONT protocols (Figure 4.5).

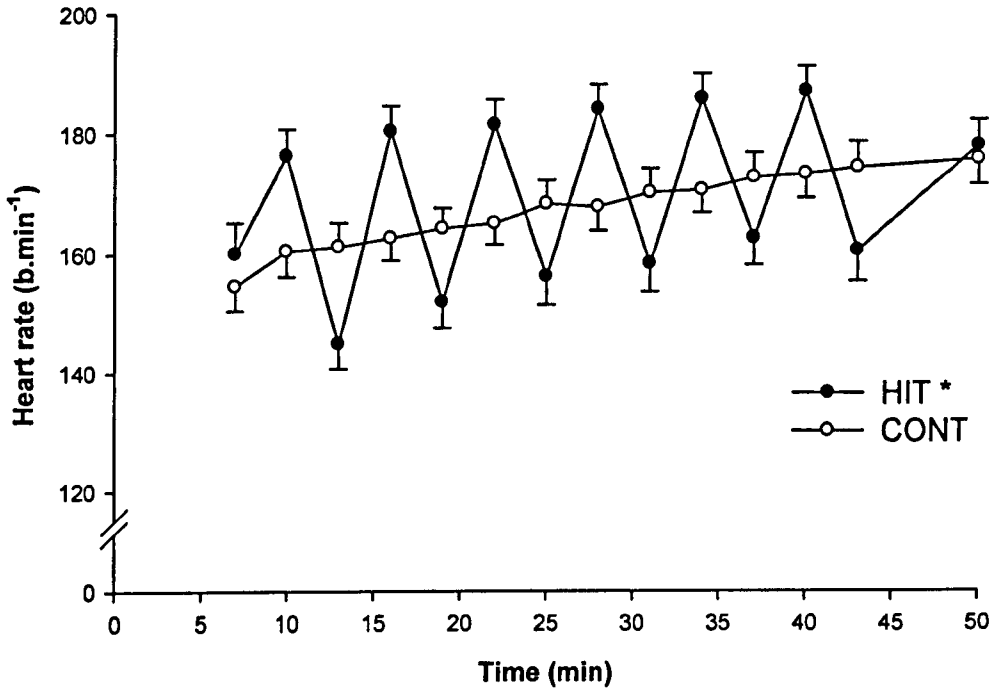

Figure 4. 2 Heart rates after each stage during the HIT and CONT running protocols. * Denotes significant main effect of time, condition and interaction ($P < 0.05$)

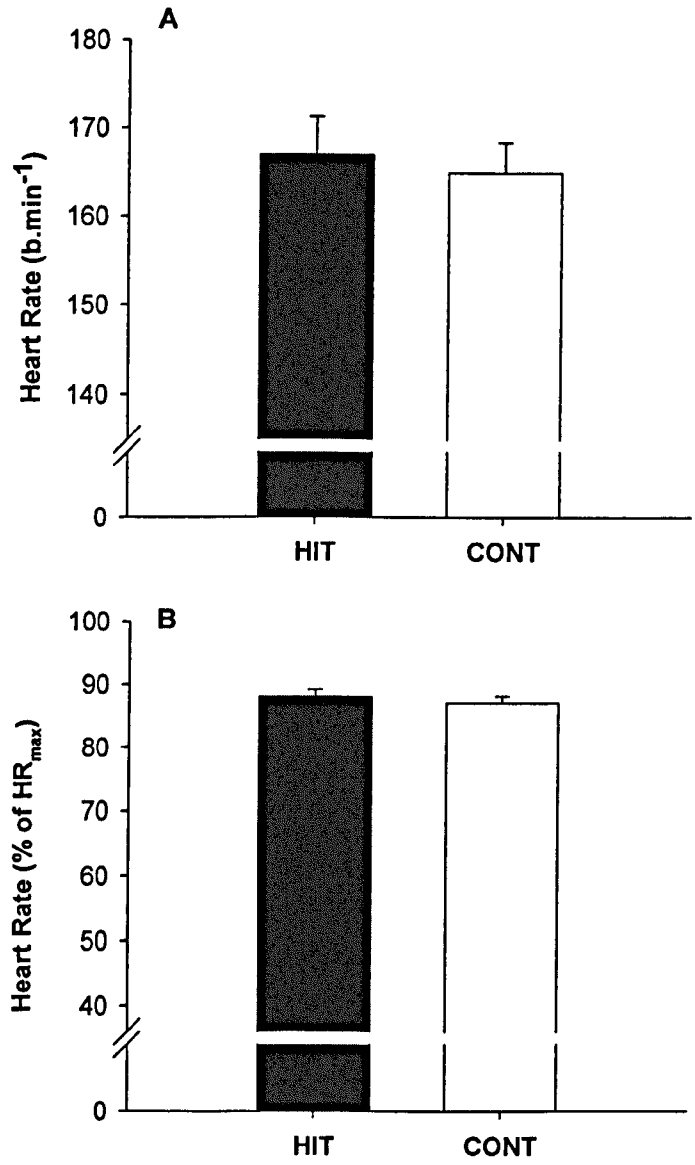

Figure 4. 3 A) Average heart rate in beats per min and B) average heart rate as a % of HRmax during the high-intensity interval (HIT) and moderate-intensity continuous (CONT) protocols.

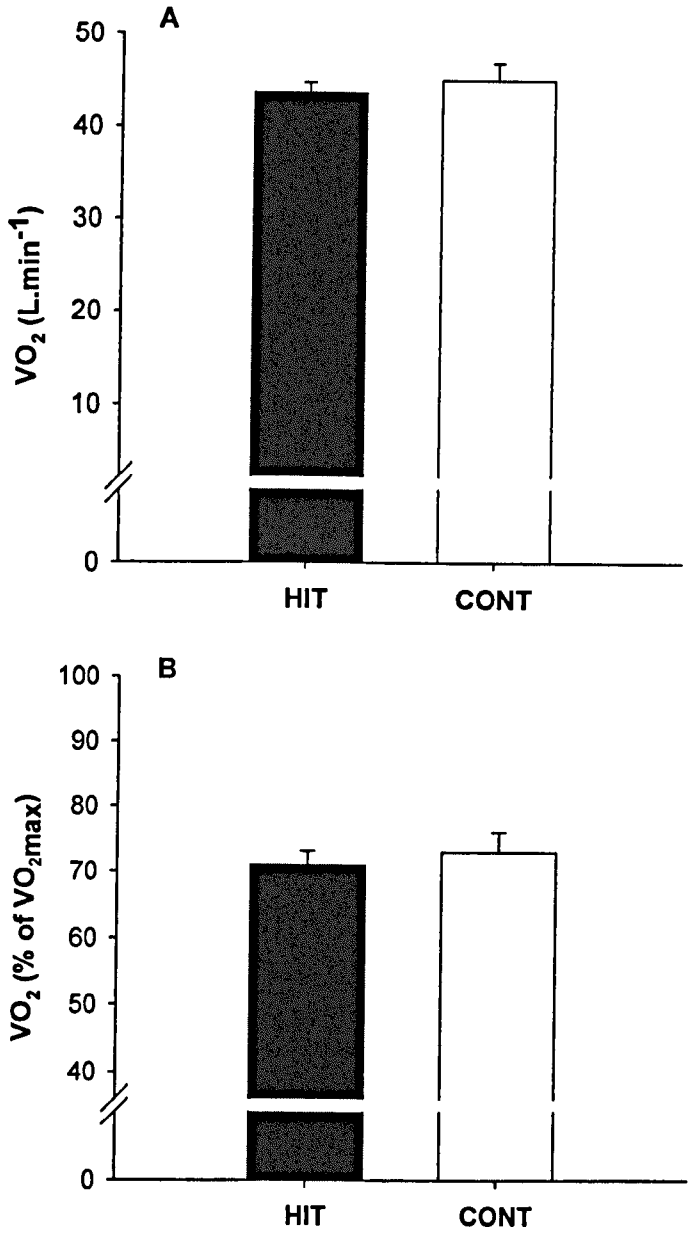

Figure 4. 4 A) Average $\dot{V}O_2$ in litres per min and B) average $\dot{V}O_2$ as a % of $\dot{V}O_{2max}$ following the high-intensity interval (HIT) and moderate-intensity continuous (CONT) protocols.

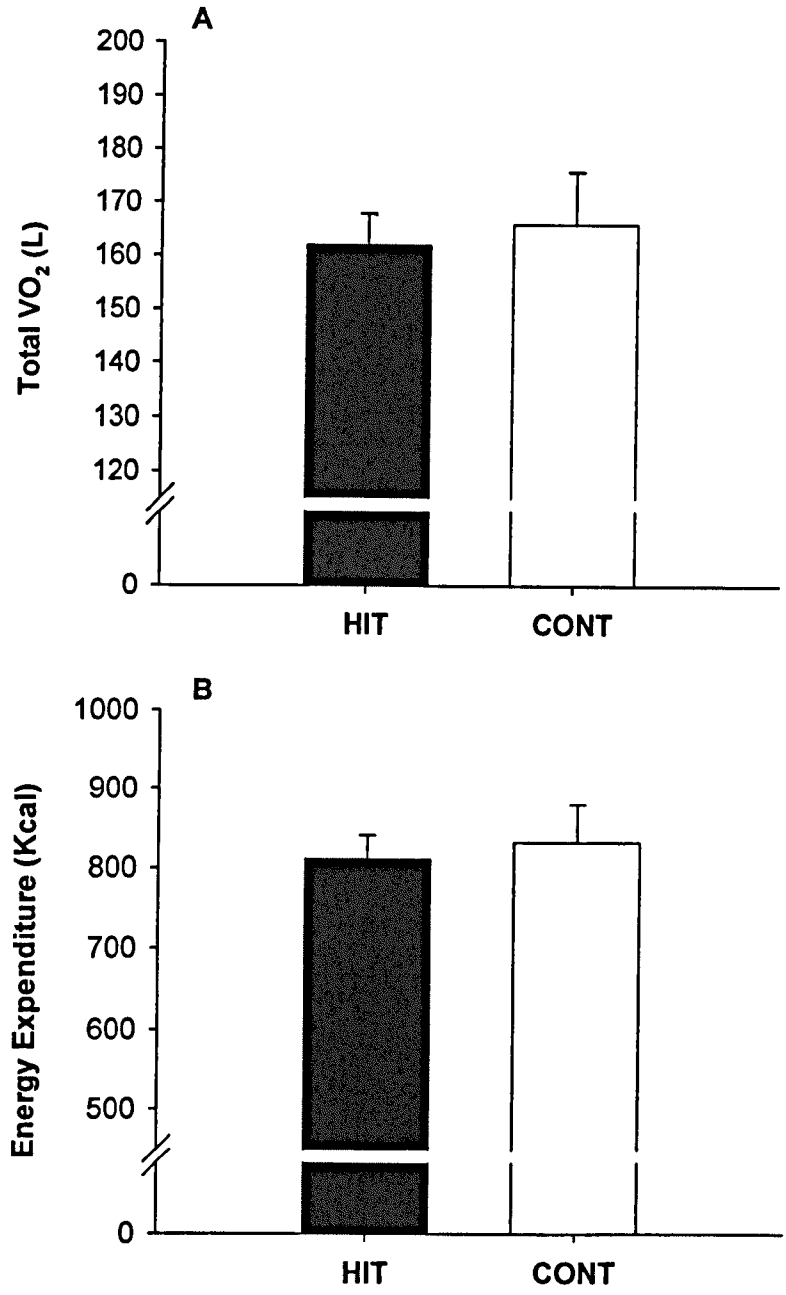

Figure 4. 5 A) Total oxygen consumption in litres and B) total energy expenditure in kcal following the high-intensity interval (HIT) and moderate-intensity continuous (CONT) protocols.

4.3.2. Psycho-perceptual responses to the HIT and CONT protocols

Despite HIT resulting in a significantly higher (P = 0.015) average RPE compared to CONT, ratings of perceived enjoyment were significantly higher (P = 0.004) in the HIT condition (Figure 4.5). Furthermore, when comparing RPE during exercise between conditions there was a significant interaction (P < 0.001), main effect of time (P < 0.001) and main effect of condition (P = 0.027) for the HIT and CONT running protocols (Figure 4.7).

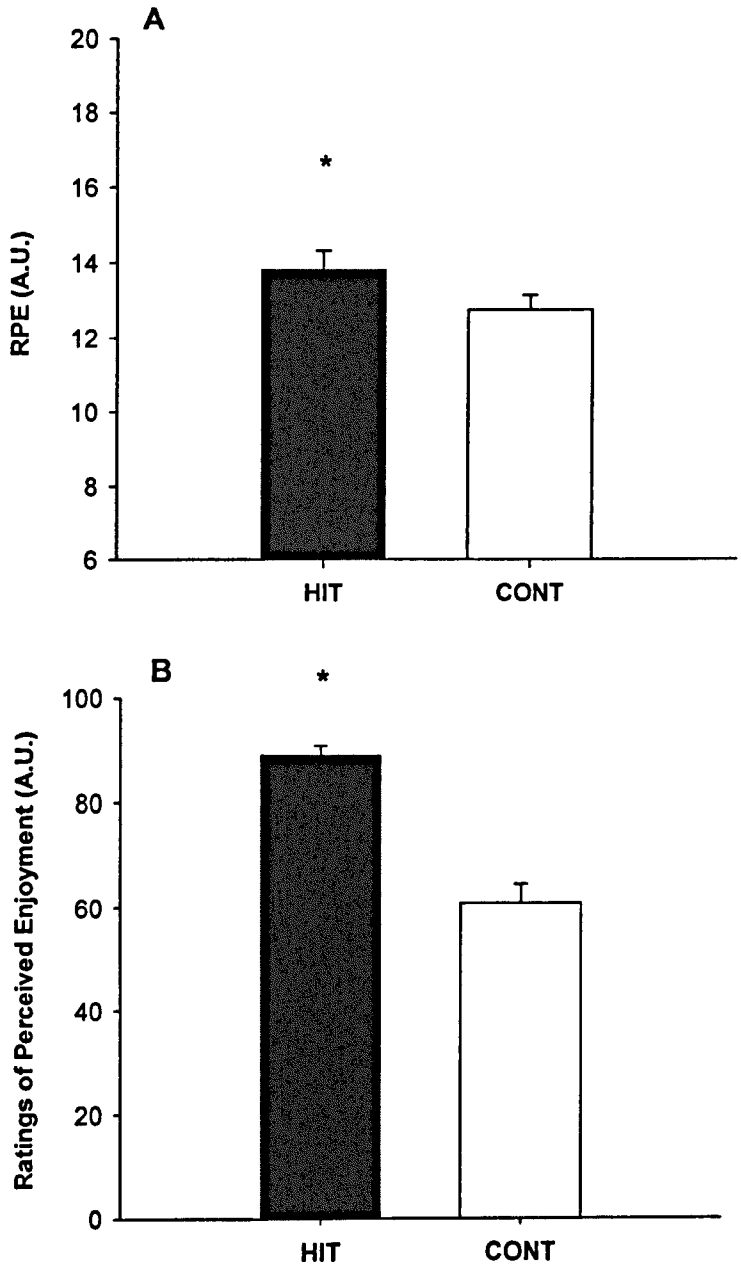

Figure 4. 6 A) Ratings of perceived exertion (RPE) and B) Ratings of perceived enjoyment during the high-intensity interval and moderate-intensity continuous (CONT) protocols. * Denotes significantly different from CONT (P < 0.05).

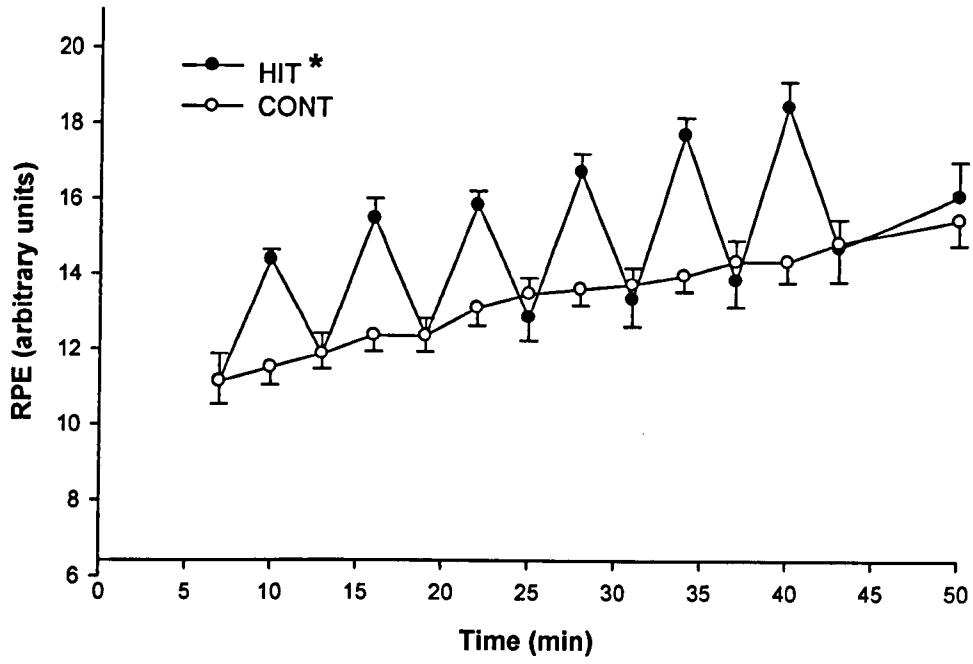

Figure 4. 7 Ratings of perceived exertion following each stage during the HIT and CONT running protocols. * Denotes significant main effect of time, condition and interaction ($P < 0.05$)

4.4. DISCUSSION

The aim of the present study was to develop two laboratory based running protocols that are matched for total oxygen consumption, average intensity, duration, energy expenditure and distance ran which can therefore be used to subsequently characterise the cell signalling responses of human skeletal muscle to HIT and CONT. Using HIT and CONT running protocols, these data demonstrate that when the two very different exercise protocols are matched for average intensity (70% $\dot{V}O_{2max}$), duration (50 min) and distance ran (9843 ± 494), the average heart rate and $\dot{V}O_2$, total oxygen consumption and energy expenditure are equal between protocols. To the author's knowledge, this is the first study that has matched HIT and CONT running protocols for all of the aforementioned indices.

Whilst existing evidence suggests HIT may be more time-efficient for inducing favourable exercise-induced mitochondrial changes these studies did not simultaneously match the exercise protocols for parameters such as average intensity, energy expenditure and duration (Gibala et al. 2006; Daussin et al. 2007; Helgerud et al. 2007; Burgomaster et al. 2008; Daussin et al. 2008). For example, Helgerud and colleagues prescribed and quantified exercise intensity during HIT and CONT running protocols on the basis of % HR_{max} (Helgerud et al. 2007). However, whilst this approach is practically advantageous, it is not without limitations, as heart rate does not always

correspond to energy production (Morton 2007). Furthermore, in order to maintain exercise intensity in the target heart rate zones, these authors had to reduce the running velocity during the training session so as to offset the effects of cardiovascular drift. However, in using this approach, the absolute workload is reduced therefore lowering the overall training stress. In an effort to provide a more controlled exercise stress, we chose to prescribe exercise intensity on the basis of running velocity corresponding to a particular % $\dot{V}O_{2max}$. In this way, the absolute workload and degree of cell signalling within the muscle was likely not compromised during exercise within or between individuals. In relation to the work of Daussin et al. (2007) and Daussin et al. (2008) who also matched their interval and continuous protocols for energy expenditure when prescribing exercise intensity on the basis of % HR_{max} the participants in their CONT group had to run an extra 7 minutes so as to ensure the energy expenditure was matched between protocols. In contrast, our data demonstrate that total O_2 consumption and energy expenditure can be matched between protocols when both exercise interventions are matched for duration, distance ran and average intensity, as quantified on the basis of running velocity corresponding to a particular % $\dot{V}O_{2max}$.

In the current study, average RPE during exercise was significantly higher during the HIT protocol compared with the CONT protocol. This finding is, of course, not unexpected given the higher cardiovascular and metabolic strain associated with each 3 min interval. However, despite such higher RPE levels, participants reported the HIT protocol to be more enjoyable than the CONT protocol, as evidenced by significantly higher ratings on the perceived enjoyment scale validated by Kendierski and DeCarlo (1991). This finding is similar to the previous research of Tjonna et al. (2008) who also reported informal comments regarding participants' (cardiovascular diseased patients) increased enjoyment during HIT compared with CONT. Furthermore, the duration and intensities of exercise in our HIT intervention is of practical relevance for aerobic conditioning programmes for those athletes involved in team sports that are characterised by intermittent activity profiles (Helgerud et al. 2001; Hoff & Helgerud 2004; Stone & Kilding 2009). Collectively, these findings are not only important from an athletic perspective in terms of improving athlete's compliance to training but also in improving exercise adherence in those individuals undertaking exercise programmes to improve aspects of human health. The latter point is particularly important given the accumulating evidence that HIT induces greater improvements in insulin sensitivity (Tjønna et al. 2008) and endothelial function (Wisløff et al. 2007) compared with CONT.

The similar average physiological responses between the HIT and CONT protocols observed here are likely to be specific to our chosen exercise intensities and work-rest ratios. Indeed, O'Brien et al. (2008) observed that 5 x 2 min interval stages at 100% $\dot{V}O_{2peak}$ (interspersed with 5 x 2 min recovery stages at 50% $\dot{V}O_{2peak}$) resulted in a significantly higher mean average $\dot{V}O_2$ than 10 x 1 min interval stages at 100 % $\dot{V}O_{2peak}$ (interspersed by 10 x 1 min stages at 50% $\dot{V}O_{2peak}$) and a 20 min continuous run, despite the average velocity in all three conditions being equal. Furthermore, Bangsbo et al. (2000) also observed that if the exercise intensity during the high-intensity intervals

is equal to or greater than that corresponding to $\dot{V}O_{2max}$, the mean O_2 consumption is typically higher during interval exercise compared with continuous exercise. Collectively, these data suggest that at intensities equal to or greater than $\dot{V}O_{2max}$, high-intensity interval exercise is associated with greater total O_2 consumption than continuous exercise. This finding is likely due to the repeated periods of elevated O_2 consumption in the post-interval period that are needed to offset the oxygen deficits created at the onset of each high-intensity interval undertaken at maximal or supra-maximal intensities. Other researchers interested in developing HIT and CONT running protocols that are matched for average physiological responses should therefore carefully design their interventions with the present and previous data in mind.

In summary, when using the chosen intensities and work to rest ratio employed in the current study, HIT and CONT running protocols can be matched for energy expenditure and total O_2 consumption when both protocols are matched for duration, distance ran, and average exercise intensity (as quantified and prescribed according to running velocity corresponding to a particular % $\dot{V}O_{2max}$). As such, these data now provide two well-controlled exercise protocols that can be used to examine the cell signalling responses of human skeletal muscle to acute high-intensity interval and moderate-intensity continuous exercise.

CHAPTER 5

SKELETAL MUSCLE CELL SIGNALLING RESPONSES TO ACUTE HIGH-INTENSITY INTERVAL EXERCISE *VS.* MODERATE-INTENSITY CONTINUOUS EXERCISE MATCHED FOR WORK DONE

Progressing on from Chapter 4, the aim of this study was to compare the cell signalling responses to high-intensity interval running versus moderate-intensity continuous running. By matching the protocols for average intensity, duration, distance ran and work done, any differences observed between protocols would therefore likely be due to fluctuations or variations in exercise intensity and/or pattern throughout exercise. This study was presented orally at the 16th Annual Congress of the European College for Sport Sciences (ECSS) (see Appendix 3) and has also been published in the Journal of Applied Physiology (see appendix 2).

5.1. INTRODUCTION

In recent years, several investigators have adopted high-intensity interval training (HIT) interventions (e.g. 4–6 x 30-s maximal cycling Wingate tests) as an alternative to more traditional continuous training (40–60 min cycling at 65% $\dot{V}O_{2max}$) approaches. This model of HIT is shown to be a potent and time-efficient stimulus for increasing the oxidative capacity of human skeletal muscle as well as improving insulin sensitivity (Babraj et al. 2009; Burgomaster et al. 2005, 2007, 2008; Gibala et al. 2006; Whyte et al. 2010). However, the practical application of Wingate-type HIT protocols may be limited due to reports of nausea and light-headedness associated with supra-maximal exercise (Richards et al. 2010) as well as the requirement for a mechanically braked cycle ergometer that is usually only found in the laboratory. To overcome these limitations, an alternative form of HIT is *running* at sub-maximal or near maximal intensity, especially considering that this mode of exercise is low cost and readily available to the general population and is the main mode of exercise in many sporting activities. Running is also beneficial given that it induces greater lipid oxidation compared with cycling at similar relative intensities (Achten & Jeukendrup, 2004), thus suggesting that running maybe a superior form of exercise for improving long-term metabolic health. Despite the applicability of this exercise mode, the acute signalling responses of human skeletal muscle to HIT *running* are not well known.

At a molecular level, mitochondrial adaptations to endurance training are thought to be due to the cumulative effects of transient increases in mRNA transcripts encoding mitochondrial proteins that follow each acute training session (Perry et al. 2010). Upon the onset of contraction, homeostatic perturbations within skeletal muscle (e.g. increased AMP/ATP ratio, Ca^{2+}, reactive oxygen species, lactate, reduced glycogen availability, etc.) result in the activation of a number of regulatory protein kinases which, in turn, phosphorylate downstream targets such as transcription factors or transcriptional co-activators (Ljubicic & Hood, 2009). Two putative kinases which have emerged as key players to the intracellular signalling cascades sensitive to muscle contraction are the adenosine monophosphate activated protein kinase (AMPK) and the p38 mitogen-activated protein kinase (p38MAPK) (Gibala et al. 2009; Little et al. 2010, 2011). These kinases converge upon the regulation of peroxisome proliferator-activated γ receptor co-activator (PGC-1α), a transcriptional co-activator repeatedly cited as the "master regulator of mitochondrial biogenesis" (Puigserver & Spiegelmann, 2003). In addition to PGC-1α, the tumour suppressor protein, p53, has also emerged as a potential regulator of mitochondrial function and several nuclear genes encoding mitochondrial proteins have p53 response elements in their promoter regions (Matoba et al. 2006). Moreover, skeletal muscle from p53 knockout mice exhibit reduced PGC-1α (Saleem et al. 2009) and mitochondrial transcription factor A protein (Matoba et al. 2006) as well as total mitochondrial content (Saleem et al. 2009) compared with wild type animals. Acute contractile activity also increases phosphorylation of p53 in rodent muscle thus highlighting an additional pathway through which muscle contraction may induce mitochondrial biogenesis (Saleem et al. 2009). Taken together, these studies suggest that p53 is an important modulator of mitochondrial content and

function, though, no study to date has examined the exercise-induced response of p53 in human skeletal muscle.

With this in mind, the aim of the present study was to characterise the acute signalling pathways activated in human skeletal by acute HIT and CONT running exercise. To isolate the effects of intermittent bursts of activity versus steady state exercise, we utilised the two exercise protocols developed in Chapter 4 that were matched for average intensity, duration and total work done. Given the potential for HIT exercise to induce a high degree of metabolic flux (e.g. increased AMP-ADP/ATP ratio, glycogen utilisation, lactate, ROS and Ca^{2+} production etc), we hypothesised that HIT would induce greater activation of those signalling pathways associated with mitochondrial biogenesis compared with CONT.

5.2. METHODS

5.2.1. Subjects

Ten recreationally active men volunteered to participate in the study (Age, 20 ± 0.5 yr; weight, 73 ± 2.5 kg; height, 1.77 ± 0.01 m; $\dot{V}O_{2max}$, 52 ± 2.1 ml. kg^{-1}. min^{-1}). The study was approved by the Ethics Committee of Liverpool John Moores University.

5.2.2. Experimental Design

In a randomized cross-over design (separated by 7 days) and after having initially been assessed for maximal oxygen consumption ($\dot{V}O_{2max}$) (see section 3.3), subjects attended the laboratory after an overnight fast and performed either high-intensity interval running (HIT) or moderate-intensity continuous running (CONT) (see section 4.2.2). Muscle biopsies (see section 3.6) were obtained from the vastus lateralis pre-, post- and 3 h after exercise and venous blood samples (see section 3.5) obtained pre- and post exercise. During the 3 h period between the post-exercise and 3 h post-exercise biopsies, subjects remained seated in the laboratory and performed light activities such as reading or working on a computer. Heart rate (section 3.2.1) was measured continuously during exercise (Polar S610i, Kempele, Finland) and ratings of perceived exertion (RPE) (section 3.4.1) were obtained at regular intervals during the protocol. Blood lactate was also assessed from fingertip capillary samples pre- and post-exercise, as well as after 13, 16, 25, 28, 40 and 43 min of exercise (Lactate Pro, Arkray, Japan). In the 30 min preceding exercise, subjects consumed 5 $ml.kg^{-1}$ body mass of water and were allowed to drink a further 3 $ml.kg^{-1}$ of water after 15 and 30 min of exercise. Subjects also consumed water *ad libitum* during the 3 h window between post-exercise and 3 h biopsies and the intake of food during this period was prohibited. Subjects completed a 3-day food diary preceding the first exercise trial and repeated the same energy intake in the 3 days prior to their second exercise trial. Subsequent dietary analysis was performed by the computer software programme Microdiet (Downlee Systems, UK) and average daily macronutrient intake in the 72 h prior to exercise was: 1910 kcal; CHO; 266 ± 43 g; 49 ± 8 %, Protein; 114 ± 46 g; 26 ± 8 % and Fat; 70 ± 16 g; 28 ± 8 %.

5.2.3. Muscle analysis

Muscle tissue was analysed for muscle glycogen concentration (see section 3.9) as well as key signalling proteins (e.g. AMPK, p38MAPK, p53) associated with mitochondrial biogenesis via the western blotting technique (see section 3.7). In addition, the RT-qPCr method (see section 3.8) was utilised to measure mRNA content of PGC-1α, HSP72 and MnSOD.

5.2.4. Statistical analysis

Statistical analysis was conducted using the Statistical Package for Social Sciences software programme (version 17). Data were analysed using a two way repeated measures General Linear Model where the within factors were time and exercise condition (HIT versus CONT). Where there were significant main effects, Bonferroni post-hoc tests were used to locate the differences. All data in text, figures and tables are presented as means (SEM) with P values ≤ 0.05 indicating statistical significance.

5.3. RESULTS

5.3.1. Physiological and metabolic responses to HIT and CONT

Heart rate, blood lactate and RPE data during exercise are shown in Table 5.1. In accordance with the differences in activity profiles between trials, HIT significantly increased blood lactate greater than CONT ($P < 0.001$). Whilst heart rate showed a trend to be higher following HIT ($P = 0.08$), there was no difference between conditions for RPE ($P = 0.74$). Muscle glycogen decreased ($P < 0.001$) by approximately 30% in both conditions with no difference ($P = 0.618$) between exercise protocols. Similarly, both exercise protocols increased plasma glucose concentration ($P = 0.002$) with no difference between conditions ($P = 0.055$). However, the exercise-induced increases in plasma fatty acids ($P < 0.001$) and glycerol ($P < 0.001$) were significantly greater in the CONT trial compared with the HIT protocol ($P = 0.04$ and $P = 0.02$, respectively) (Table 5.2).

Table 5. 1. Heart rate, RPE and plama lactate during the HIT and CONT protocols. * Denotes significant main effect of exercise. # Denotes significant main effect of condition. † Denotes significant interaction ($P < 0.05$).

		Time (min)							
		0	10	13	22	25	40	43	50
Heart Rate (b.min^{-1}) * †	*HIT*	89 ± 7	184 ± 9	163 ± 15	188 ± 8	163 ± 13	189 ± 8	163 ± 12	178 ± 9
	CONT	89 ± 7	171 ± 11	172 ± 10	177 ± 9	179 ± 8	182 ± 8	182 ± 12	186 ± 9
RPE (AU) *	*HIT*	6 ± 0	13 ± 2	12 ± 2	16 ± 2	13 ± 1	18 ± 1	15 ± 2	16 ± 2
	CONT	6 ± 0	12 ± 1	13 ± 1	14 ± 1	15 ± 1	17 ± 1	17 ± 1	17 ± 1
Plasma Lactate (mmol.L^{-1}) * # †	*HIT*	1.3 ± 0.5	8.0 ± 1.74	6.4 ± 2.0	9.4 ± 2.9	7.1 ± 2.3	9.4 ± 2.6	7.3 ± 2.1	6.2 ± 2.8
	CONT	1.7 ± 0.9	4.8 ± 1.94	5.0 ± 2.2	6.4 ± 3.4	5.6 ± 2.3	5.9 ± 3.2	6.4 ± 3.9	5.5 ± 2.4

Table 5. 2. Muscle glycogen and plasma glucose, NEFA and glycerol before and after completion of the HIT and CONT protocols. * Denotes significant main effect of exercise. # Denotes significant main effect of condition. † Denotes significant interaction ($P < 0.05$).

	HIT		CONT	
	Pre-exercise	Post-exercise	Pre-exercise	Post-exercise
Muscle glycogen ($mmol.kg^{-1}$ dw)	307 ± 54	191 ± 62 *	301 ± 59	190 ± 69 *
Glucose ($mmol.L^{-1}$)	5.4 ± 0.4	6.5 ±0.6 *	5.7 ± 0.7	6.7 ± 0.7 *
Fatty acids ($mmol.L^{-1}$)	0.37 ± 0.22	0.64 ± 0.28 *	0.44 ± 0.19	1.1 ± 0.43 *#
Glycerol ($\mu mol.L^{-1}$)	54 ± 20	179 ± 59 *	49 ± 25	246 ± 60 *#†

5.3.2. Exercise-induced kinase activation

Representative western blots are shown in Figure 5.1. Phosphorylation of $AMPK^{Thr172}$ increased 1.5-fold post exercise ($P = 0.04$) with no difference ($P = 0.44$) between conditions and returned to basal levels at 3 h post-exercise (Figure 5.2A). Similarly, phosphorylation of $p38MAPK^{Thr180/Tyr182}$ increased 1.9- and 1.5-fold immediately following exercise for HIT and CONT, respectively ($P < 0.001$), though there was no significant difference between exercise protocols ($P = 0.47$) (Figure 5.2B).

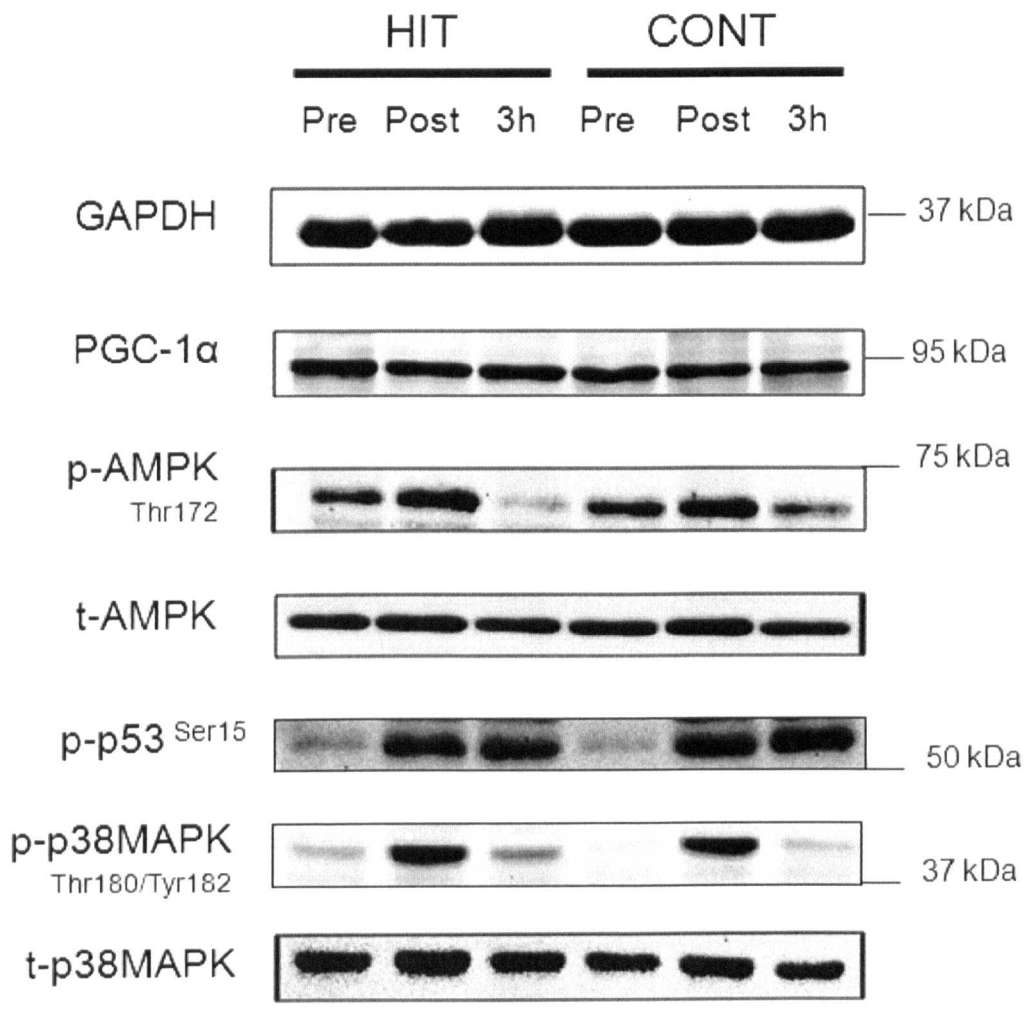

Figure 5. 1 Representative western blots before (Pre), after (Post) and 3 hours (3h) after the HIT and CONT protocols.

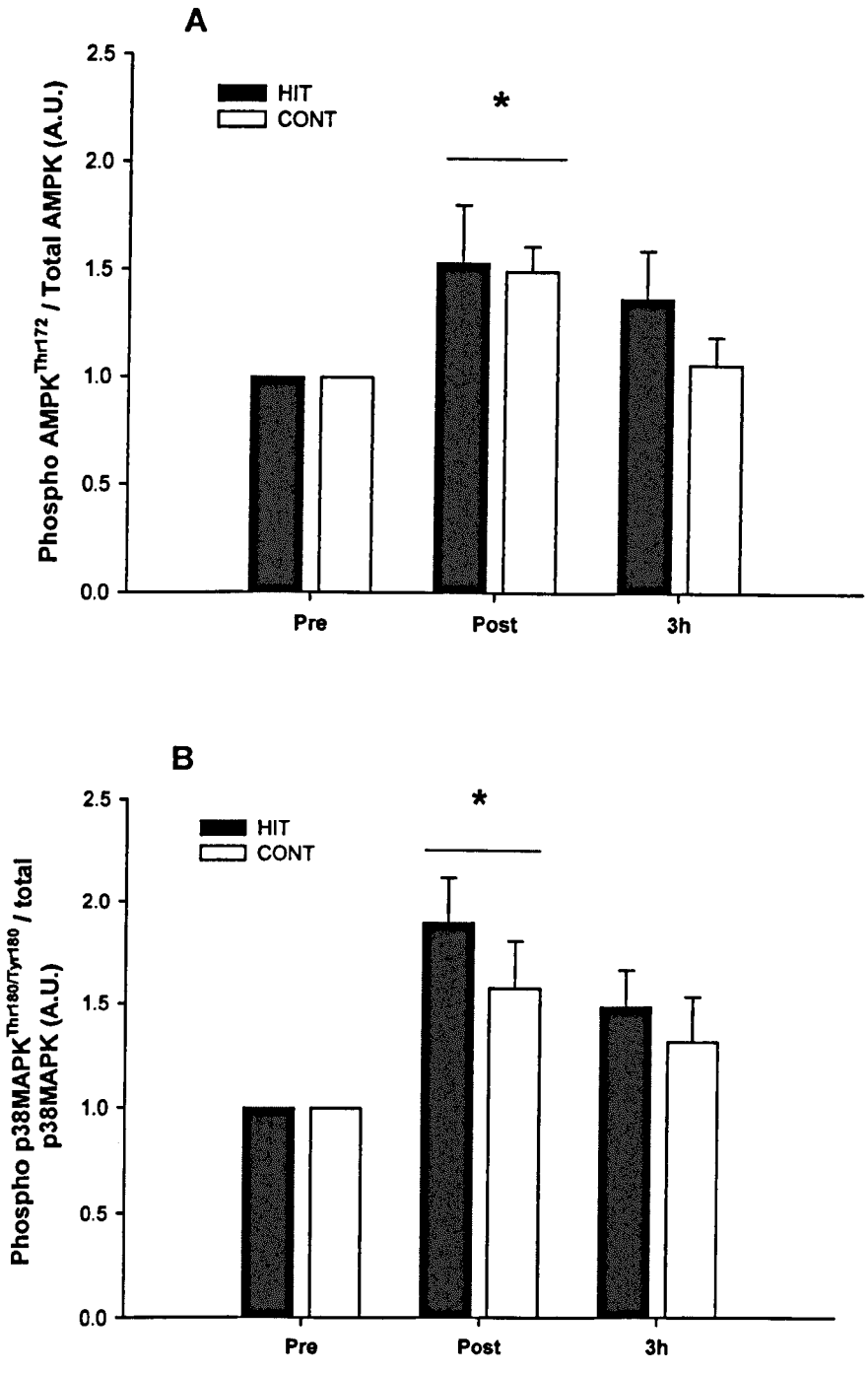

Figure 5. 2 A) Phosphorylation (P-) of AMPK^{Thr172} and B) P-p38MAPK$^{Tyr180/Thr182}$ expressed relative to total AMPK immediately before (Pre), after (Post) and 3 h after (3h) the HIT and CONT protocols. * Denotes significant difference from pre-exercise ($P < 0.05$).

5.3.3. PGC-1α mRNA and protein content

Muscle PGC-1α mRNA content increased 4-fold at 3 h following exercise (P = 0.01) with no difference between conditions (P = 0.80; Figure 5.3A). There were no changes in total PGC-1α protein content at any time during the HIT or CONT trials (P = 0.20; Figure 5.3B).

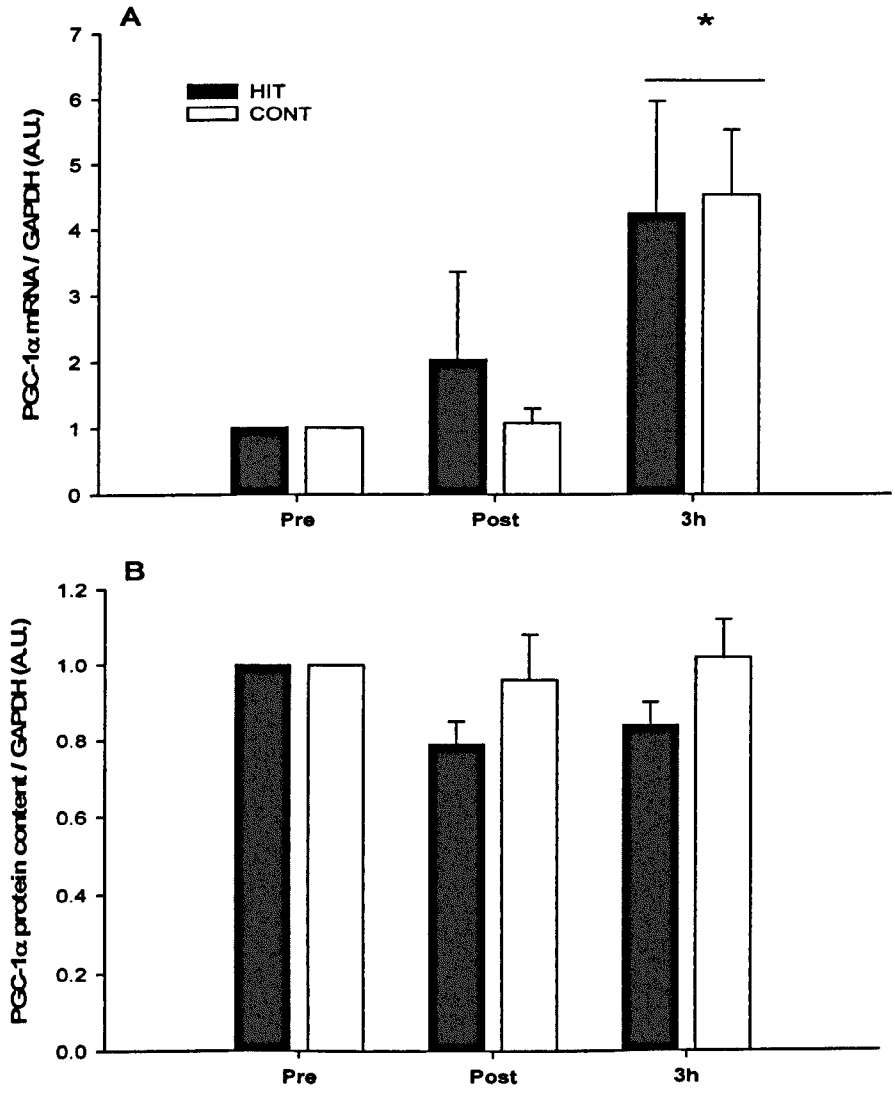

Figure 5. 3 A) PGC-1α mRNA content and, B) PGC-1α total protein content expressed relative to GAPDH immediately before (Pre), after (Post) and 3 hours (3h) after the HIT and CONT protocols. * Denotes significant difference from pre-exercise (P < 0.05).

5.3.4. p53 phosphorylation

$p53^{Ser15}$ phosphorylation showed a tendency to increase immediately post exercise (P = 0.07), but did not reach statistical significance until 3 h following exercise. At this time point, p53 phosphorylation was increased 2.7-fold and 2.1-fold in HIT and CONT trials, respectively (P = 0.01). There was no difference in $p53^{Ser15}$phosphorylation between exercise protocols (P = 0.91; Figure 5.4).

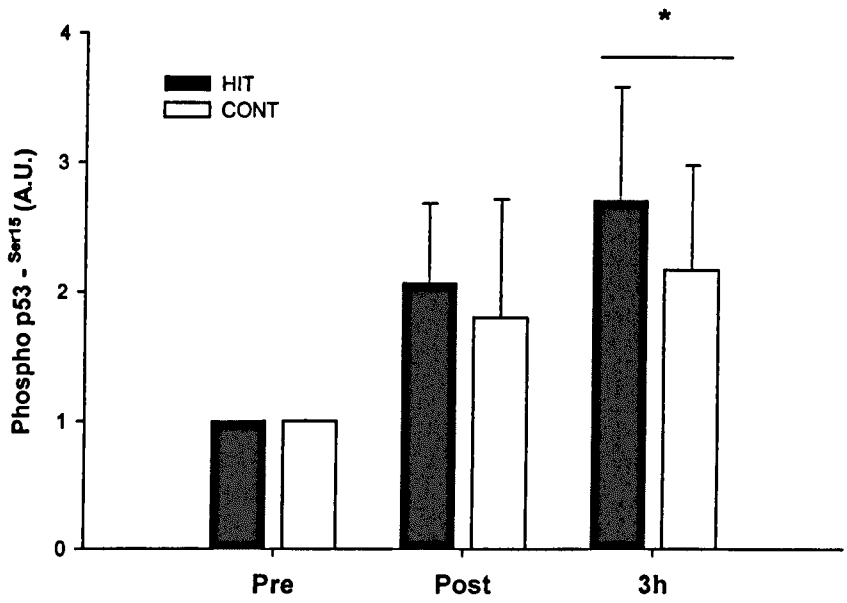

Figure 5. 4 Phosphorylation of $p53^{Ser15}$ immediately before (Pre), after (Post) and 3 hours (3h) after the HIT and CONT protocols. * Denotes significant difference from pre-exercise (P < 0.05).

5.3.5. HSP72 and MnSOD mRNA content

Although there was a 3-4-fold increase in HSP72 mRNA immediately post-exercise this did not reach statistical significance (P = 0.10). At 3 h post-exercise however, there was an approximate 4-fold increase in both trials (P = 0.04) with no difference (P = 0.87) between conditions (Figure 5.5A). In contrast, neither exercise protocol increased MnSOD mRNA content at any time point post-exercise (p = 0.44) (Figure 5.5B).

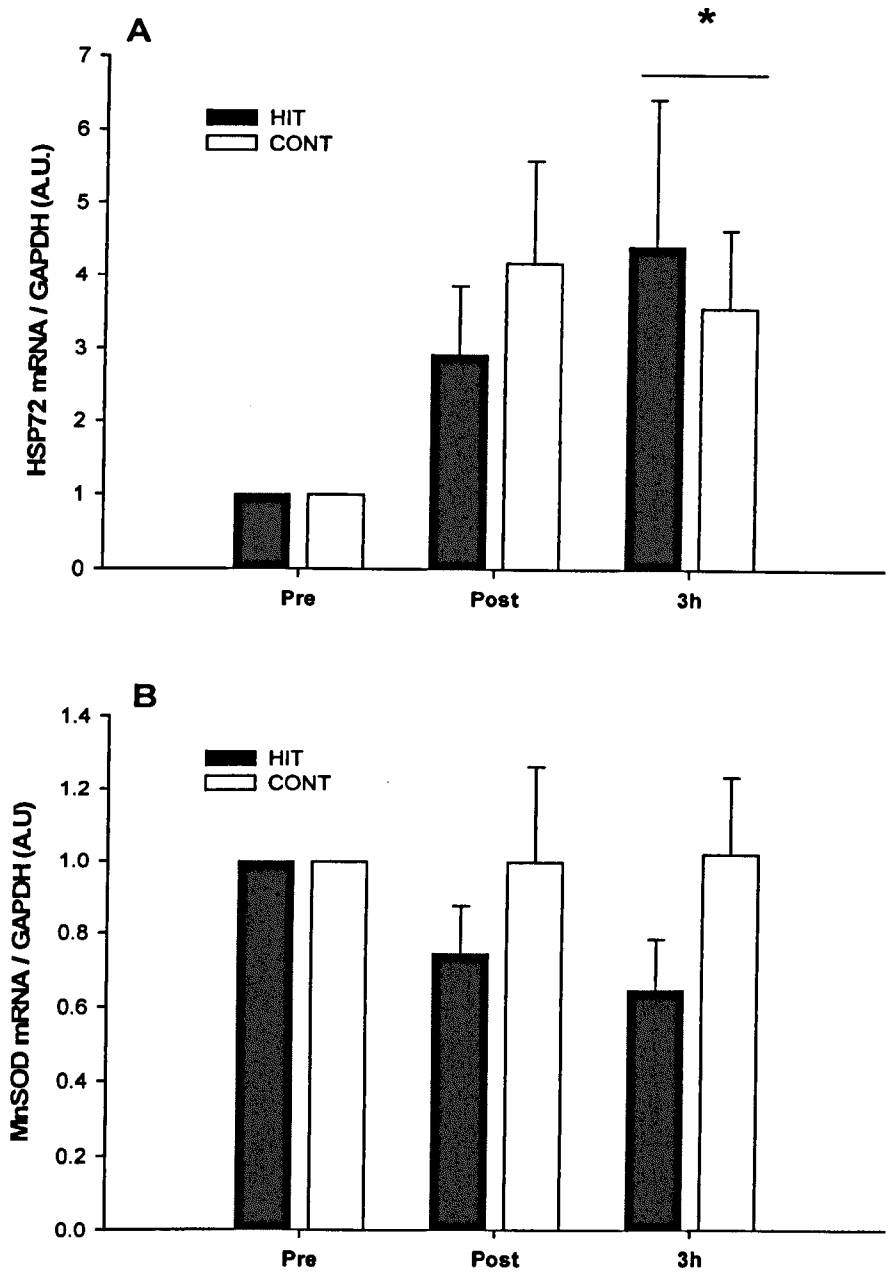

Figure 5. 5 A) HSP72 mRNA content, and B) MnSOD mRNA content immediately before (Pre), after (Post) and 3 hours (3h) after the HIT and CONT protocols. * Denotes significant difference from pre-exercise ($P < 0.05$).

5.4. DISCUSSION

The aim of the current study was to characterise the acute signalling responses of human skeletal muscle to HIT and CONT running exercise and test the hypothesis that HIT would induce greater mitochondrial signalling than CONT. Contrary to our hypothesis, we observed no differences in activation of the above signalling cascades between HIT and CONT running when both protocols are matched for average intensity, duration and distance ran. We provide novel data by demonstrating that both HIT and CONT running induces comparable AMPK and p38MAPK phosphorylation immediately post-exercise with similarly increased PGC-1α mRNA at 3 hours post-exercise. Additionally, this is the first report of exercise-induced p53 phosphorylation in human skeletal muscle.

Exercise-induced phosphorylation of AMPK is well documented in human skeletal muscle following cycling based protocols (Cochran et al. 2010, Gibala et al. 2009, Little et al. 2010, 2011). To the authors' knowledge, however, this is the first report of AMPK phosphorylation in response to running exercise. Given that exercise increases AMPK phosphorylation in an intensity-dependent manner (Egan et al. 2010; Rose et al. 2009), we hypothesised that HIT would augment AMPK signalling to a greater extent compared with CONT. Despite these reports, our data demonstrate no differences in exercise-induced AMPK phosphorylation between HIT and CONT running. We suggest that these results are due to the fact that both protocols were matched for average intensity, duration and distance ran therefore suggesting that the intensity-dependant activation of AMPK is driven by the absolute intensity and perhaps not the average intensity across a given bout of exercise. Our chosen model of HIT consisted of 6 x 3-min periods at 90 % $\dot{V}O_{2max}$ interspersed with 3-min active recovery periods at 50 % $\dot{V}O_{2max}$, as well as a combined 14 min period of warm-up and cool-down at 70 % $\dot{V}O_{2max}$. In this way, the majority of exercise in the HIT protocol (32 minutes) was performed at intensities equal to or greater than 70 % $\dot{V}O_{2max}$. However, it appears that the repeated 3 min intervals at 90 % $\dot{V}O_{2max}$ within this time-scale (i.e. 18 minutes) offers no additional augmentation with regards to AMPK phosphorylation when compared to that of 50 minutes of CONT running at 70 % $\dot{V}O_{2max}$. Support for this hypothesis stems from the data of Howlett et al. (1998) where it can be estimated that important allosteric regulators of AMPK signalling, AMP and ADP concentration, are similar when comparing 3 minutes of exercise at 90% versus 70 % $\dot{V}O_{2max}$. It is possible, therefore, that a difference in relative exercise intensity of 20 % $\dot{V}O_{2max}$ between exercise protocols which are already relatively intense may not be sufficient to cause further metabolic signalling responses. Furthermore, the 6 x 3-min recovery periods at 50 % $\dot{V}O_{2max}$ may not offer a metabolic stress severe enough to induce AMPK signalling given that exercise at comparable intensities does not induce AMPK phosphorylation (Egan et al. 2010). In contrast, CONT running for 50 minutes at 70 % $\dot{V}O_{2max}$ likely offers a sustained stimulus for AMPK phosphorylation as opposed to the transient periods of stress induced by the intermittent bursts of activity inherent to HIT running.

Muscle glycogen availability and utilisation, another regulator of AMPK (Yeo et al. 2010), was also not different between trials that may have contributed to the similar AMPK phosphorylation observed in our study. The similar decreases in muscle glycogen content may have been due to the alternating bouts of exercise in HIT between 90% $\dot{V}O_{2max}$ (thus heavily reliant on CHO oxidation) and 50% $\dot{V}O_{2max}$ (more reliant on lipid oxidation), as opposed to 50 minutes of CONT running at 70% $\dot{V}O_{2max}$ which likely induces sustained glycogen utilisation. It is possible that the matched duration and average intensity is more important in regards to muscle glycogen utilisation, rather than the continuous versus intermittent nature of the running. Both resting glycogen concentration (> 300 mmol.kg^{-1} dw) and utilisation (> 100 mmol.kg^{-1} dw) was similar to that observed by us (Morton et al. 2009) and others (Arkinstall et al. 2001; Tsintzas et al. 1995) whilst using similar average exercise intensities and subject populations to that studied here.

The p38MAPK is a stress-activated kinase that has repeatedly shown to be phosphorylated following acute cycling (Cochran et al. 2010; Gibala et al. 2009; Little et al. 2010, 2011) and marathon running (Boppart et al. 2000; Yu et al. 2001). Consistent with these data, we also observed increased p38MAPK$^{Thr182/Tyr180}$ phosphorylation following more practically applicable running exercise protocols, though the magnitude of phosphorylation was not different between HIT and CONT. This finding is similar to recent data demonstrating that p38MAPK phosphorylation is not affected by exercise intensity, even when a large difference between exercise protocols exists such as 80% versus 40% $\dot{V}O_{2max}$ (Egan et al. 2010). Similar to AMPK (Hawley et al. 2005; Irrcher et al. 2008; Yu et al. 2001), p38MAPK phosphorylation is also sensitive to cytosolic calcium (Wright et al. 2008), glycogen availability (Chan et al. 2004) and reactive oxygen species (ROS) (Gomez-Cabrera et al. 2005). Given the difficulties in directly assessing ROS and also due to the limited tissue obtained by our chosen biopsy technique, we attempted to indirectly ascertain the degree of redox signalling induced by our exercise protocols by examining changes in expression of two redox sensitive genes, HSP72 (Fischer et al. 2006; Khassaf et al. 2003) and MnSOD (Gomez-Cabrera et al. 2005). Although we observed no changes in MnSOD mRNA at any time-point, we observed similar four-fold increases in HSP72 expression after HIT and CONT at 3 hours post-exercise. This magnitude and time-course of adaptation is in agreement with previous literature in which similar subject populations and CONT exercise protocols have been studied (Puuntschart et al. 1996). These are the first data, however, to observe increased expression of members of the heat shock family in response to HIT running.

In accordance with the role of AMPK and p38MAPK as upstream signalling kinases regulating PGC-1α expression (Canto & Auwerx, 2010; Wright et al. 2007), we observed a similar four-fold increase in PGC-1α mRNA at 3 hours post- both exercise protocols with no concomitant changes in PGC-1α protein content. This time-course of PGC-1α expression is well documented in human muscle following cycling protocols (Gibala et al. 2009; Little et al. 2010, 2011). In contrast, it appears that changes in PGC-1α protein are typically only observed within days of exercise (Baar et al. 2002; Perry et al. 2010) although, controversially one study reports increased PGC-1α protein

immediately post-exercise (Mathai et al. 2008). To the authors' knowledge, only one study has measured PGC-1α mRNA changes in response to acute running (45 minute of CONT running at 75% $\dot{V}O_{2max}$) where five-fold and four-fold changes (albeit not statistically significant) were observed in the soleus and vastus lateralis muscle, respectively, at 4 hours post-exercise (Harber et al. 2009). Our data extend these findings by providing the first significant report of PGC-1α expression in response to running exercise whilst also demonstrating that the magnitude of response is comparable between HIT and CONT when matched for work done suggesting the importance of matched duration and average intensity when comparing modes of exercise.

The tumour suppressor protein, p53, has recently emerged as a regulator of mitochondrial function (Kruse & Gu, 2006; Matoba et al. 2006; Park et al. 2009). p53 has been shown to modulate the activity of nuclear-encoded synthesis of cytochrome-*c* oxidase (SCO2), which is critical for the proper assembly of the cytochrome-*c* oxidase (COX) enzyme complex in the electron transport chain (Matoba et al. 2006). Furthermore, PGC-1α also contains a p53-binding site in its promoter region (Irrcher et al. 2009) and skeletal muscles from p53 knockout mice display reduced PGC-1α protein content (Saleem et al. 2009) and mitochondrial content (Park et al. 2009; Saleem et al. 2009) compared with wild type animals. Findings from a variety of cell types have shown that AMPK (Jones et al. 2005) and p38MAPK (She et al. 2001) can phosphorylate p53 on serine 15, and acute contractile activity has been demonstrated to increase phosphorylation on this amino acid residue in rodent muscle (Saleem et al. 2009). The present data confirm and extend these findings by demonstrating for the first time that $p53^{Ser15}$ phosphorylation increases in human skeletal muscle at 3 h post-exercise. Based on the time-course of this response and similar fold-changes, it is tempting to speculate that AMPK and p38MAPK signalling during exercise leads to a temporal and co-ordinated downstream p53 phosphorylation followed by p53 mediated transcription of PGC-1α in the hours following exercise. Future studies are needed, however, to confirm the presence and physiological relevance of this signalling cascade. Furthermore, p53 has also been shown to regulate mitochondrial transcription factor A (Park et al. 2009) and as such, the sub-cellular location of p53 following muscle contraction also warrants investigation.

Progressing the protocols on from Chapter 4, from a metabolic perspective these data have resulted in slightly different observations. Despite heart rate showing a trend to be higher following HIT (possibly due to the lower training status of these subjects compared to Chapter 4), RPE was not different between protocols. However, as expected the intermittent bursts of effort induced a higher blood lactate profile than CONT. A further reason for comparable signalling responses between HIT and CONT is the elevated steady-state of blood lactate in CONT. The CONT protocol resulted in a lactate response that ranged between 4.0 - 6.4 mmol. L^{-1} for 40 min highlighting the subjects was working above their lactate threshold, therefore, possibly resulting in the comparable cell signalling responses with HIT. The effect of increased blood lactate in HIT also likely mediated its effects on the decreased circulating fatty acids during and after HIT compared with CONT as it has previously been shown that lactate may inhibit lipolysis (Liu et al. 2009). It should be pointed out that despite the differences in blood metabolites, i.e. higher plasma glycerol and fatty acids in

the CONT protocol it is difficult to ascertain whether a greater proportion of lipids was oxidised during CONT compared with HIT especially given muscle glycogen utilisation was similar between protocols.

In summary, the present study characterises for the first time, the responses of the acute molecular cell signalling pathways in human skeletal muscle in response to two physiologically relevant and practically applicable running exercise protocols. We demonstrate that acute HIT and CONT running (when matched for average intensity, duration and work done) induces comparable increases in AMPK and p38MAPK phosphorylation as well as PGC-1α mRNA content in human skeletal muscle. Furthermore, this is the first report of exercise-induced p53 phosphorylation in humans, consistent with the notion that this protein may be involved in exercise-induced mitochondrial biogenesis. These data have practical implications for the design of physiologically relevant interventions to induce beneficial improvements in human health and athletic performance.

CHAPTER 6

THE EFFECT OF REDUCED CARBOHYDRATE AVAILABILITY ON HIGH-INTENSITY INTERVAL EXERCISE-INDUCED RESPONSES IN HUMAN SKELETAL MUSCLE

Progressing from Chapter 5 where the skeletal muscle cell signalling responses to acute HIT and CONT running were comparable, the aim of the current study was to evaluate the role of carbohydrate availability in modulating and augmenting the exercise-induced cell signalling response. Although we observed comparable responses in Chapter 5, we chose HIT as our chosen exercise protocol given that individuals perceive it to be more enjoyable, it is applicable for improving both human health and performance and also its relevance to an array of team and endurance sports. This work was orally presented at the 17th Annual Congress of the European College of Sport Science (ECSS), Bruges, and was awarded the Young Investigator Award. Furthermore, this work is currently under review for publication.

6.1. INTRODUCTION

In considering possible contractile-induced stressors for activating cell signalling pathways, reductions in carbohydrate (CHO) availability is now emerging as one of the most potent signals (Philp et al. 2012). For example, both AMPK (Wojtaszewski et al. 2003; McBride et al. 2009; Yeo et al. 2010) and p38MAPK (Chan et al. 2004; Cochran et al. 2010) are activated to a greater extent when glycogen availability is low and transcription of several metabolic related genes such as PDK4, CPT-1, CD36, GLUT-4, UCP-3 and HSP72 are also enhanced when exercise is commenced with reduced CHO availability (Febbraio et al. 2002; Pilegaard et al. 2002; Cluberton et al. 2005; Civitarese et al. 2005). Furthermore, both our laboratory (Morton et al. 2009) and others (Hansen et al. 2005; Yeo et al. 2008; Hulston et al. 2010; Van Proeyen et al. 2011) have demonstrated that endurance training deliberately commenced with reduced endogenous and exogenous carbohydrate availability results in enhanced skeletal muscle oxidative capacity. Such nutritional modulation of training-induced adaptations has been termed as the *'train-low: compete-high'* model surmising that selected training sessions be deliberately commenced with reduced carbohydrate availability but yet competition always be performed with high CHO availability (Burke 2010).

Whilst both AMPK and p38MAPK signalling have been shown to be increased with CHO restriction (Wojtaszewski et al. 2003; McBride et al. 2009; Yeo et al. 2010; Cochran et al. 2010), their subsequent effects on downstream regulation of PGC-1α remain equivocal. For example, Pilegaard et al., (2005) demonstrated that restricting CHO in the recovery phase following acute exercise (so as to minimise muscle glycogen resynthesis) augments expression of PGC-1α compared with provision of CHO to promote muscle glycogen resynthesis. In contrast, restricting CHO before, during and after exercise (Russell et al. 2005; Cluberton et al. 2005; Cochran et al. 2010) did not enhance PGC-1α mRNA expression compared with provision of CHO feeding. Furthermore, we observed that the training-induced increases in PGC-1α content in both the vastus lateralis and gastrocnemius of human skeletal muscle occurs independent of both endogenous and exogenous CHO availability (Morton et al. 2009). Taken together, these data suggest that CHO availability does not modulate transcriptional regulation of PGC-1α and therefore may not be central to facilitating the enhanced training stimulus associated with training in conditions of reduced CHO availability.

In this regard, an emerging regulator of mitochondrial biogenesis which may be sensitive to CHO availability is the tumour suppressor protein, p53 (Saleem et al. 2011). Indeed, Saleem et al. (2009) demonstrated that p53 KO mice display reduced mitochondrial function and aerobic capacity and we observed in Chapter 5 for the first time in human skeletal muscle that p53 phosphorylation increases in a signalling time-course that appears related to upstream signalling through AMPK and p38MAPK. Given that both AMPK and p38MAPK have been shown to directly phosphorylate p53 in cell culture models (She et al. 2001; Jones et al. 2005) and also considering they are up-regulated during training with reduced CHO availability (Wojtaszewski et al. 2003; Cochran et al. 2010), it is therefore tempting to speculate that p53 is sensitive to reduced CHO availability in

contracting human skeletal muscle and therefore potentially enhancing the signalling response for a given bout of exercise.

With this in mind, the aim of the present study was to therefore test the hypothesis that reduced CHO availability enhances p53 signalling and expression of metabolic genes associated with regulation of mitochondrial biogenesis and substrate utilisation in human skeletal muscle. Although these data may initially be considered in the context of enhancing the acute signalling response to a given exercise bout, the potential of p53 as a signalling axis regulating mitochondrial biogenesis also has obvious health implications given the function of the mitochondrion and the emerging role of p53 in the pathology of cancer, ageing and insulin resistance (Lago et al. 2011; Vousden & Lane 2007; Vousden & Ryan 2009).

6.2. METHODS

6.2.1. Subjects

Eight recreationally active men volunteered to participate in the study (Age, 25 ± 1.5 yr; weight, 78 ± 2.9 kg; height, 1.77 ± 0.01 m; $\dot{V}O_{2max}$, 55 ± 1.9 ml. kg^{-1}. min^{-1}). The study was approved by the Ethics Committee of Liverpool John Moores University.

6.2.2. Experimental design

In a randomised crossover design separated by a minimum of 7-days and following an initial assessment of maximal oxygen consumption ($\dot{V}O_{2max}$) (see section 3.3), subjects performed an acute bout of high-intensity interval (HIT) running (see section 4.2.2) in conditions of high CHO availability (HIGH) or low CHO availability (LOW). In the LOW trial subjects reported to the laboratory at 6:30 pm the evening before the acute bout of HIT running and performed an intermittent glycogen depleting cycling protocol. In the 30 minutes following the glycogen depletion protocol the subjects consumed a low carbohydrate snack (< 50 g CHO) so as to minimise any muscle glycogen resynthesis (Morton et al. 2009). On the morning of testing subjects reported to the laboratory in a glycogen depleted and fasted state (LOW) and performed an acute bout of HIT running. In the HIGH trial subjects reported to the laboratory only on the morning following a high carbohydrate diet (8 $g.kg^{-1}$ bm) the day before and a high carbohydrate breakfast (2 $g.kg^{-1}$ bm) on the morning of testing which ensured they commenced the acute bout of HIT running with high CHO availability. In addition, subjects in HIGH were fed 1 $g.min^{-1}$ CHO before and during exercise and 1.2 $g.kg^{-1}.h^{-1}$ bm in the 3 h following exercise. In the LOW trial, subjects consumed a diet low in CHO (3 $g.kg^{-1}$ bm) in the 24 h leading up to the acute bout of HIT running and completely restricted CHO intake during the course of testing. Muscle biopsies (see section 3.6) were obtained pre-, post- and 3 h post the morning HIT protocol and venous blood samples (see section 3.5) were obtained at regular intervals during the course of testing so as to profile the metabolite responses to HIT running exercise with low or high carbohydrate availability. Heart rate (see section 3.2.1) and RPE (see section 3.4.1) were monitored continuously throughout exercise and

CHO and lipid oxidation rates were monitored continuously through indirect calorimetry (see section 3.2.4).

6.2.3. Glycogen depletion

Due to the increased recruitment of the vastus lateralis during cycling compared with running (Arkinstall et a. 2001) a cycling depletion protocol was chosen so as to maximally deplete the vastus lateralis as much as possible prior to the morning HIT running protocol. Prior to the start of the study subjects were required to perform a maximal incremental cycling test to volitional fatigue on a Lode ergometer (Daum Electronic Premium 8i, Furth, Germany) for determination of peak power output (PPO). The maximal incremental protocol commenced at 50 W for 2-min and the work rate was increased by 35 W every minute thereafter until exhaustion (Hawley & Noakes 1992). The exercise protocol utilised to deplete muscle glycogen is an adapted version of the protocol used by Pedersen et al. (2008). Following a 5-min warm up at 50 W, subjects commenced cycling at 90 % of PPO followed immediately by 2-min recovery at 50 % of PPO. This work-recovery protocol was maintained until the subjects were unable to complete 2-min at 90 % PPO, determined as an inability to maintain a cadence of 60 rpm for 15 s. As the subjects had limited cycling experience their ability to perform 2-min at 90 % of PPO was restricted therefore, when subjects could not maintain 2-min, the work bouts reduced to 1-min whilst maintaining the 2-min recovery bouts at 50 % of PPO. When the subjects were unable to maintain the 1-min work-recovery bouts the work period was reduced to 30 seconds. Once the subjects could not maintain 90 % of PPO for 30-s the intensity was lowered to 80 % of PPO and the exercise bouts returned to 2-min before lowering to 1-min and 30-s. When the subjects could not maintain 80 % of PPO at 30-s the intensity was lowered to 70 % of PPO and finally 60 % of PPO. Exercise was terminated when subjects could not complete 30-s of cycling at 60 % of PPO at a cadence corresponding to > 60 RPM. This protocol was chosen so as to maximally deplete both Type I and Type II muscle fibres of the subjects' glycogen stores (Kuipers et al. 1987). The activity pattern and total time to exhaustion was recorded and water was consumed *ad libitum* throughout exercise.

6.2.4. Diet control

In the 24 h preceding the main experimental trial, subjects consumed a low CHO (3 $g.kg^{-1}$ bm) or high CHO (8 $g.kg^{-1}$ bm) diet. On the day of testing, subjects in HIGH were also provided with a high CHO breakfast containing 2 $g.kg^{-1}$ bm 2 h prior to commencing the HIT protocol. In addition, in the 10-min before exercise, subjects consumed 8 $ml.kg^{-1}$ of a CHO beverage (6.4 %, Lucozade Sport, GSK, UK) as well as 3 $ml.kg^{-1}$ during active recovery periods 2 (19-min) and 5 (31-min). Immediately post-exercise (after muscle biopsy) and every subsequent hour until the 3 h biopsy was obtained subjects consumed 1.2 $g.kg^{-1}$ bm in the form of CHO drinks and snacks. In LOW, subjects were totally restricted from CHO and thus commenced the HIT protocol in both a glycogen depleted and overnight fasted state. Subjects were allowed water ad libitum only in the 3 h recovery period leading to the final biopsy and thus remained fasted before, during and after exercise.

6.2.5. Muscle analysis

Muscle tissue was analysed for muscle glycogen concentration (see section 3.9) as well as key signalling proteins (e.g. AMPK, p38MAPK, p53) associated with mitochondrial biogenesis via the western blotting technique (see section 3.7). In addition, the RT-qPCr method (see section 3.8) was utilised to measure mRNA content of PGC-1α, COXIV, SCO2, PDK-4, CPT-1, and TFAM.

6.2.6. Statistical analysis

Statistical analysis was conducted using the Statistical Package for Social Sciences software programme (version 17). Metabolic responses (i.e. blood metabolites and muscle glycogen data), western blot and mRNA data were analysed using a two way repeated measures General Linear Model where the within factors were time and condition (HIGH versus LOW). A comparison of the average physiological responses during exercise were analysed using students t-tests for paired samples. All data in text, figures and tables are presented as means (SEM) with P values ≤ 0.05 indicating statistical significance.

Table 6. 1 Low carbohydrate diet for the before exercise based on a 75-kg man consuming 3 $g.kg^{-1}$ bm.

Food and drink schedule	Time	Description	Nutrient
Breakfast	8:00	Large bowl of cornflakes (40 g) with 200 ml semi skimmed milk Large glass of orange juice (200 ml)	391 Kcal; 81 g CHO; 12 g Pro; 3 g Fat
Mid-morning snack	10:30-11:00	One banana	118 Kcal: 28 g CHO; 1.6 g Pro; 0.4 g Fat
Lunch	13:00-13:30	One ham salad (lettuce, tomato) sandwich made with 2 slices thick white bread Muller rice with strawberry yogurt	664 Kcal: 111 g CHO; 26 g Pro; 15 g Fat
Mid-afternoon snack	15:00	Snack a jacks x 1	83 Kcal; 17 g CHO; 1.5 g Pro; 1.5 g Fat
Dinner	17:30-18:00	One large grilled chicken fillet Medium portion of pasta (110 g) Tomato based sauce (100 g)	438 Kcal; 40 g CHO; 50 g Pro; 9.5 g Fat
Evening snack	21:00	One tin of tomato soup (400 g)	215 Kcal; 24 g CHO; 3 g Pro; 12 g Fat
TOTAL			1560 Kcal; 233 g CHO; 90 g Pro; 36 g Fat (56 % CHO, 23 % Pro, 21 % Fat)

Table 6. 2 High carbohydrate diet for the day before exercise based on a 75-kg man consuming 8 $g.kg^{-1}$ bm.

Food and drink schedule	Time	Description	Nutrient
Breakfast	8:00	Large bowl of cornflakes (40 g) with 200 ml semi skimmed milk Four slices of white toast with butter and jam Large glass of orange juice (400 ml)	739 Kcal; 143 g CHO; 20 g Pro; 13 g Fat
Mid-morning snack	10:30-11:00	One banana Lucozade Original	441 Kcal; 114 g CHO; 1.6 g Pro; 0.4 g Fat
Lunch	13:00-13:30	Two ham salad (lettuce, tomato) sandwiches made with 4 slices thick white bread Muller rice with strawberry yogurt Lucozade Original	1123 Kcal; 211 g CHO; 35 g Pro; 20 g Fat
Mid-afternoon snack	15:00	Snack a jacks x 2	167 Kcal; 33 g CHO; 3 g Pro; 3 g Fat
Dinner	17:30-18:00	One large grilled chicken fillet Large portion of pasta (220 g) Tomato based sauce (100 g) Lucozade Original	613 Kcal; 75 g CHO; 57 g Pro; 11 g Fat
Evening snack	21:00	Large bowl of cornflakes (40 g) with 200 ml semi skimmed milk	239 Kcal; 44 g CHO; 10 g Pro; 3 g Fat
TOTAL			3322 Kcal; 623 g CHO; 128 g Pro; 52 g Fat (70 % CHO, 15 % Pro, 14 % Fat)

Table 6. 3 Food and drink schedule for the HIGH trial on the day of testing based on a 75-kg man. Contains high carbohydrate breakfast providing 2 $g.kg^{-1}$ bm.

Food and drink schedule	Time	Description	Nutrient
Breakfast	2 h pre-exercise	Large bowl of cornflakes (60 g) with 240 ml semi skimmed milk Four slices think white toast with butter and jam Large glass of orange juice (400 ml)	813 Kcal; 160 g CHO; 23 g Pro; 14 g Fat (73 % CHO, 11 % Pro, 16 % Fat)
Exercise	5-min pre-exercise	Lucozade Sport - 8 ml/kg (600 ml)	144 Kcal; 38 g CHO
	15-min into exercise	Lucozade Sport – 3 ml/kg (225 ml)	54 Kcal; 14 g CHO
	30-min into exercise	Lucozade Sport – 3 ml/kg (225 ml)	54 Kcal; 14 g CHO
Recovery	Immediately post-exercise	Five Jaffa Cakes and 250 ml Lucozade Original	330 Kcal; 88 g CHO; 3 g Pro; 5 g Fat
	+1 h into recovery	Five Jaffa Cakes and 250 ml Lucozade Original	330 Kcal; 88 g CHO; 3 g Pro; 5 g Fat
	+ 2 h into recovery	Five Jaffa Cakes and 250 ml Lucozade Original	330 Kcal; 88 g CHO; 3 g Pro; 5 g Fat
TOTAL			2226 Kcal; 490 g CHO; 32 g Pro; 29 g Fat

6.3. RESULTS

6.3.1. Exercise variables

There was no difference in average heart rate (P = 0.08), $\dot{V}O_2$ (P = 0.38) and RPE (P = 0.19) during exercise between the HIGH and LOW conditions (see Table 2). In contrast, there was a significant difference in RER during exercise in HIGH compared with LOW (P = 0.008). As such, total CHO oxidation was significantly greater in HIGH compared with LOW (P = 0.001) whereas total lipid oxidation was greater in LOW compared with HIGH (P = 0.004) (Table 6.4).

6.3.2. Blood analyses

An overview of the blood metabolites are shown in Figure 6.1. Plasma glucose, lactate and serum insulin were significantly higher in HIGH compared to LOW (P < 0.05). In contrast, plasma glycerol (*P* < 0.001) and fatty acid concentrations (P = 0.001) were significantly higher during the LOW condition compared to the HIGH condition.

Table 6. 4 Exercise variables during 50-min HIT running. † Denotes significantly different from HIGH (P < 0.05).

	HIGH	LOW
HR (bpm)	163 ± 11	157 ± 11
$\dot{V}O_2$ (ml kg^{-1} min^{-1})	37.6 ± 3.9	36.6 ± 3.9
% $\dot{V}O_{2max}$	69 ± 4	67 ± 3
RER	0.98 ± 0.02	0.90 ± 0.05 †
CHO oxidation (g)	177 ± 17	122 ± 26 †
Fat oxidation (g)	8 ± 1	23 ± 10 †
RPE (A.U.)	14.4 ± 1.29	14.9 ± 0.96

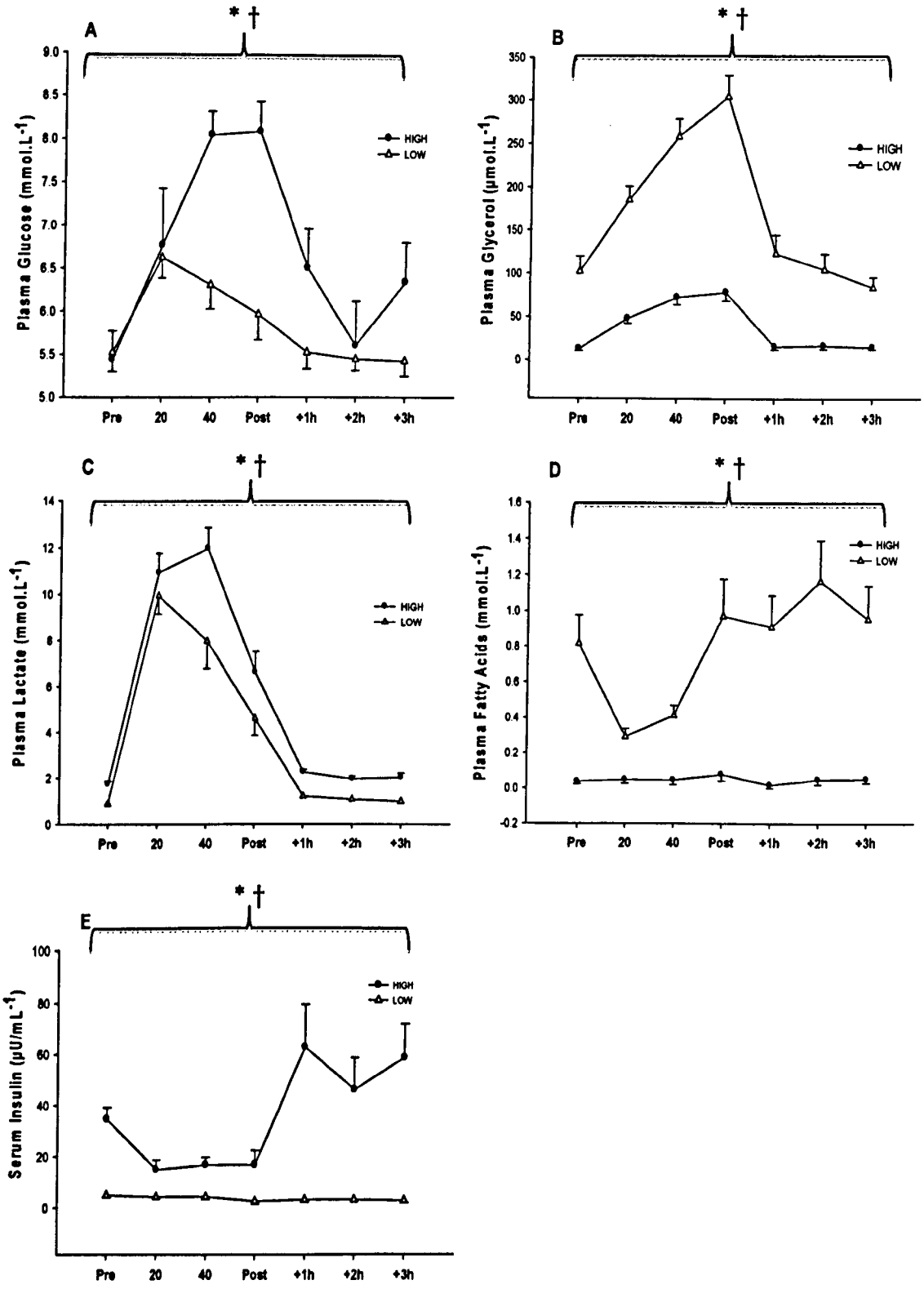

Figure 6. 1 A) Blood glucose, B) glycerol, C) lactate, D) fatty acids and E) serum insulin responses to HIGH and LOW trials at various time points during testing. * Denotes significant main effect of time. † Denotes significantly different from HIGH ($P < 0.05$).

6.3.3. Muscle glycogen

Cycle time to exhaustion during the glycogen depletion protocol in the LOW trial was 68 ± 10 min. Pre-exercise muscle glycogen was higher in HIGH compared with LOW (NORM: 467 ± 19; LOW: 103 ± 9, $P < 0.001$) and this difference persisted immediately post-exercise and 3 h post exercise. Furthermore, the exercise-induced decreases in muscle glycogen ($P = 0.02$) were greater in HIGH compared with LOW ($P = 0.01$) whereby total glycogen utilisation during exercise was 142 ± 34 and 30 ± 12 mmol.kg^{-1} dw, respectively (Figure 6.2).

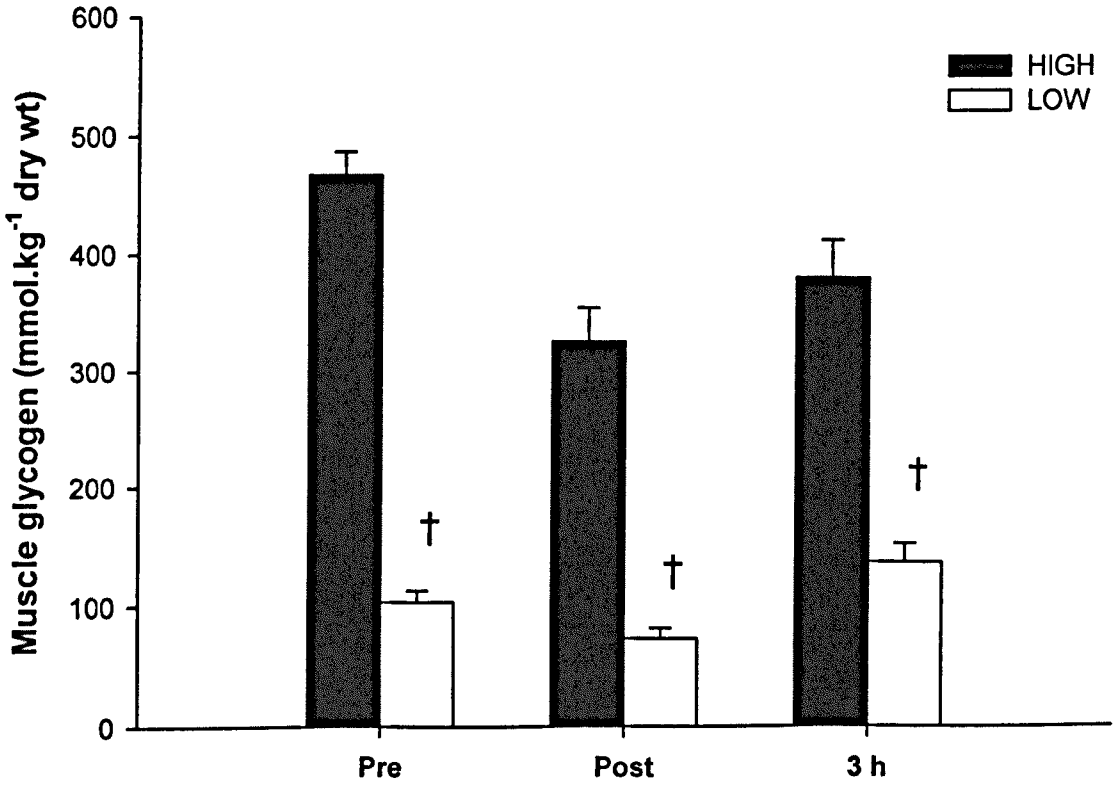

Figure 6. 2 Muscle glycogen before (Pre), after (Post) and 3 hours (3h) after the HIGH and LOW trials. † Denotes significant difference from HIGH ($P < 0.05$).

6.3.4. Muscle signalling

Phosphorylation (P-) of ACC, a marker for AMPK activity was significantly different with LOW demonstrating a 2.8-fold increase immediately post-exercise compared to no change in HIGH ($P = 0.03$) (Figure 6.3). Similarly, P-p53 displayed a significant difference between conditions ($P = 0.02$) with LOW demonstrating a 2.7-fold increase 3 h post-exercise compared to no change in HIGH (Figure 6.4). In contrast, there was no difference in time ($P = 0.35$) or condition ($P = 0.54$) for P-p38MAPK$^{Tyr180/Thr182}$ following exercise (Figure 6.5).

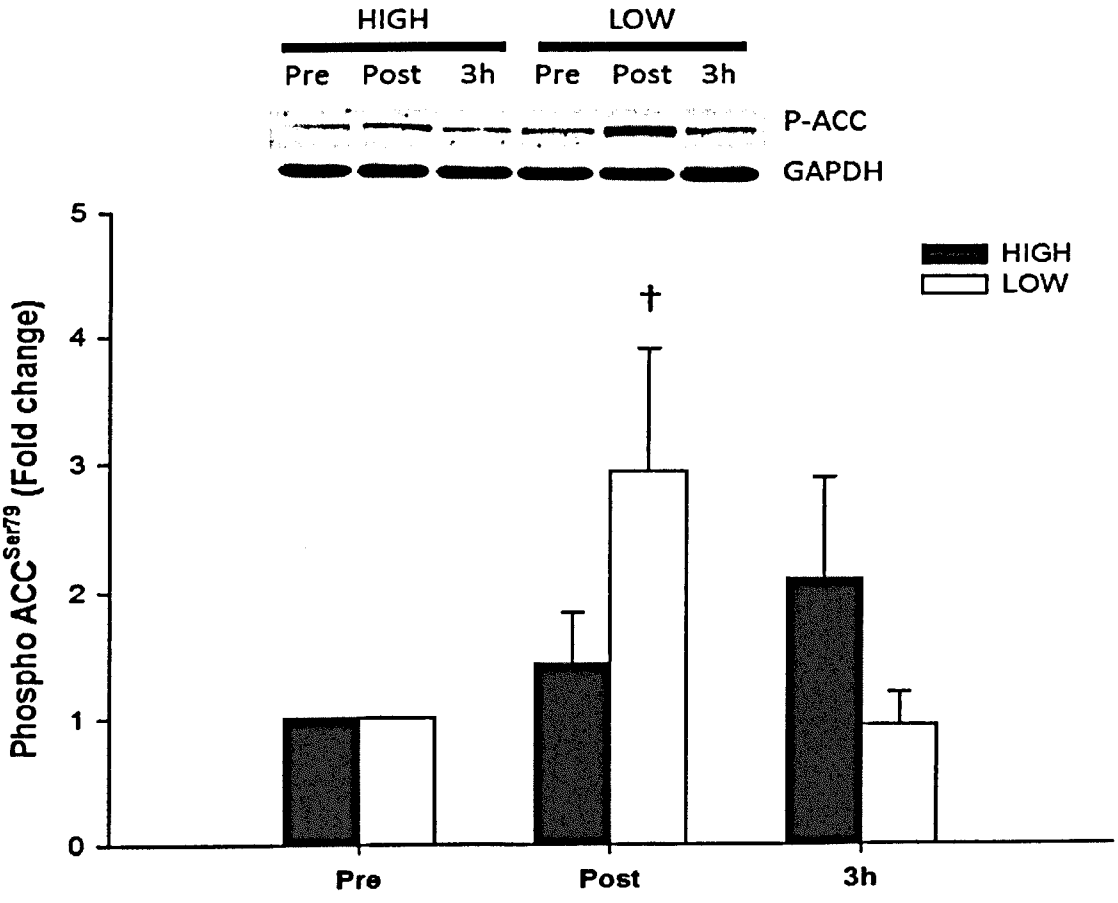

Figure 6. 3 Phosphorylation of ACC^{Ser79} immediately before (Pre), after (Post) and 3 hours (3h) after the HIGH and LOW trials. † Denotes significantly different from HIGH ($P < 0.05$).

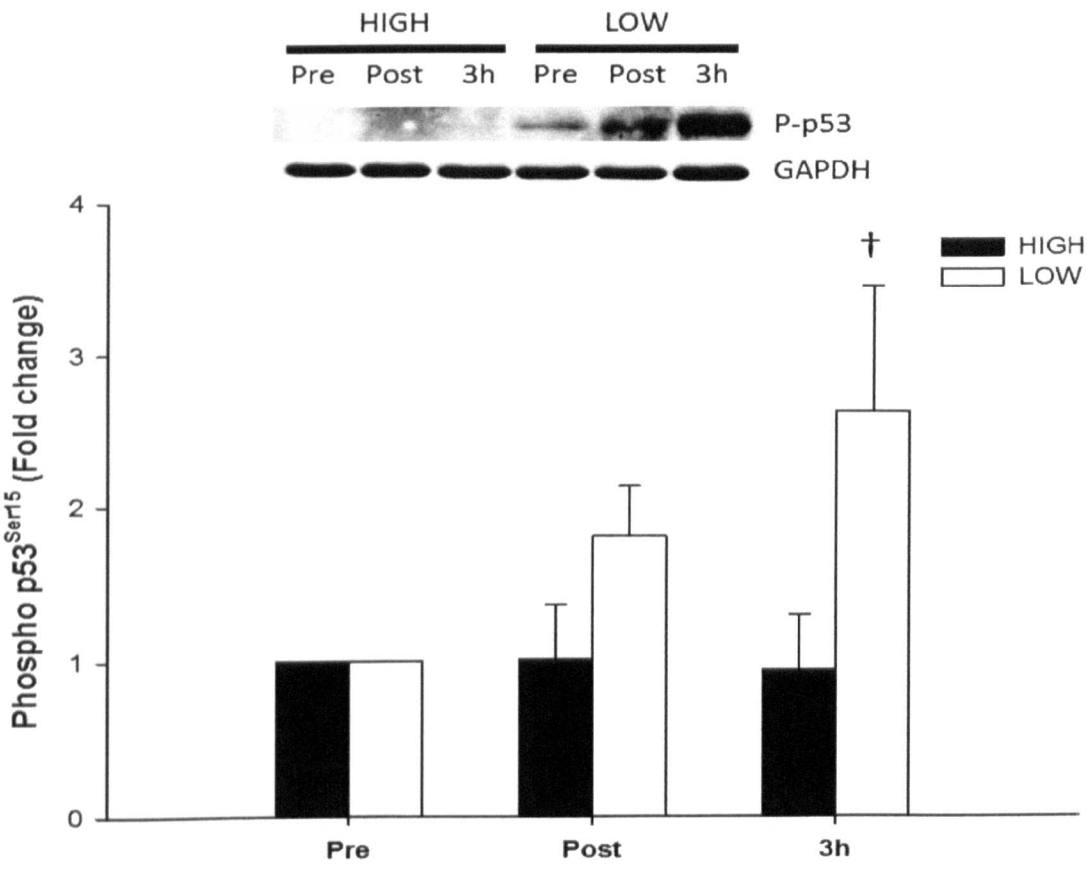

Figure 6. 4 Phosphorylation of $p53^{Ser15}$ immediately before (Pre), after (Post) and 3 hours (3h) after the HIGH and LOW trials. † Denotes significantly different from HIGH ($P < 0.05$).

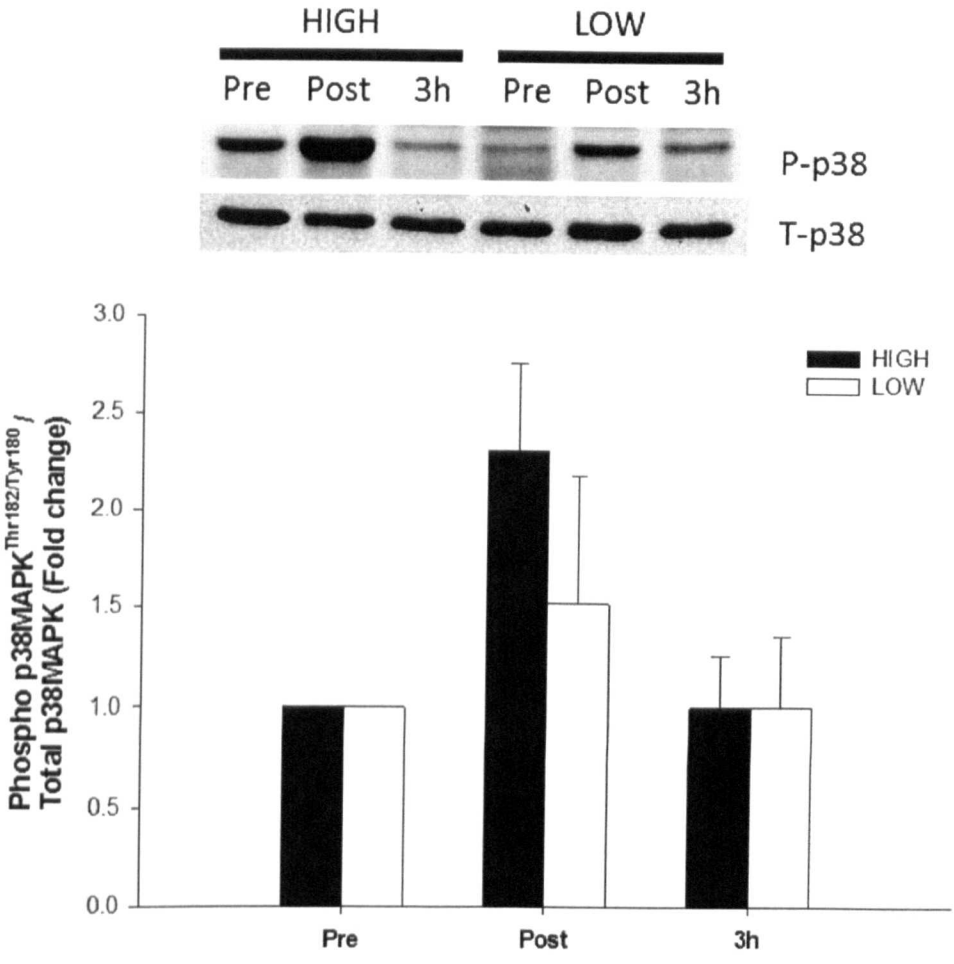

Figure 6. 5 Phosphorylation of p38MAPK$^{Thr180/Tyr182}$ expressed relative to total p38MAPK immediately before (Pre), after (Post) and 3 h (3h) after the HIGH and LOW trials.

6.3.5. mRNA analysis

There was a significant effect of condition for mRNA content of PGC-1α (P = 0.05), COXIV (P = 0.05), Tfam (P = 0.02), PDK4 (P = 0.016) and a trend for CPT1 (P = 0.09) such that both pre- and post-exercise values were higher in LOW compared with HIGH (see Figure 6.6). SCO2 mRNA displayed no significant effect of CHO availability (P = 0.10). Exercise increased PGC-1α expression in both HIGH and LOW (P = 0.01), however, this increase was independent of CHO availability (P = 0.96). In contrast, no effects of exercise was observed for COXIV (P = 0.26), Tfam (P = 0.18), PDK4 (P = 0.92), CPT1 (P = 0.29) and SCO2 (P = 0.18) in either the HIGH or LOW trials (see Figure 6.6).

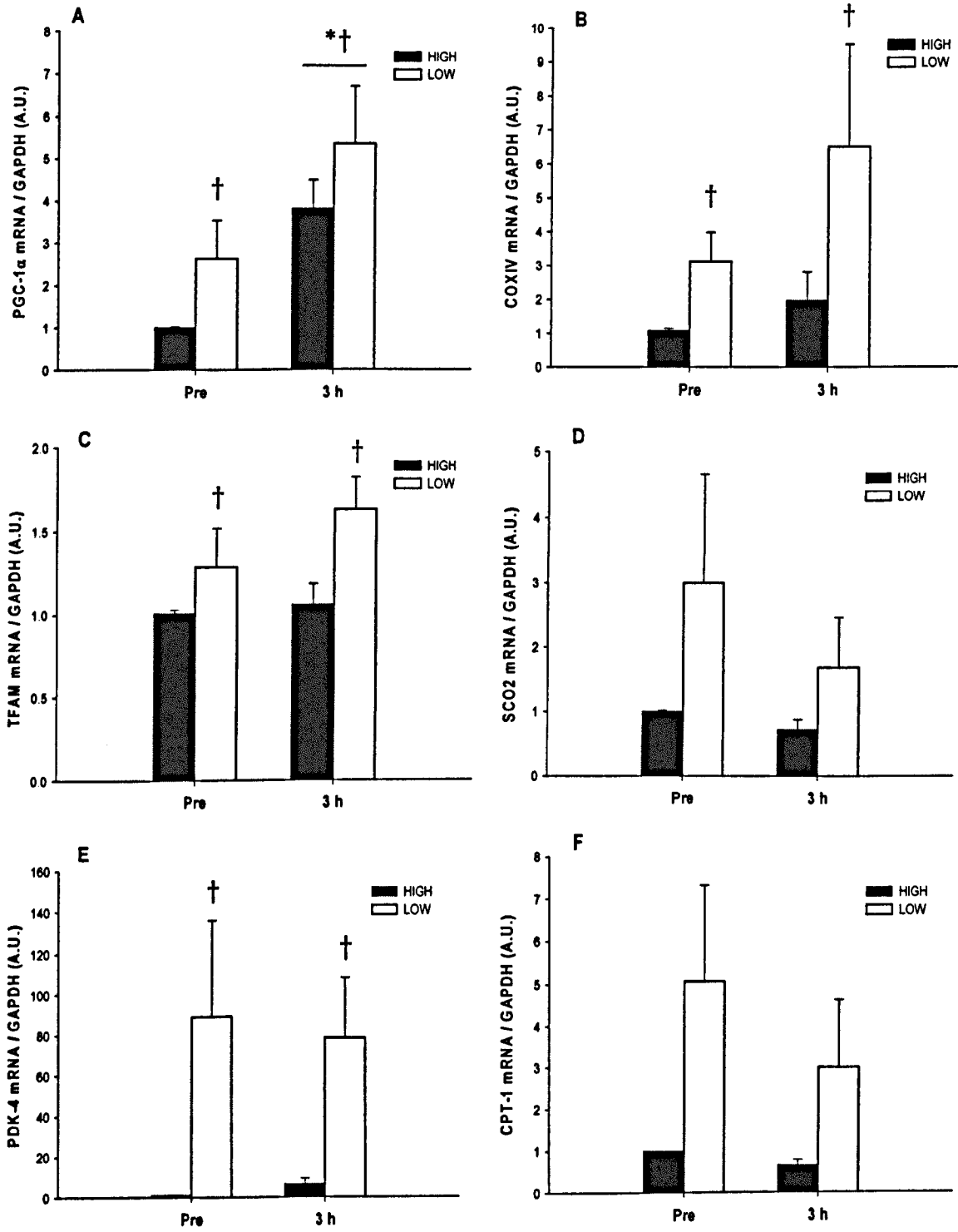

Figure 6. 6 mRNA content of A) PGC-1α, B) COXIV, C) Tfam, D) SCO2, E) PDK-4 and F) CPT-1 expressed relative to GAPDH immediately before (Pre) and 3 h (3 h) after exercise. * Denotes significant difference in time ($P < 0.05$). † Denotes significant difference between conditions ($P < 0.05$).

6.4. DISCUSSION

The aim of the present study was to test the hypothesis that exercising with reduced CHO availability enhances p53 signalling and expression of metabolic genes associated with regulation of mitochondrial biogenesis and substrate utilisation in human skeletal muscle. To the author's knowledge, this is the first report in contracting human skeletal muscle, that p53 phosphorylation is enhanced during conditions of reduced CHO availability. Furthermore, by altering the nutrient availability of the cell, AMPK signalling through ACC was also enhanced in conditions of reduced CHO availability. Given the emerging role of p53 in mitochondrial biogenesis, i.e. promoting exercise capacity (Matoba et al. 2006; Saleem et al. 2009) and in the pathology of metabolic disorders such as cancer, ageing and insulin resistance (Vousden & Ryan 2009) it is considered that these data have relevance for both the athletic and clinical populations.

In order to assess whether low CHO availability modulates the acute cell signalling response in human skeletal muscle, we adopted a model whereby the LOW trial consisted of exercise performed in a glycogen depleted, overnight fasted and CHO restricted state (a protocol which could effectively be referred to as sleep-train-recover low). In contrast, exercise in the HIGH condition was performed after a 24 h CHO loading strategy as well as consuming CHO before, during and after exercise in accordance with typical doses advised to athletic populations (ACSM et al. 2009). This model of exercise and nutritional manipulation was successful in achieving two extremes of endogenous and exogenous CHO availability, as evidenced by differences in muscle glycogen and plasma glucose across all time-points studied. Such differences in substrate availability induced pronounced differences in substrate utilisation and metabolic regulation during exercise. Indeed, consistent with other authors (Hargreaves et al. 1995; Arkinstall et al. 2004; Roepstorff et al. 2004), we also observed resting muscle glycogen and utilisation was greater in HIGH compared with LOW (as was plasma lactate concentrations) whereas plasma NEFA availability was greater in LOW, the latter likely due to low circulating insulin levels when in the fasted state (Horowitz et al. 1997). In agreement with previous reports (Pilegaard et al. 2002; Pilegaard et al. 2005), we also observed both resting and post-exercise PDK4 and CPT1 mRNA were increased in LOW compared with HIGH thus reflecting early transcriptional regulation of two key enzymes regulating both CHO and lipid metabolism. When examined at the whole body level, exercise in LOW resulted in lower RER values and three times greater total lipid oxidation compared with HIGH, despite identical work done and the relatively intense nature of our exercise protocol. These data agree with many previous reports (Arkinstall et al. 2004; Bosch et al. 1993; Coyle et al. 1997; Hargreaves et al. 1995; Roepstorff et al. 2004; Spencer et al. 1992; Weltan, Bosch, Dennis & Noakes 1998a; Weltan, Bosch, Dennis & Noakes 1998b), that demonstrate the potency of restricting CHO availability on regulating metabolic stress and substrate utilisation in humans.

The AMPK has been purported as an important regulatory signalling kinase that leads to activation of downstream transcription factors and co-activators thought to regulate mitochondrial biogenesis. It is thought to be activated by perturbations in the AMP-ADP:ATP ratio (Hardie & Sakamoto 2006;

Oakhill et al. 2011) but also by low muscle glycogen (Wojtaszewski et al. 2003), the latter possibly due to the presence of a glycogen binding domain in the β-subunit (McBride et al. 2009). As a downstream marker of AMPK activity in vivo (Park et al. 2002; Nielsen et al. 2003; Jäger et al. 2007; Little, Safdar, et al. 2011b), we examined the phosphorylation status of ACC^{Ser79} and despite no evidence of greater AMPK activity pre-exercise, we observed a 3-fold increase in ACC^{Ser79} in LOW immediately post-exercise compared with no change in HIGH, a finding in agreement with other authors (Wojtaszewski et al. 2003; McBride et al. 2009; Yeo et al. 2010). Unfortunately, it is difficult to determine from our chosen experimental design if the apparent enhanced AMPK signalling in LOW is due to low glycogen *per se* and/or low circulating glucose availability. In this regard, it is noteworthy that provision of glucose during exercise also attenuates AMPK activity (Akerstrom et al. 2006) but this effect is only apparent when glucose feeding spares muscle glycogen utilisation (Lee-Young et al. 2006), thus suggesting that glycogen availability may be the predominant mechanism.

In addition to its role as a tumour suppressor protein regulating the interplay between glycolysis and oxidative phosphorylation in cancer cells (Bensaad et al. 2006; Lago et al. 2011), an emerging function of p53 is its apparent role in the regulation of mitochondrial biogenesis in skeletal muscle (Matoba et al. 2006; Saleem et al. 2009). Similar to the magnitude and time-course of change observed in Chapter 5 (i.e. 3-fold at 3 h post-exercise) we show in the current study a near 3-fold increase in phosphorylation of p53. However, this effect was only apparent in LOW compared to no change in HIGH. Given the aim of this study was to maximise the training response for a given bout of exercise these data have clear implications for the athletic population where the continual aim is to enhance the training stimulus. Furthermore, in considering glucose deprivation results in p53 phosphorylation in cell culture in a manner that is dependent upon upstream AMPK activation (Jones et al. 2005), it is possible that this signalling axis may also be regulating p53 activation in contracting skeletal muscle. Such a hypothesis is particularly attractive considering that we only observed phosphorylation of ACC^{Ser79} in the LOW trial. Moreover, although p38MAPK can also regulate p53 phosphorylation in cell culture (She et al. 2001), this mechanism may not underpin p53 regulation in contracting skeletal muscle given that we observed no consistent effects of exercise or CHO availability on $p38MAPK^{Tyr180/Thr182}$ phosphorylation. In relation to the latter, it is noteworthy that the effects of CHO availability on p38MAPK remains inconsistent with some authors observing no effect (Yeo et al. 2010) yet others observing enhanced $p38MAPK^{Tyr180/Thr182}$ phosphorylation when pre-exercise CHO availability is reduced (Cochran et al. 2010). Although we acknowledge that other currently unknown upstream kinases may be regulating p53 activation during exercise, AMPK appears a feasible candidate based on available data at present.

In interpreting the present data, it is also important to consider the potential role of increased FFA availability as signalling molecules regulating training adaptation given that when CHO availability is reduced, FFA availability is concomitantly increased. Indeed, Yeo et al. (2008) observed that five days of a high fat diet followed by one day of CHO restoration increased resting AMPK α_1 and α_2 activity as well as the magnitude of exercise-induced increase in $pACC^{Ser221}$. As such, it is

possible that increased circulating FFA availability may be regulating increased AMPK activity (through a currently unknown mechanism) during exercise thereby leading to downstream p53 phosphorylation. It is therefore difficult to ascertain from the present data whether it is low CHO or increased fatty acid availability that is the primary signalling mechanism. However, conflicting data for a beneficial role of lipid availability in regulating oxidative adaptation of skeletal muscle also exist given that three days of high fat feeding reduces expression of genes involved in oxidative phosphorylation (Sparks et al. 2005) and that pharmacological inhibition of plasma fatty acid availability does not impair exercise-induced increases of those genes involved in regulation of mitochondrial biogenesis or substrate utilisation (Fillmore et al. 2010; Tunstall et al. 2007; Watt et al. 2004).

To investigate the enhanced phosphorylation of p53 further we measured downstream targets of p53 related to mitochondrial biogenesis. In this regard we measured changes in mRNA content of both SCO2 and Tfam. Although we observed no effect of exercise or condition on SCO2 expression, it is possible that measurements at 3 hours post-exercise is not sufficient to detect exercise-induced changes in gene expression (to the authors' knowledge, this is the first study to measure SCO2 mRNA in human skeletal muscle). Similarly, we also observed no effect of exercise on Tfam expression in either HIGH or LOW which is also likely due to time-course issues given that 4-6 hours post-exercise appears necessary to detect exercise-induced increases in mRNA content in human skeletal muscle (Pilegaard et al. 2003; Perry et al. 2010). Despite the apparent lack of an exercise effect, we did observe an effect of condition such that Tfam mRNA levels were higher both before and after exercise in LOW compared with HIGH. However, based on our chosen experimental design, this enhanced gene expression may be due to low CHO availability *per se* and/or a residual effect of the glycogen depleting exercise protocol the evening before. Indeed, the magnitude of elevation of Tfam in LOW compared with HIGH (i.e. approximately 1.5 fold differences) is consistent with time-course studies which show significant increases between 4-24 hours post-exercise which is similar to the time-course of our depletion protocol given that our pre-exercise biopsy was sampled within 10-12 hours upon exercise completion. This potential carryover effect of the evening depletion protocol may also be contributing to the effects of condition on both PGC-1α and COXIV expression in that both genes were also elevated in LOW compared with HIGH. However, in relation to PGC-1α expression, it is noteworthy that exercise increased mRNA levels by similar magnitudes in both trials (approximately 3-fold increases) thus providing further evidence (Cluberton et al. 2005; Russell et al. 2005; Cochran et al. 2010) that acute transcriptional regulation of the PGC-1α gene may not be influenced by CHO availability. Nevertheless, future studies would benefit from measuring both nuclear and mitochondrial translocation of PGC-1α (Safdar et al. 2011) in conditions of both high and low CHO availability so as to provide a more definitive assessment of regulation of this pathway.

Given the emergence of p53 as a regulator of mitochondrial biogenesis in skeletal muscle (Saleem et al. 2009; Park et al. 2009), the nutritional modulation of contraction-induced p53 activation is

likely to have implications for both athletic and clinical populations. Indeed, numerous investigators have demonstrated a functional role of p53 in improving exercise capacity as demonstrated by improved exercise capacity in wild type animals compared with p53 KO animals during fatiguing swimming (Matoba et al. 2006), treadmill running (Park et al. 2009) and electrical stimulation (Saleem et al. 2009) protocols. Furthermore, considering the role of p53 in promoting a shift from glycolysis towards lipid metabolism (Bensaad et al. 2006) and the fact that training with reduced CHO availability promotes lipid utilisation during sub-maximal exercise (Yeo et al. 2008; Hulston et al. 2010), early signalling through p53 may therefore represent an additional potential signalling pathway which regulates oxidative adaptations to endurance training (i.e. increased mitochondrial volume and shifts in substrate utilisation) thereby exerting potent effects on subsequent endurance performance. Moreover, because p53 is associated with tumour suppression (Madan et al. 2011), insulin resistance (Armata et al. 2010) and longevity (Matheu et al. 2007), the combined effects of exercise and low CHO availability on p53 activity therefore represents potential exercise interventions to improve various indices of human health. Clearly, further longitudinal studies examining the physiological function of p53 in human cells and tissues are now warranted.

In summary, these data demonstrate that exercise-induced p53 is modulated by CHO availability in that restriction of CHO enhances p53 phosphorylation in human skeletal muscle which may be regulated, in part, through upstream signalling by AMPK. This model of exercise and nutritional modulation provides a novel signalling axis through which mitochondria may adapt to an acute bout of exercise. Future studies should now focus on the measurement of downstream gene targets of p53 within a more physiologically relevant time-course.

CHAPTER 7

Synthesis of findings

7. Synthesis of Findings

The present Chapter presents an initial overview of the findings in relation to the original aims and objectives of the thesis set out in Chapter 1. A general discussion is then presented where specific attention is given to how the present data has advanced the understanding of the molecular adaptations to endurance exercise training. In addition, this Chapter also contains a discussion on the limitations of the chosen experimental designs and recommendations for future research are also provided.

7.1. Achievement of Aims and Objectives

The aim of this thesis was to characterise the skeletal muscle cell signalling responses thought to regulate mitochondrial biogenesis following an acute bout of high-intensity interval exercise versus moderate-intensity continuous exercise. Having established which mode of exercise provides the greatest training stimulus in terms of activation of acute cell signalling pathways, a further aim was to investigate the role of carbohydrate availability in modulating these responses. These aims were to be achieved via the completion of three objectives, each of which is now discussed in turn.

Aim 1

a) The development of moderate-intensity continuous and high-intensity interval exercise protocols that are matched for total oxygen consumption, average intensity, duration, energy expenditure and distance ran (Study 1).

This objective was addressed via the completion of Study 1 (Chapter 4). In order for this thesis to have a strong methodological basis, it was important for the HIT and CONT protocols to be matched for work done, average intensity, duration and distance ran. With this in mind, the HIT and CONT protocols were initially designed with the aim of being matched for average intensity (70 % $\dot{V}O_{2max}$) and duration (50-min) thereby resulting in similar cumulative distance ran. Subjects subsequently performed the two protocols whilst being assessed for total oxygen consumption and energy expenditure. As a result of matching the average intensity and duration, physiological responses and work done were comparable between the two protocols. Despite higher ratings of perceived exertion in HIT, subjects also associated this protocol with higher ratings of perceived enjoyment than CONT. This study therefore provided a sound methodological platform for which to study the acute cell signalling responses of human skeletal muscle to acute HIT and CONT running whereby any potential differences could be attributed to fluctuations in activity and intensity profile.

Aim 2

b) Characterise the acute skeletal muscle cell signalling responses (associated with the regulation of mitochondrial biogenesis) of human skeletal muscle to an acute bout of high-intensity interval exercise and moderate-intensity continuous exercise that is previously matched for energy expenditure and total oxygen consumption (Study 2).

This objective was addressed via the completion of Study 2 (Chapter 5). As a result of matching the HIT and CONT protocols, comparable skeletal muscle cell signalling responses (i.e. AMPK, p38MAPK phosphorylation and PGC1 expression) were observed. Additionally, this study also provided the first evidence than p53 phosphorylation is increased in contracting human skeletal muscle thereby suggesting a novel-signalling pathway. Due to the observation of comparable signalling responses between HIT and CONT, we chose HIT as the model to further study in Chapter 6 given it is associated with greater feelings of perceived enjoyment than CONT but also its relevance to team sport activity and its application to improve indices of human health.

Aim 3

c) Examining the effects of carbohydrate availability on exercise-induced cell signalling responses of human skeletal muscle (Study 3).

This objective was addressed via the completion of Study 3 (Chapter 6). In addition to modification of the training stimulus, manipulation of nutrient availability has also been shown to enhance the training response. With this in mind, we devised an experimental design in which subjects commenced, completed and recovered from acute HIT with either high or low CHO availability. Interestingly, we observed an enhanced phosphorylation of AMPK and p53 signalling when exercising with reduced carbohydrate availability. In contrast, when in a state of high carbohydrate availability this signalling response was attenuated suggesting that restriction of carbohydrate is a potent method for inducing an enhanced acute training stimulus.

7.2. General Discussion of Findings

The last 10-15 years have significantly advanced our understanding of the molecular signalling pathways, which regulate how skeletal muscle adapts to endurance training. However, the optimal exercise and nutritional stimulus to maximise the training response remains to be determined. In this regard, recent data based on cycling protocols have demonstrated that supra-maximal HIT is a more time efficient method to induce skeletal muscle oxidative adaptations compared with moderate intensity CONT exercise. Despite the quantification of work done on a cycle ergometer being methodologically advantageous in that power output can be readily measured, the practical application of this mode of exercise is limited due to the requirement of a specially braked ergometer that is usually only found in the laboratories. Although work done during running exercise is methodologically more difficult to assess, this form of exercise at sub-maximal or near maximal intensities may be a more practical model of training. The limited available evidence on running exercise is somewhat surprising given that exercise mode is critical to exercise adherence (Daley & Maynard 2003; Glass & Chvala 2001) but also the relevance of running to an array of sporting situations. As such, the present thesis has provided novel data for the literature by

providing the first report to examine the acute cell signalling responses to running exercise. Importantly, we utilised two running protocols of CONT and HIT that are matched for work done but are also relevant to the general population (as 'keep fit' activities) and the elite athlete (both endurance and team sport athletes). Herein, we report comparable signalling for AMPK, p38MAPK and PGC-1α between HIT and CONT, which is likely due to the matching of average intensity, duration and work done. This is the first comparison of acute cell signalling responses following HIT and CONT when matched for these parameters, however, the signalling events observed are similar to that seen in other models of endurance exercise such as knee extensor and cycling exercise. In considering a potential approach to augmenting the signalling events, the intermittent bursts within the interval protocol may have to be performed at supra-maximal intensity with a shorter duration as shown previously by Gibala and colleagues (2009). Whilst the present data are based on an acute bout of HIT and CONT running, it is currently unknown as to the cumulative effect (i.e. repeated training sessions) of these bouts of exercise on skeletal muscle adaptation and thus longitudinal training studies incorporating our chosen model of HIT and CONT are now warranted.

Perhaps the most significant contribution to the literature is the finding of exercise-induced phosphorylation of p53. This is the first set of data in humans implying a role for p53 in the regulation of mitochondrial biogenesis and also appear in agreement with a recent study in mice demonstrating a temporal pattern of p53 signalling following AMPK and p38MAPK phosphorylation (Saleem et al. 2009). Given that knockout of p53 impairs aerobic capacity and exercise performance and results in greater fatigue (Saleem et al. 2009) and taken with the current data, there is now growing evidence to suggest that p53 may be directly implicated in exercise-induced mitochondrial adaptations. Furthermore, evidence is also available that supports a role for p53 in regulating PGC-1α by the discovery of a p53 binding site on the PGC-1α promoter (Irrcher et al. 2008). However, functional relevance of this promoter is unknown considering we observed in Chapter 6 a divergent signalling of p53 and expression of PGC-1α mRNA thus adding to the complexities of the cell signalling network and the chain of events responsible for mitochondrial biogenesis. In addition, given that p53 is both a nuclear and mitochondrial targeted transcription factor, further work should also aim to examine the subcellular localisation of this protein following exercise.

In addition to exercise stimulus, emerging data also demonstrates that modification of nutrient availability is a potent method to augment the training stimulus. In study 3, we employed an experimental model whereby subjects were glycogen depleted and restricted of carbohydrate before, during and after exercise. Although previous studies have demonstrated that reduced carbohydrate availability enhances AMPK signalling, the downstream effect of this enhanced AMPK activity was unclear as PGC-1α mRNA appears unaffected by carbohydrate availability (Cluberton et al. 2005; Russell et al. 2005). In considering the findings of p53 in study 2, we therefore felt it pertinent to further investigate the role of carbohydrate availability on p53

phosphorylation and in doing so, we demonstrate that p53 signalling is enhanced with reduced carbohydrate availability. This is the first study in skeletal muscle to report such findings, although these data appear consistent with cell culture models where glucose deprivation results in the phosphorylation of p53. In considering the lack of effect of carbohydrate availability on PGC-1α, we therefore present a novel working hypothesis for contraction induced mitochondrial biogenesis (see Figure 7.1) where contraction with reduced carbohydrate availability enhances AMPK signalling and therefore culminates in enhanced phosphorylation of p53. Whilst data in the current thesis cannot add to the literature for the downstream gene targets of p53 (possibly due to the short time course studied), we have drawn upon data from animal and cell culture models to hypothesise the emerging role of p53 in regulating mitochondrial biogenesis.

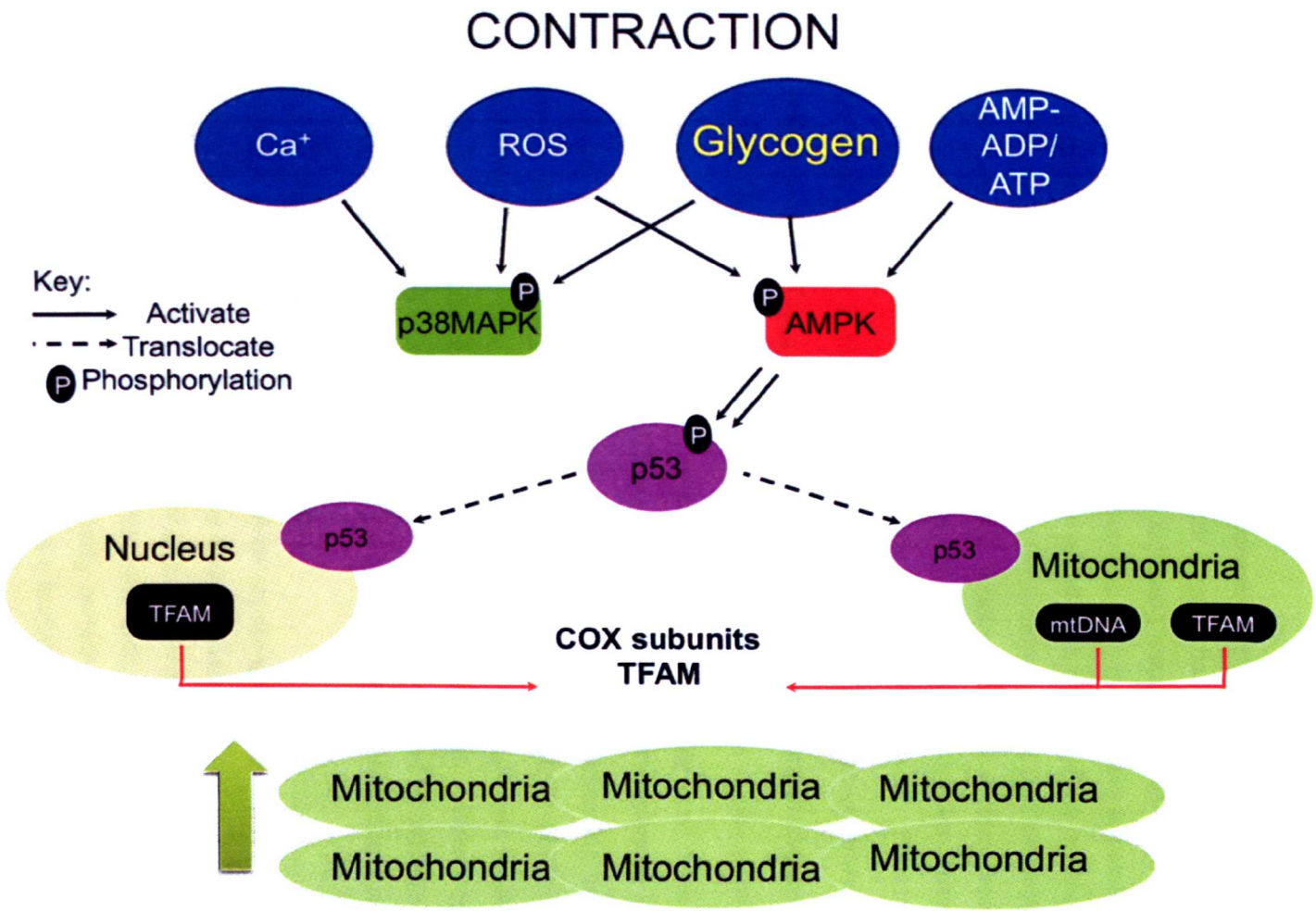

Figure 7. 1 Proposed model of p53 mediated mitochondrial biogenesis in human skeletal muscle. Contraction-induced glycogen signalling in a reduced state enhances AMPK signalling. Phosphorylation of AMPK results in p53 phosphorylation, which subsequently translocates to the nucleus and mitochondria. The expression of TFAM is up regulated in the nucleus before translocating to the cytosol and then the mitochondria. Here p53 modulates the activity of TFAM, which regulates mtDNA, which subsequently increases protein of the COX subunits resulting in an increase in mitochondrial content.

7.3. Limitations

7.3.1. Specificity of work / rest ratios

The metabolic and cell signalling responses to the exercise protocols employed in the current thesis are likely to be specific to the work – rest periods that make up the HIT and CONT protocols. Whilst it is difficult to compare the cell signalling responses to other HIT and CONT protocols (this is the first time a direct acute comparison has been made), from a metabolic perspective it is likely our responses are specific to the 3-min on/off work bouts and the continuous nature of the CONT protocol. For example, Edwards et al. (1973) demonstrated that intermittent (10-30 s work / 30 s rest) and continuous (12 min) protocols matched for total duration and average power output resulted in higher mean values for heart rate, ventilation, respiratory exchange ratio, total oxygen consumption and blood lactate during the intermittent protocol. Similarly, O'Brien et al. (2008) observed that 5 x 2 min interval stages at 100% $\dot{V}O_{2peak}$ (interspersed with 5 x 2 min recovery stages at 50% $\dot{V}O_{2peak}$) resulted in a significantly higher mean average $\dot{V}O_2$ than 10 x 1 min interval stages at 100 % $\dot{V}O_{2peak}$ (interspersed by 10 x 1 min stages at 50% $\dot{V}O_{2peak}$) and a 20 min continuous run (75 % $\dot{V}O_{2peak}$), despite the average velocity in all three conditions being equal. Furthermore, Bangsbo et al. (2000) also observed that if the exercise intensity during the high-intensity intervals is equal to or greater than that corresponding to $\dot{V}O_{2max}$, the mean O_2 consumption is typically higher during interval exercise compared with continuous exercise. Taken collectively, these data demonstrate how the metabolic responses following varying work – rest periods are specific to the exercise protocols employed. Other researchers interested in developing HIT and CONT running protocols that are matched for average physiological responses should therefore carefully design their interventions with the present and previous data in mind.

7.3.2. Muscle fibre specific responses

Although in Chapter 5 we observed similar signalling and metabolic responses following HIT and CONT, it is important to note that these data are taken from whole muscle homogenates. Indeed, considering that our CONT protocol would predominantly recruit type I fibres (Tsintzas et al. 1995) whereas our HIT protocol would likely stimulate both type II (during the interval periods) and type I fibre recruitment (during the active recovery periods), it is possible that the comparable signalling observed in Chapter 5 may have arisen as a function of specific responses in individual fibres types that are reflective of recruitment patterns, but which ultimately surmise in comparable responses in mixed muscle biopsy samples. This is especially true considering that the contractile induced phosphorylation of AMPK (Lee-Young et al. 2009) and p38MAPK (Tannerstedt et al. 2009) as well as training-induced increases in PGC-1α protein content (Russell et al. 2003) are particularly evident in type II fibres when the intensity of contraction is >65% $\dot{V}O_{2max}$. Furthermore, given that p53 may be involved in a temporal activation following AMPK and p38MAPK signalling it is also

important to characterise the fibre specific regulation of p53, as logic would suggest a similar pattern of type II dependent p53 phosphorylation.

7.3.3. Subject specific characteristics

The current thesis recruited subjects on the basis that they were recreationally active in terms of frequency of engaging in physical activity and number of training sessions per week. However, the training status of subjects may differ due to the specific nature (i.e. mode, intensity, frequency) of their training history e.g. the inclusion of subjects who perform three bouts of resistance exercise a week but no endurance exercise and the inclusion of subjects who perform three bouts of endurance exercise a week but no resistance exercise would still be classed as recreational despite differences in training history. In relation to cell signalling, there are apparent divergent signalling responses according to training history (Coffey et al. 2006; Vissing et al. 2011) thus demonstrating the need for tightly controlled criteria for subject inclusion. When considering the subject population for future studies researcher(s) should therefore pay particular attention to the inclusion criteria so as to minimise the effect of divergent signalling responses. It is these subtle differences between subject characteristics that may underpin some of the variability observed within human investigations.

7.3.4. Depletion protocols

As a result of the experimental model employed in Chapter 6, only one group was required to perform the depletion protocol the evening before. This subsequently resulted in higher resting mRNA content for PGC-1α, TFAM, COXIV, and PDK-4 in the LOW group. It is therefore unclear as to whether the higher mRNA contents are a result of the low carbohydrate availability *per se* or due to the residual effects of the glycogen depleting protocol the evening before. An alternative approach may be to deplete both groups in the morning of the day before so as to reduce any transcriptional activity induced by the depletion ride followed by a model of high or low CHO feeding to restore muscle glycogen in the high CHO group and maintain low muscle in the LOW group. This would then ensure that if there were any differences in resting mRNA contents of mitochondrial genes it would likely be due to the condition effect, i.e. carbohydrate availability, as opposed to the additional exercise bout. However, whilst this approach may be methodologically advantageous, there may still be a degree of muscle glycogen re-synthesis in the low CHO group and/or the inclusion of additional macronutrients (e.g. fat and protein feeding) that may also affect gene expression.

7.3.5. Subcellular measurements

Recent evidence from a range of acute studies (Wright et al. 2007; Little, Safdar, Cermak, et al. 2010a; Little, Safdar, et al. 2011b; Safdar et al. 2011) demonstrates PGC-1α translocates to both the nucleus and the mitochondria. Whilst measuring the mRNA content is a useful tool to examine exercise-induced activation of a pathway, increased mRNA content for a given protein may not always translate into increased protein (Baar et al. 2002) or provide evidence of protein functional activity. With this in mind, a more accurate assessment of measuring the acute PGC-1α activity may be to examine the subcellular localisation of PGC-1α. This would allow for a more functional

assessment of the PGC-1α protein but may also be a more accurate tool in determining the enhanced adaptive response for a given bout of acute exercise. Accordingly, p53 is a transcription factor that also translocates to the nucleus (Philp et al. 2011) and mitochondria (Zhao et al. 2005; Endo et al. 2006) and therefore examining the subcellular localisation of p53 would also allow for more accurate assessment of the role that p53 plays in modulating the activity of downstream gene targets such as Tfam.

7.4. Practical Implications

Data an athletic perspective, the present data have several practical applications in terms of training programme design. Athletes currently combine moderate-intensity continuous exercise with high-intensity interval exercise both within the same session and within a block period of training. Although we observed no difference in cell signalling responses between HIT and CONT, others (Helgerud et al. 2007; Tjonna et al. 2008; Wisloff et al. 2009) have observed that HIT is more superior to CONT for augmenting $\dot{V}O_{2max}$. Furthermore, given that substrate utilisation can be acutely modified by the mode of exercise (Chapter 5), i.e. HIT vs. CONT, and/or CHO availability (Chapter 6), this highlights the importance of specificity and selecting the most pertinent training session and nutrition protocol to meet the athletes training goals. Moreover, the integration of training with low CHO availability should be carefully structured into the athletes programme as it has previously been shown that restriction of CHO in sequential bouts of high-intensity exercise reduces the self-selected intensity of exercise (Yeo et al. 2008; Hulston et al. 2010), therefore potentially risking a de-training effect. Taken together, planning of training programme design should take into account previous and current data when prescribing training plans for athletes such that manipulation of intensity, duration, work done or nutritional availability will collectively affect the adaptive response. In addition to training programme design, these data may also have implications for meeting body compositional goals of athletes. Given that excess intake of carbohydrate is associated with weight gain (van Dam & Seidell 2007) and a common goal of athletes is attain low body fat, the adopted model of exercise and CHO restriction in Chapter 6 (whereby utilisation of fat as an energy source is augmented) may provide an additional avenue for sport scientists and coaches to achieve the body compositional goals of their athletes.

From a health perspective, the benefits of regular endurance exercise in decreasing the risk of type II diabetes, obesity and cardiovascular disease is well documented. However, the role endurance exercise plays in reducing tumour growth is unknown. As such, the exercise and nutritional manipulation of p53 activation has obvious therapeutic potential. Indeed, it has recently been shown that a diet low in carbohydrate reduces the progression of cancer development (Ho et al. 2011) and knockout of p53 also induces insulin resistance and facilitate the process of aging (Vousden & Lane 2007; Vousden & Ryan 2009). The enhanced phosphorylation of p53 during HIT completed with reduced CHO availability may provide another avenue for offsetting metabolic related disorders health.

7.5. Recommendations for Future Research

Following are recommendations for potential future work so as to gain a better insight into the molecular signalling events that regulate skeletal muscle adaptations to endurance exercise and to further augment training adaptation.

Recommendation 1: Does performing exercise of different intensities or work – rest periods with varying intensity (sub-maximal/supra-maximal) and durations affect p53 signalling?

PGC-1α is regulated in an intensity-dependent manner through the AMPK signalling cascade (Egan et al. 2010). In considering that AMPK is also found to be intensity-dependent (Chen et al. 2003) and that p53 phosphorylation follows activation of AMPK (Jones et al. 2005; Saleem et al. 2009; Bartlett et al. 2012) then it is tempting to speculate that p53 may also be regulated in an intensity-dependent manner. A basic exercise-intensity relationship therefore needs to be established.

Recommendation 2: How does exercise modulate the sub-cellular localisation of p53 and does CHO availability also modulate sub-cellular location of p53?

Similar to PGC-1α, p53 translocates to the nucleus and mitochondria following episodes of stress (Yoshida et al. 2003; Park et al. 2009) though no data are currently available in humans. In this regard, the interaction of p53 with the Tfam gene in the mitochondria (Park et al. 2009) would be of particular interest given the well documented role of Tfam in regulating mtDNA expression.

Recommendation 3: How does endurance training modulate whole p53 protein content and does CHO availability affect whole muscle p53 protein content?

The studies contained in this thesis are based on acute interventions on the premise that acute responses may be predictable of actual training responses. Clearly, longitudinal training studies are now needed to verify the validity of this assumption. In the context of p53, no data are currently available on how endurance training modifies p53 content or on the role of nutrient availability or training stimulus in determining the extent of this adaptation.

Recommendation 4: What is the time-course of p53 signalling following acute endurance exercise in human skeletal muscle?

Several studies have elucidated the time-course responses of key proteins involved in the adaptation to exercise training. For example, Morton et al. (2006) reported the time-course responses of HSPs following endurance exercise and Leick et al. (2009) characterised the time at which expression of PGC-1α peaked following exercise. In the context of p53, 3 h (as measured in

this thesis) may still miss the time in which its transcriptional activity peaks; therefore a basic time-course of p53 signalling in response to endurance exercise is required.

CHAPTER 8

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APPENDIX

APPENDIX 1 – Bartlett, JD, Close, GL, Drust, B, Morton, JP. (2010). The development of laboratory-based intermittent and continuous running protocols that are matched for various physiological parameters. *British Journal of Sports Medicine*, 44, i9.

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The development of laboratory-based intermittent and continuous running protocols that are matched for various physiological parameters

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Key words: maximal oxygen uptake, intermittent exercise, endurance training

Introduction Data from cycling based protocols demonstrate that high-intensity interval training (HIT) is a more efficient mode of exercise training compared with traditional moderate-intensity continuous (CONT) approaches (Gibala et al, J Physiol 2006,575,901-911; Burgomaster et al, J Physiol 2008,586,151-160). The use of cycling based protocols is methodologically advantageous as total work done can be quantified by measuring subject's power output while pedalling on a cycle ergometer. However, examining the efficacy of HIT versus CONT running is more challenging as measurements of heart rate *per se* may not always reflect energy production. It may therefore be more accurate to prescribe, quantify and match exercise intensity during HIT and CONT running by matching protocols for average velocity corresponding to a particular % $\dot{V}O_{2max}$ (Morton, Med Sci Sports Exerc 2007,39,1885).

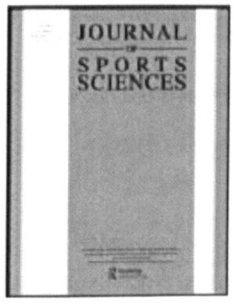
Aims The aim of the current study was to develop and match laboratory-based HIT and CONT running protocols for energy expenditure, average intensity, duration and distance ran.

Methods Eight recreationally active men performed two running protocols consisting of HIT (6 x 3 min 90 % $\dot{V}O_{2max}$ interspersed with 6 x 3 min active recovery at 50 % $\dot{V}O_{2max}$ with 7 min warm-up and cool down at 70 % $\dot{V}O_{2max}$) and CONT (50 min continuous running exercise at 70 % $\dot{V}O_{2max}$). Both protocols were matched for average intensity (70% $\dot{V}O_{2max}$), duration (50-min) and distance ran (9843 ± 494 m).

Results There was no difference ($p>0.05$) in average heart rate (88 ± 3 *versus* 87 ± 3 % HR_{max}), average $\dot{V}O_2$ (71 ± 6 *versus* 73 ± 4 % $\dot{V}O_{2max}$), total $\dot{V}O_2$ (162 ± 16 *versus* 166 ± 27 L) or energy expenditure (811± 83 *versus* 832 ± 136 kcal) between HIT and CONT protocols, respectively. Although ratings of perceived exertion were higher ($p<0.05$) during HIT compared to CONT (14 ± 1 *versus* 13 ± 1), HIT was characterised by higher ratings of perceived enjoyment ($p<0.05$) (88 ± 6 *versus* 61 ± 12).

Conclusion To the authors' knowledge, this is the first study to have matched HIT and CONT running for all the aforementioned variables. These data provide a controlled experimental model to subsequently study the efficacy of HIT versus CONT running. Furthermore, HIT is perceived as more enjoyable than CONT which may have implications for improving exercise adherence in individuals undertaking exercise training to improve human health and/or performance.

APPENDIX 2 – Bartlett, JD, Close, GL, MacLaren, DPM, Gregson, W, Drust, B, Morton JP. (2011). High-intensity interval running is perceived to be more enjoyable than moderate-intensity continuous exercise: Implications for exercise adherence. *Journal of Sport Sciences*, 29, 547-553.



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High-intensity interval running is perceived to be more enjoyable than moderate-intensity continuous exercise: Implications for exercise adherence

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High-intensity interval running is perceived to be more enjoyable than moderate-intensity continuous exercise: Implications for exercise adherence

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Abstract

The aim of this study was to objectively quantify ratings of perceived enjoyment using the Physical Activity Enjoyment Scale following high-intensity interval running versus moderate-intensity continuous running. Eight recreationally active men performed two running protocols consisting of high-intensity interval running (6 × 3 min at 90% $\dot{V}O_{2max}$ interspersed with 6 × 3 min active recovery at 50% $\dot{V}O_{2max}$ with a 7-min warm-up and cool down at 70% $\dot{V}O_{2max}$) or 50 min moderate-intensity continuous running at 70% $\dot{V}O_{2max}$. Ratings of perceived enjoyment after exercise were higher ($P < 0.05$) following interval running compared with continuous running (88 ± 6 vs. 61 ± 12) despite higher ($P < 0.05$) ratings of perceived exertion (14 ± 1 vs. 13 ± 1). There was no difference ($P < 0.05$) in average heart rate (88 ± 3 vs. 87 ± 3% maximum heart rate), average $\dot{V}O_2$ (71 ± 6 vs. 73 ± 4% $\dot{V}O_{2max}$), total $\dot{V}O_2$ (162 ± 16 vs. 166 ± 27 L) or energy expenditure (811 ± 83 vs. 832 ± 136 kcal) between protocols. The greater enjoyment associated with high-intensity interval running may be relevant for improving exercise adherence, since running is a low-cost exercise intervention requiring no exercise equipment and similar relative exercise intensities have previously induced health benefits in patient populations.

Keywords: *Maximal oxygen uptake, intermittent exercise, endurance training*

Introduction

The prevalence of the metabolic syndrome (a cluster of cardiovascular risk factors, including high blood pressure, dyslipidaemia, insulin resistance, and obesity) has grown to epidemic proportions and is reported to have affected 300 million people worldwide (Tjønna et al., 2008). The proliferation of this epidemic is the result of excess calorie intake and the adoption of a sedentary lifestyle (Hawley & Gibala, 2009). It is increasingly evident that an increase in physical activity is an effective low-cost treatment for the prevention of such symptoms associated with metabolic disorders (Goodyear & Kahn, 1998; Hawley 2004; Hawley & Lessard, 2008; Pedersen & Saltin, 2006; Seals, Hagberg, Hurley, Ehsani, Holloszy, 1984). However, despite the obvious therapeutic potential of exercise interventions, many people are reluctant to engage in exercise, often citing "lack of time" and "lack of enjoyment" as contributing factors (Leslie et al., 1999; Stutts, 2002; Trost, Owen, Bauman, Sallis, & Brown, 2002).

To this end, several investigators (Babraj et al., 2009; Burgomaster, Hughes, Heigenhauser, Bradwell, & Gibala, 2005, Burgomaster et al., 2007, 2008; Gibala et al., 2006; Whyte, Gill, & Cathcart, 2010) have recently adopted short-term high-intensity interval training interventions (4–6 × 30-s maximal cycling Wingate test protocols) as an alternative to more traditional continuous training (40–60 min cycling at 65% $\dot{V}O_{2max}$) approaches, which are more time-consuming. These authors collectively reported that high-intensity interval training is a time-efficient strategy to induce rapid adaptations in skeletal muscle, as demonstrated by changes in oxidative enzyme protein content/activity as well as improved insulin sensitivity. Although these results have proved insightful, their practical application in improving exercise adherence is limited due to reports of nausea and light-headedness experienced during the exercise protocol (Richards et al., 2010) as well as the requirement for a mechanically braked cycle ergometer, usually found only in the laboratory.

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Moreover, it is also difficult to determine if individuals would prefer this type of training intervention due to its short duration *per se* or because they actually perceive the varied activity profile as more *enjoyable* than prolonged approaches, a factor that could potentially improve exercise adherence in the long term.

With this in mind, one potential approach to overcome the barriers associated with lack of exercise may be to develop high-intensity interval *running* interventions that are undertaken at sub-maximal or near maximal intensity. This emphasis upon running is important given that running requires no specialist exercise equipment and because preference of exercise mode is also critical to exercise adherence (Daley & Maynard, 2003; Glass & Chvala, 2001). In this regard, Helgerud et al. (2007) demonstrated that high-intensity interval running (4 × 4 min at 95% maximum heart rate) is a more efficient training method than moderate-intensity continuous training (45 min at 70% maximum heart rate), as demonstrated by superior improvements in maximum oxygen uptake ($\dot{V}O_{2max}$), cardiac output, and stroke volume. Such findings are particularly important given that low whole-body $\dot{V}O_{2max}$ is closely associated with those at an increased risk of metabolic disorders (Mootha et al., 2003). Furthermore, high-intensity interval exercise has also been shown to reduce risk factors associated with the metabolic syndrome compared with moderate-intensity continuous exercise in a variety of patient populations (Haram et al., 2009; Moholdt et al., 2009; Stensvold et al., 2010; Tjønna et al., 2008, 2009; Wisløff et al., 2007). Interestingly, the exercise intensity associated with high-intensity interval exercise in these populations has been well tolerated despite the low initial fitness of the patients studied. Indeed, Tjønna et al. (2008) reported informal comments from participants performing high-intensity interval running and found it more motivating to have a varied procedure to follow during each training session, whereas those in the moderate-intensity continuous group found it "quite boring" to exercise continuously for the whole duration. Such comments appear to support the notion that high-intensity interval training is perceived to be more enjoyable than moderate-intensity continuous exercise, although this remains to be tested formally.

The aim of this study, therefore, was to test the hypothesis that high-intensity interval running is perceived to be more enjoyable than moderate-intensity continuous running. To objectively quantify ratings of perceived enjoyment, we employed a previously validated questionnaire (Kenzierski & DeCarlo, 1991), the Physical Activity Enjoyment Scale, which has been shown to be a valid measure to quantify perceived enjoyment and is deemed suitable for use as a mediator variable in designing interventions to increase physical activity participation (Motl et al., 2001). Furthermore, our particular model of high-intensity interval running has been shown previously in our laboratory to improve $\dot{V}O_{2max}$ and oxidative adaptations (protein content of COX-IV, PGC-1α, and SDH activity) of the vastus lateralis and gastrocnemius muscles (Morton et al., 2009).

Methods

Eight healthy recreationally active men volunteered to participate in the study (mean ± *s*: age, 25 ± 5 years; weight, 73 ± 5 kg; height, 1.74 ± 0.02 m; body mass index, 24.2 ± 2.2; $\dot{V}O_{2max}$, 57 ± 4 ml · kg^{-1} · min^{-1}). All participants undertook exercise two to three times a week and gave written informed consent to participate after details of the study had been explained in full. Participants refrained from additional exercise outside of the study requirements as well as alcohol and caffeine intake for at least 24 h prior to any of the test sessions. The study received approval from the Ethics Committee of Liverpool John Moores University.

All participants were initially assessed for $\dot{V}O_{2max}$ using an incremental exercise test performed on a motorized treadmill (HP Cosmos, Germany). Oxygen uptake was measured continuously during exercise using an on-line gas analysis system (Metamax, Cortex). The test began with a 2-min stage at a treadmill speed of 10 km · h^{-1} followed by 2-min stages at 12 km · h^{-1}, 14 km · h^{-1}, and 16 km · h^{-1}. On completion of the 16 km · h^{-1} stage, the treadmill was inclined by 2% every 2 min until volitional exhaustion despite strong verbal encouragement. Maximum oxygen uptake was taken as the highest $\dot{V}O_2$ value obtained in any 10-s period and was taken as having been achieved when meeting the following end-point criteria in accordance with the guidelines of the British Association of Sport and Exercise Sciences: (1) heart rate within 10 beats · min^{-1} of age-predicted maximum (220 − age); (2) respiratory exchange ratio > 1.15; and (3) plateau of $\dot{V}O_2$ despite increased workload (Bird & Davison, 1997). At least 48 h after assessment of $\dot{V}O_{2max}$, participants undertook a running test that consisted of similar time points at each stage (3 min) as the high-intensity interval running protocol so as to provide a clearer profile of $\dot{V}O_2$, which was used to accurately match the participants' running velocities with their respective exercise intensities. The exercise protocol commenced at a treadmill speed of 8 km · h^{-1} and was increased by 1 km · h^{-1} every 3 min until at least 95% $\dot{V}O_{2max}$ had been reached (taken from previous $\dot{V}O_{2max}$ test). Expired gases were collected using the same on-line gas analysis system (Metamax, Cortex).

At least 5 days after the assessment of $\dot{V}O_{2max}$ and running economy, participants completed two running exercise protocols consisting of high-intensity interval running and moderate-intensity continuous running in a fully randomized and crossover design (Altman, 1991). The exercise protocols were performed 7 days apart after an overnight fast and at the same time of day to eliminate any circadian effects on the physiological responses to the exercise protocols (Reilly & Brooks, 1986). Both protocols were performed on the same motorized treadmill (HP Cosmos, Germany). The high-intensity interval running protocol commenced with a 7-min warm-up at a running velocity corresponding to 70% $\dot{V}O_{2max}$ followed by six 3-min bouts at a running velocity corresponding to 90% $\dot{V}O_{2max}$. The high-intensity intervals were separated by 3-min active recovery periods at a running velocity corresponding to 50% $\dot{V}O_{2max}$. Following the interval and recovery periods, participants then performed a 7-min cool down at a running velocity corresponding to 70% $\dot{V}O_{2max}$. The exercise protocol therefore consisted of 18 min of high-intensity exercise and 18 min of active recovery time, giving a total interval exercise time of 36 min. When including the warm-up and cool-down, the total duration of the exercise protocol was 50 min. The average intensity during the high-intensity interval running protocol, when quantified according to average running velocity, equated to 70% $\dot{V}O_{2max}$. The moderate-intensity continuous running protocol consisted of 50 min continuous running at a running velocity corresponding to 70% $\dot{V}O_{2max}$. In this way, the two exercise protocols were matched for exercise duration (50 min), average intensity (70% $\dot{V}O_{2max}$), and distance run (9843 ± 494 m). Given that there is no applied external load with horizontal motorized treadmill running at 0% incline (as is the case in conventional ergometry), current biomechanical methods are not well enough established to accurately measure work done. Nevertheless, in this type of activity, work done is best quantified by the total energy cost of each protocol, as assessed by total oxygen consumption (which was also matched in both exercise protocols).

Oxygen uptake (Metamax, Cortex) and heart rate (Polar S610i, Kempele, Finland) were measured continuously during each exercise protocol and participants also reported ratings of perceived exertion (RPE) at 1-min intervals during exercise (Borg, 1970). Energy expenditure was estimated during exercise via indirect calorimetry assuming a non-protein respiratory exchange ratio (Zuntz, 1901). Immediately upon completion of each protocol, participants also reported ratings of perceived enjoyment according to the Physical Activity Enjoyment Scale validated by Kendzierski and DeCarlo (1991). Each item was rated on a 7-point bipolar scale with 4 representing a neutral point in terms of how much the respondent enjoyed the exercise (Table I).

Statistical analysis was conducted using the Statistical Package for Social Sciences software program (version 15). All data were initially analysed for normality according to the Shapiro-Wilks test. Once verified, a comparison of the average physiological responses between exercise protocols was assessed using Student's *t*-tests for paired samples, and a two-way general linear

Table I. A reproduction of the Physical Activity Enjoyment Scale questionnaire demonstrating the 7-point bipolar scale.

* I enjoy it	1	2	3	4	5	6	7	I hate it
I feel bored	1	2	3	4	5	6	7	I feel interested
I dislike it	1	2	3	4	5	6	7	I like it
* I find it pleasurable	1	2	3	4	5	6	7	I don't find it pleasurable
* I am very absorbed in this activity	1	2	3	4	5	6	7	I am not at all absorbed in this activity
It's no fun at all	1	2	3	4	5	6	7	It's a lot of fun
* I find it energizing	1	2	3	4	5	6	7	I find it tiring
It makes me depressed	1	2	3	4	5	6	7	It makes me happy
* It's very pleasant	1	2	3	4	5	6	7	It's very unpleasant
* I feel good physically while doing it	1	2	3	4	5	6	7	I feel bad physically while doing it
* It's very invigorating	1	2	3	4	5	6	7	It's not at all invigorating
I am very frustrated by it	1	2	3	4	5	6	7	I am not at all frustrated by it
* It's very gratifying	1	2	3	4	5	6	7	It's not at all gratifying
* It's very exhilarating	1	2	3	4	5	6	7	It's not at all exhilarating
It's not at all stimulating	1	2	3	4	5	6	7	It's very stimulating
* It gives me a strong sense of accomplishment	1	2	3	4	5	6	7	It doesn't give me a strong sense of accomplishment
* It's very refreshing	1	2	3	4	5	6	7	It's not at all refreshing
I felt as though I would rather be doing something else	1	2	3	4	5	6	7	I felt as though there is nothing else I would rather be doing

* Denotes reversal when scoring.

model was used to determine whether there was an interaction or any significant differences over time and between conditions for heart rate and RPE. A Pearson's rank correlation was run to assess whether a relationship existed between perceived enjoyment and $\dot{V}O_{2max}$. All data in the text, figures, and tables are presented as means and standard deviations (s) with P-values ≤ 0.05 indicating statistical significance.

Results

Psycho-perceptual responses to high-intensity interval and moderate-intensity continuous protocols

Ratings of perceived enjoyment were significantly higher ($P = 0.004$) in the high-intensity interval running condition compared with the moderate-intensity continuous running condition (Figure 1A). In spite of the higher enjoyment during the high-intensity interval running protocol, average ratings of perceived exertion were also significantly higher ($P = 0.015$) during high-intensity interval running compared with the moderate-intensity continuous running protocol (Figure 1B). In addition, when comparing RPE during exercise between conditions there was a significant interaction ($P = 0.000$), main effect of time ($P = 0.000$), and main effect of condition ($P = 0.027$) for the high-intensity interval running and moderate-intensity continuous running protocols (Figure 2A).

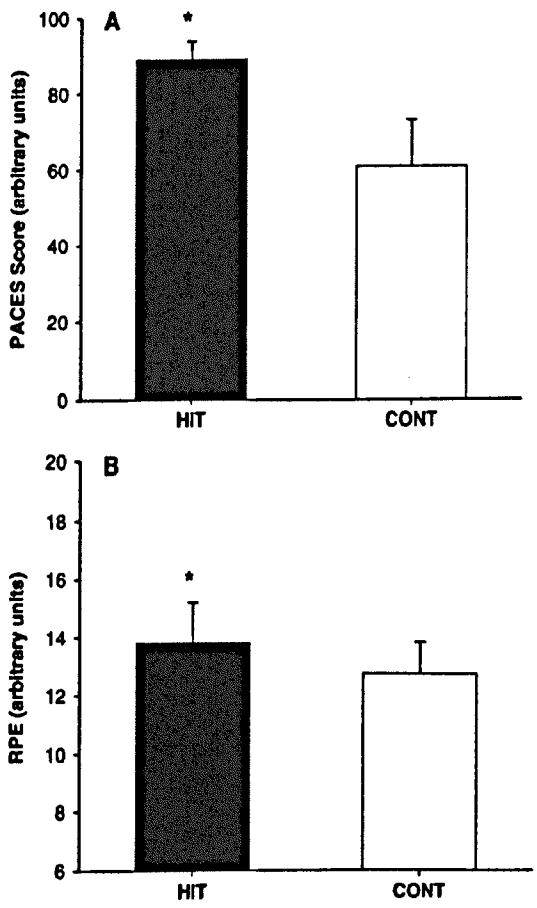

Figure 1. (A) Ratings of perceived enjoyment (PACES score) after completing the high-intensity interval (HIT) protocol and moderate-intensity continuous (CONT) protocol, as quantified using the Physical Activity Enjoyment Scale (PACES). (B) Average ratings of perceived exertion (RPE), as quantified using the Borg scale. *Significantly different from CONT protocol ($P < 0.05$).

Physiological responses to high-intensity interval and moderate-intensity continuous protocols

There was no significant difference in average heart rate ($P = 0.547$), average percent maximum heart rate ($P = 0.528$), total $\dot{V}O_2$ ($P = 0.358$), average $\dot{V}O_2$ ($P = 0.389$), average $\%\dot{V}O_{2max}$ ($P = 0.305$) or energy expenditure ($P = 0.383$) between the high-intensity interval running and moderate-intensity continuous running protocols (Table II). However,

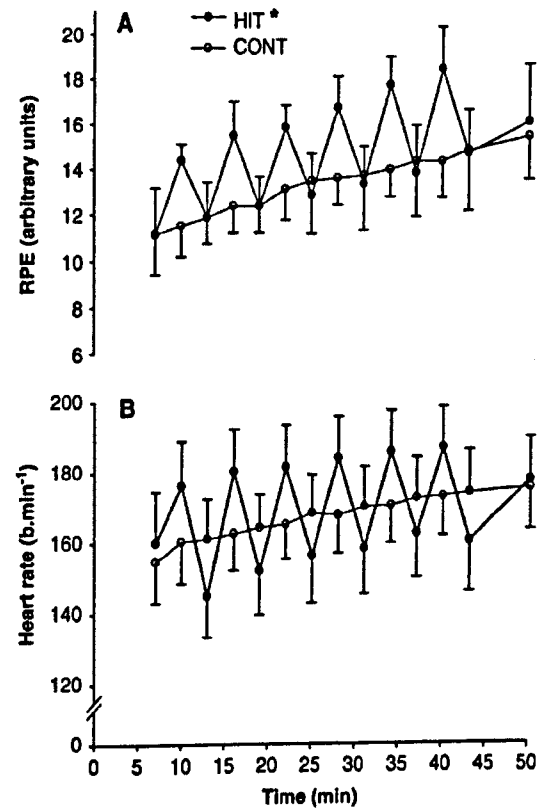

Figure 2. (A) Ratings of perceived exertion (RPE) and (B) heart rates after each stage during the high-intensity interval (HIT) protocol and moderate-intensity continuous (CONT) protocol. *Significant main effect of time, condition and interaction ($P < 0.05$).

Table II. A comparison of the average physiological responses induced by the two running protocols (mean ± *s*).

	Protocol	
	High-intensity interval protocol	Moderate-intensity continuous protocol
Heart rate (beats · min^{-1})	167 ± 12	165 ± 9
Heart rate (%HR_{max})	88 ± 3	87 ± 3
Total VO_2 (L)	162 ± 16	166 ± 27
Average VO_2 (L · min^{-1})	3.23 ± 0.32	3.32 ± 0.53
Average VO_2 (%VO_{2max})	71 ± 6	73 ± 4
Energy expenditure (kcal)	811 ± 83	832 ± 136

Note: HR_{max} = maximum heart rate.

there was a significant interaction and main effect for time on both heart rate and $\dot{V}O_2$ when comparing these variables between protocols throughout the 50 min of exercise ($P = 0.000$ for all effects; heart rate data shown in Figure 2B), thus highlighting the obvious difference in activity profile between exercise protocols.

Discussion

The aim of the present study was to quantify subjective feelings of enjoyment generated by undertaking an acute bout of high-intensity interval running and moderate-intensity continuous running. Our data demonstrate that recreationally active males perceive high-intensity interval running to be more enjoyable than moderate-intensity continuous running, despite higher ratings of RPE during interval exercise. In addition, this experimental approach represents a methodological development, as it is the first study to match high-intensity interval running and moderate-intensity continuous running for duration, distance run, average intensity, and total oxygen consumption and energy expenditure (i.e. work done).

We quantified ratings of perceived enjoyment according to the Physical Activity Enjoyment Scale and observed higher scores following high-intensity interval running compared with moderate-intensity continuous running. We acknowledge that our participants were recreationally active ($\dot{V}O_{2max}$: 57 ml · kg^{-1} · min^{-1}) and that the perception of effort/enjoyment experienced by these individuals may be different from that of individuals who do not regularly engage in physical activity. Nevertheless, our results are in agreement with subjective reports from previous authors examining cardiovascular diseased ($\dot{V}O_{2max}$: 13 ml · kg^{-1} · min^{-1}) and metabolic syndrome patients ($\dot{V}O_{2max}$: 32–36 ml · kg^{-1} · min^{-1}) who reported informal comments of increased enjoyment during high-intensity interval running compared with moderate-intensity continuous running (Tjønna et al., 2008; Wisløff et al., 2007). These authors also observed that high-intensity interval exercise induced greater improvements in endothelial function and aerobic capacity, and improved insulin signalling in skeletal muscle compared with moderate-intensity continuous exercise training. While we cannot comment on the health-promoting effects of our specific exercise protocol, both of the aforementioned investigations used similar relative training intensities (4 × 4-min intervals at 95% maximum heart rate) to those studied here, thus highlighting the potential for our exercise model to induce health benefits. Furthermore, we have previously observed that our model of high-intensity interval running increases protein content of PGC-1α of the vastus lateralis and gastrocnemius muscles (Morton et al., 2009) within the physiological range associated with improved insulin sensitivity in humans (Lira, Benton, Yan, & Bonen, 2010).

Comments from the patients studied by Wisløff et al. (2007) and Tjønna et al. (2008) suggested that greater post-exercise feelings of enjoyment are due to the varied nature of the activity profile inherent to high-intensity training compared with the "boring" steady-state continuous approach, a view also reported by the current participants. It is presently difficult to therefore distinguish if enhanced feelings of enjoyment associated with high-intensity interval running are specifically due to the varied activity, periods of increased exercise intensity or a combination of both factors. In this regard, it is possible that enjoyment may also be enhanced with varied activity profiles that are undertaken at lower intensities, although the clinical relevance of such protocols are questionable given that exercise intensity appears to be a key determinant of physiological adaptations (Egan et al., 2010).

In spite of higher ratings of enjoyment associated with high-intensity interval running, ratings of perceived exertion were significantly higher following high-intensity interval running than moderate-intensity continuous running. A positive relationship between higher ratings of enjoyment and higher ratings of perceived exertion has been demonstrated previously in recreationally active participants (Raedeke, 2007), suggesting that individuals who engage in regular exercise and thus are aerobically fitter, feel a greater sense of challenge, stimulation or accomplishment after training sessions that are perceived to be more demanding. However, Pearson's correlation test demonstrated no significant relationship between $\dot{V}O_{2max}$ and Physical Activity Enjoyment Scale scores during high-intensity interval running ($P = 0.823$) or moderate-intensity continuous running ($P = 0.977$), suggesting that initial fitness does

not seem to be a major determinant of exercise enjoyment. In keeping with this thesis, it might be surmised that the varied activity profiles inherent to the Wingate cycling training protocols may also result in enhanced enjoyment (Babraj et al., 2009; Burgomaster et al., 2005, 2007, 2008; Gibala et al., 2006; Whyte et al., 2010), although unfortunately these studies did not address or comment on this issue. However, the 30-s maximal efforts inherent to these protocols are associated with intense feelings of nausea and light-headedness (Richards et al., 2010). In contrast, in relation to the participants studied in our laboratory both here and previously (Morton et al., 2009), as well as the patient populations studied by other groups (Tjønna et al., 2008, 2009; Wisløff et al., 2007), there are no reports of ill-feeling during or after the high-intensity interval running protocols undertaken at near maximal or sub-maximal intensities.

To isolate the effects of "exercise activity profile" on enjoyment as opposed to duration *per se*, we evaluated the perceived enjoyment responses after two exercise protocols that were matched for duration, distance run, average intensity, oxygen consumption, and energy expenditure. To the authors' knowledge, this experimental approach is unique, as it is the first study to match high-intensity interval and moderate-intensity continuous running protocols for all the aforementioned indices. Indeed, when prescribing and quantifying exercise intensity during high-intensity interval running and moderate-intensity continuous running protocols on the basis of percent maximum heart rate, Helgerud et al. (2007) and Tjønna et al. (2008) matched their interventions for energy expenditure but not duration, distance run or average intensity. While this approach of prescribing, quantifying, and monitoring exercise intensity on the basis of heart rate measurements is practically advantageous it is not without limitations, as heart rate *per se* does not always correspond to energy production (Gilman, 1996). Furthermore, to maintain exercise intensity in the target heart rate zones, these authors had to reduce the running velocity during the training session so as to offset the effects of cardiovascular drift. However, in this way, the absolute workload is reduced, therefore lowering the overall training stress. In an effort to provide a more controlled exercise stress, we chose to prescribe exercise intensity on the basis of running velocity corresponding to a particular $\%\dot{V}O_{2max}$, whereby the absolute workload and degree of metabolic signalling within the muscle was not likely to be compromised during exercise within or between individuals (Morton, 2007). It should be noted, however, that the matched physiological responses observed here are likely to be highly specific to our chosen exercise intensities and work-to-rest ratios (O'Brien, Wibskov, Knez, Paton, & Harvey, 2008).

Conclusions

In conclusion, the present results demonstrate that ratings of perceived enjoyment are greater after undertaking an acute bout of high-intensity interval running compared with moderate-intensity continuous running. Our results are of practical relevance given that running is a low-cost exercise intervention requiring no specialist exercise equipment and that similar relative exercise intensities have been shown to improve cardiovascular and metabolic health in clinical populations. High-intensity interval running may therefore prove an effective strategy to increase long-term exercise participation and improve human health and future studies (longitudinal in design) are required to test this hypothesis.

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APPENDIX 3 – Bartlett, JD, Jeong, TS, Gregson, W, Drust, B, Close GL, Cochran, A, Gibala MJ, Morton, JP. Signalling responses of human skeletal muscle to high-intensity interval running versus moderate-intensity continuous running. Presented as an Oral at ECSS 2011, Liverpool, UK.

SIGNALLING RESPONSES OF HUMAN SKELETAL MUSCLE TO HIGH-INTENSITY INTERVAL RUNNING VERSUS MODERATE-INTENSITY CONTINUOUS RUNNING

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Introduction
We recently demonstrated that high-intensity interval (HIT) running is perceived as more enjoyable than moderate-intensity continuous (CONT) running (Bartlett et al. 2011). Such data have implications for improving exercise adherence and long-term health, especially considering that HIT cycling is a more time-efficient training stimulus than CONT cycling for improving skeletal muscle oxidative capacity (Gibala et al. 2006). Despite running being the most readily available form of exercise to the general population, comparable data for this exercise mode are lacking. The aim of this study was to therefore test the hypothesis that acute HIT running is a more potent exercise stimulus than CONT running for activating key signal transduction pathways associated with mitochondrial biogenesis.

Method
Muscle biopsies (vastus lateralis) were obtained from six active men who performed two running protocols consisting of HIT or CONT in a randomized manner separated by 7-days. HIT consisted of 6 x 3 min at 90 % VO_{2max} interspersed with 3 min recovery periods at 50 % VO_{2max}, plus a 7 min warm-up and cool down at 70 % VO_{2max}. CONT consisted of 50 min of continuous running exercise at 70 % VO_{2max}. The protocols were matched for energy expenditure, average intensity, average heart rate, duration and distance ran (Bartlett et al. 2011).

Results
Phosphorylation (P-) of $p38MAPK^{Thr180/Tyr182}$ increased after both trials ($P<0.05$), but the increase in HIT (2.2 ± 0.1-fold) was greater than CONT (1.5 ± 0.2-fold) ($p<0.05$). Similarly, $P\text{-}AMPK^{Thr172}$ increased immediately after exercise ($P<0.05$), with a tendency for the change to be greater in HIT vs CONT (1.8 ± 0.3 vs 1.4 ± 0.1-fold; $P=0.07$). Exercise did not change the total protein content of PGC-1α or GAPDH in either condition ($p>0.05$).

Discussion
These preliminary data suggest that HIT running may be a more potent stimulus than CONT running for activating key signaling kinases involved in stimulating mitochondrial biogenesis. Despite both protocols being matched for work done, our data suggest that it is the variations in exercise intensity as opposed to energy expenditure *per se* which ultimately determines the metabolic responses of skeletal muscle. The precise mechanisms underpinning the enhanced p38MAPK phosphorylation remain unknown and biopsy samples are currently being analysed for markers of metabolic and oxidative stress and downstream markers linked to mitochondrial biogenesis.

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APPENDIX 4 – Bartlett, JD, Joo, CH, Jeong, TS, Louhelainen, J, Cochran, AJ, Gibala, MJ, Gregson, W, Close, GL, Drust, B, Morton, JP. (2012). Matched work high-intensity interval and continuous running induce similar increases in PGC-1α mRNA, AMPK, p38 and p53 phosphorylation in human skeletal muscle. *Journal of Applied Physiology*, 112, 1135-1143.

Matched work high-intensity interval and continuous running induce similar increases in PGC-1 α mRNA, AMPK, p38, and p53 phosphorylation in human skeletal muscle

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Matched work high-intensity interval and continuous running induce similar increases in PGC-1α mRNA, AMPK, p38, and p53 phosphorylation in human skeletal muscle

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Bartlett JD, Hwa Joo C, Jeong TS, Louhelainen J, Cochran AJ, Gibala MJ, Gregson W, Close GL, Drust B, Morton JP. Matched work high-intensity interval and continuous running induce similar increases in PGC-1α mRNA, AMPK, p38, and p53 phosphorylation in human skeletal muscle. *J Appl Physiol* 112: 1135–1143, 2012. First published January 29, 2012; doi:10.1152/japplphysiol.01040.2011.—The aim of the present study was to test the hypothesis that acute high-intensity interval (HIT) running induces greater activation of signaling pathways associated with mitochondrial biogenesis compared with moderate-intensity continuous (CONT) running matched for work done. In a repeated-measures design, 10 active men performed two running protocols consisting of HIT [6 × 3-min at 90% maximal oxygen consumption ($\dot{V}O_{2max}$) interspersed with 3-min recovery periods at 50% $\dot{V}O_{2max}$ with a 7-min warm-up and cool-down period at 70% $\dot{V}O_{2max}$] or CONT (50-min continuous running at 70% $\dot{V}O_{2max}$). Both protocols were matched, therefore, for average intensity, duration, and distance run. Muscle biopsies (vastus lateralis) were obtained preexercise, postexercise, and 3 h postexercise. Muscle glycogen decreased ($P < 0.05$) similarly in HIT and CONT (116 ± 11 vs. 111 ± 17 mmol/kg dry wt, respectively). Phosphorylation (P-) of p38MAPK$^{Thr180/Tyr182}$ (1.9 ± 0.1- vs. 1.5 ± 0.2-fold) and AMPK^{Thr172} (1.5 ± 0.3- vs. 1.5 ± 0.1-fold) increased immediately postexercise ($P < 0.05$) in HIT and CONT, respectively, and returned to basal levels at 3 h postexercise. P-p53^{Ser15} (HIT, 2.7 ± 0.8-fold; CONT, 2.1 ± 0.8-fold), PGC-1α mRNA (HIT, 4.2 ± 1.7-fold, CONT, 4.5 ± 0.9-fold) and HSP72 mRNA (HIT, 4.4 ± 2-fold; CONT, 3.5 ± 1-fold) all increased 3 h postexercise ($P < 0.05$) although neither parameter increased ($P > 0.05$) immediately postexercise. There was no difference between trials for any of the above signaling or gene expression responses ($P > 0.05$). We provide novel data by demonstrating that acute HIT and CONT running (when matched for average intensity, duration, and work done) induces similar activation of molecular signaling pathways associated with regulation of mitochondrial biogenesis. Furthermore, this is the first report of contraction-induced p53 phosphorylation in human skeletal muscle, thus highlighting an additional pathway by which exercise may initiate mitochondrial biogenesis.

high-intensity training; endurance exercise; vastus lateralis; glycogen

REGULAR ENDURANCE EXERCISE induces an increase in skeletal muscle mitochondrial density (29). At a molecular level, mitochondrial adaptations to endurance training are thought to be due to the cumulative effects of transient increases in mRNA transcripts encoding mitochondrial proteins that follow each acute training session (46). Upon the onset of contraction, homeostatic perturbations within skeletal muscle [e.g., increased AMP/ATP ratio, Ca^{2+}, reactive oxygen species (ROS), lactate; reduced glycogen availability, etc.] result in the activation of a number of regulatory protein kinases that, in turn, phosphorylate downstream targets such as transcription factors or transcriptional coactivators (41). Two putative kinases that have emerged as key players inherent to the intracellular signaling cascades sensitive to muscle contraction are the adenosine monophosphate-activated protein kinase (AMPK) and the p38 mitogen-activated protein kinase (p38MAPK) (22, 38, 39). These kinases converge upon the regulation of peroxisome proliferator-activated γ receptor coactivator (PGC-1α), a transcriptional coactivator repeatedly cited as the "master regulator of mitochondrial biogenesis" (47). The importance of PGC-1α in regulating mitochondrial content and function is evident from rodent studies demonstrating that overexpression increases oxidative enzyme activity (37), improves insulin sensitivity (5), protects against sarcopenia (61), and also improves exercise capacity (11).

In addition to PGC-1α, the tumor suppressor protein, p53, has also emerged as a potential regulator of mitochondrial function, and several nuclear genes encoding mitochondrial proteins have p53 response elements in their promoter regions (42). Moreover, skeletal muscle from p53 knockout mice exhibit reduced PGC-1α (54) and mitochondrial transcription factor A protein (45) as well as total mitochondrial content (54) compared with wild-type animals. Acute contractile activity also increases phosphorylation of p53 in rodent muscle thus highlighting an additional pathway through which muscle contraction may induce mitochondrial biogenesis (54). Taken together, these studies suggest that p53 is an important modulator of mitochondrial content and function; however, no study to date has examined the exercise-induced response of p53 in human skeletal muscle.

Although the molecular mechanisms underpinning contraction-induced mitochondrial biogenesis are now beginning to be understood, the optimal exercise stimulus to induce mitochondrial adaptations remains to be determined. In this regard, several investigators have adopted high-intensity interval training (HIT) interventions (e.g., 4–6 × 30-s supramaximal cycling Wingate tests) as an alternative to more traditional continuous training [40–60 min cycling at 65% maximal oxygen consumption ($\dot{V}O_{2max}$)] approaches. This model of HIT is a potent and time-efficient stimulus for increasing the oxidative capacity of human skeletal muscle as well as improving insulin sensitivity (3, 8, 9, 10, 21, 49, 62). In addition, an alternative form of HIT that is receiving increased research

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attention is running at high but submaximal intensities (e.g., 80–90% $\dot{V}O_{2max}$) for 4–5 repeated intervals each of 2- to 4-min duration (28, 43, 44, 58, 63). Although this form of HIT is somewhat longer than the Wingate type protocols (a typical training session could last 30–60 min in total, as opposed to 15 min for Wingate-type sessions), this mode, duration, and relative exercise intensity are also applicable to clinical populations and has been well-tolerated by both cardiovascular (63) and metabolically diseased patients (58). Performing HIT in this way also induces greater feelings of perceived enjoyment compared with CONT (even when both protocols are matched for average intensity, duration, and work done), thereby demonstrating the potential for improved exercise adherence with this intervention (4). However, despite the apparent applicability and popularity of running among the general population, the acute signaling responses of human skeletal muscle to both HIT and CONT running are not well known.

With this in mind, the aim of the present study was to therefore characterize the acute signaling pathways activated in human skeletal muscle by acute HIT and CONT running exercise. Although both protocols were matched for average intensity, duration, and total work done (4), we hypothesized that HIT running would induce greater activation of signaling pathways associated with mitochondrial biogenesis compared with CONT, thereby increasing the efficiency of the training stimulus for a given exercise duration and quantity of work done. This hypothesis was based on the rationale that many of the contractile-induced stressors thought to signal kinase activation (e.g., increased AMP/ATP ratio, glycogen utilization, lactate, ROS, and Ca^{2+} production) would be expected to increase during brief periods of high-intensity exercise, to a greater extent compared with moderate-intensity exercise (24, 30, 52, 55). In this way, we postulated that performing repeated brief periods of high-intensity exercise (6 × 3-min at 90% $\dot{V}O_{2max}$ interspersed with 3-min recovery periods at 50% $\dot{V}O_{2max}$) would result in a greater summative metabolic stress and Ca^{2+} flux compared with sustained exercise at the same average intensity (i.e., 70% $\dot{V}O_{2max}$). Our chosen model of HIT has long-term physiological relevance as it has also been shown in our laboratory to induce training-induced oxidative adaptations (increased content of PGC-1α, COXIV, and SDH activity) of the vastus lateralis and gastrocnemius muscles (43).

MATERIALS AND METHODS

Subjects. Ten recreationally active men volunteered to participate in the study (mean ± SD: age, 20 ± 1 yr; weight, 73 ± 8 kg, height, 1.77 ± 0.03 m; $\dot{V}O_{2max}$, 52 ± 7 ml·kg^{-1}·min^{-1}). The experimental procedures and potential risks associated with the study were explained, and subjects gave written informed consent prior to participation. Subjects refrained from additional exercise outside of the study requirements as well as from alcohol and caffeine intake for at least 48 h before any of the testing sessions. None of the subjects had history of neurological disease or musculoskeletal abnormality, and none was under pharmacological treatment during the course of the study. The study was approved by the Ethics Committee of Liverpool John Moores University.

Experimental design. In a randomized crossover design (separated by 7 days) and after having initially been assessed for $\dot{V}O_{2max}$, subjects attended the laboratory after an overnight fast and performed either high-intensity interval running (HIT) or moderate-intensity continuous running (CONT). Muscle biopsies were obtained from the vastus lateralis preexercise, postexercise, and 3 h after exercise and venous blood samples obtained pre- and postexercise. During the 3-h period between the postexercise and 3 h postexercise biopsies, subjects remained seated in the laboratory and performed light activities such as reading or working on a computer. Heart rate was measured continuously during exercise (Polar S610i, Kempele, Finland) and ratings of perceived exertion (RPE) were obtained at regular intervals during the protocol (7). Blood lactate was also assessed from fingertip capillary samples pre- and postexercise, as well as after 13, 16, 25, 28, 40, and 43 min of exercise (Lactate Pro, Arkray, Japan). In the 30 min preceding exercise, subjects consumed 5 ml/kg body mass of water only and were allowed to drink a further 3 ml/kg of water after 15 and 30 min of exercise (no other form of beverage such as energy containing or caffeinated drinks was permitted at any time). Subjects also consumed water ad libitum during the 3-h window between postexercise and 3-h biopsies, and intake of food during this period was prohibited. The pattern of fluid intake was recorded in the initial trial and repeated for the subsequent trial. Subjects completed a 3-day food diary preceding the first exercise trial and repeated the same energy intake in the 3 days before their second exercise trial. Subsequent dietary analysis was performed by the computer software program Microdiet (Downlee Systems, UK), and average daily macronutrient intake in the 72 h prior to exercise was 1,910 kcal: carbohydrate (CHO) 49 ± 8%, protein 26 ± 8%, and fat 28 ± 8%.

Assessment of $\dot{V}O_{2max}$. All participants were initially assessed for $\dot{V}O_{2max}$ using an incremental exercise test performed on a motorized treadmill (HP Cosmos, Germany). Oxygen uptake was measured continuously during exercise using an on-line gas analysis system (Metamax, Cortex, Germany). The test began with a 3-min stage at a treadmill speed of 10 km/h followed by 3-min stages at 12, 14, and 16 km/h. Upon completion of the 16-km/h stage, the treadmill inclined by 2% every 3 min thereafter until volitional exhaustion. The $\dot{V}O_{2max}$ was stated as being achieved by the following end-point criteria: *1*) heart rate within 10 beats/min of age-predicted maximum, *2*) respiratory exchange ratio > 1.1, and *3*) plateau of oxygen consumption despite increased workload (23).

Exercise protocols. Both exercise protocols were performed on a motorized treadmill (HP Cosmos, Germany). The HIT protocol commenced with a 7-min warm up at a running velocity corresponding to 70% $\dot{V}O_{2max}$ followed by six 3-min bouts at a running velocity corresponding to 90% $\dot{V}O_{2max}$. The high-intensity intervals were separated by 3-min active recovery periods at a running velocity corresponding to 50% $\dot{V}O_{2max}$. Following the interval and recovery periods, participants then performed a 7-min cool down at a running velocity corresponding to 70% $\dot{V}O_{2max}$. The exercise protocol gave a total of 18 min of high-intensity exercise and 18 min of active recovery time, thus giving a total interval exercise time of 36 min. When including the warm-up and cool-down times, the total duration of the exercise protocol was 50 min. The CONT protocol consisted of 50 min continuous running at a running velocity corresponding to 70% $\dot{V}O_{2max}$. When completed in this way, we have previously observed that the HIT and CONT protocols are matched for average intensity, energy expenditure, duration, and distance run (4).

Muscle biopsies. Muscle biopsies were obtained from separate incision sites (2–3 cm apart) from the lateral portion of the vastus lateralis muscle pre-, post-, and 3 h after the exercise protocol using a Bard Monopty Disposable Core Biopsy Instrument 12 gauge × 10 cm length (Bard Biopsy Systems, Tempe, AZ). Samples were obtained (~60 mg) under local anesthesia (0.5% marcaine) and immediately frozen in liquid nitrogen and stored at −80°C for later analysis. Samples were analyzed for muscle glycogen, $AMPK^{Thr172}$, $p38MAPK^{Thr180/Tyr182}$, $p53^{ser15}$, and PGC-1α, HSP72, and MnSOD mRNA content.

Muscle analysis. Approximately 3–6 mg of freeze dried sample was powdered, dissected free of all visible nonmuscle tissue, and subsequently hydrolyzed by incubation in 500 μl of 1 M HCl for 3–4 h at 100°C. After cooling to room temperature, samples were neutralized by the addition of 250 μl 0.12 mol/l Tris-2.1 mol/l KOH

saturated with KCl. Following centrifugation, 150 μl of the supernatant was analyzed in duplicate for glucose concentration according to the hexokinase method using a commercially available kit (GLUC-HK, Randox Laboratories, Antrim, UK). Glycogen concentration is expressed as mmol/kg dry weight (dw), and intra-assay coefficient of variation was < 5%.

Western blotting. Approximately 20–30 mg of frozen muscle was ground to powder and homogenized in 120 μl of ice-cold lysis buffer [25 mM Tris·HCl (pH 7.4), 50 mM NaF, 100 mM NaCl, 5 mM EGTA, 1 mM EDTA, 10 mM Na-pyrophosphatase, 1 mM Na_3VO_4, 0.27 M sucrose, 1% Triton X-100, 0.1% 2-mercaptoethanol] and supplemented with a protease inhibitor tablet (Complete mini, Roche Applied Science, West Sussex, UK). Homogenates were centrifuged at 14,000 *g* for 10 min at 4°C, and the supernatant was collected. The protein content of the supernatant was determined using a bicinchoninic acid assay (Sigma, UK). Each sample was diluted with an equal volume of 2X Laemmli buffer (National Diagnostics) and boiled for 5 min at 100°C. For each blot, a standard and internal control was loaded along with 50 μg of protein from each sample and then separated in Tris-glycine running buffer (10× Tris/glycine, Geneflow, Staffordshire, UK) using self-cast 4% stacking and 10% separating gels (National Diagnostics). Gels were transferred semidry onto nitrocellulose membrane (Geneflow) for 2 h at 200 V and 45 mA per gel in transfer buffers (*anode 1*: 0.3 M Tris, 20% methanol, pH 10.4; *anode 2*: 0.25 M Tris, 20% methanol, pH 10.4; cathode: 0.4 M 6-amino hexanoic acid, 20% methanol, pH 7.6). After transfer, membranes were blocked for 1 h at room temperature in Tris-buffered saline (TBST: 0.19 M Tris pH 7.6, 1.3 M NaCl, 0.1% Tween-20) with 5% nonfat milk. The membranes were then washed for 3 × 5 min in TBST before being incubated overnight at 4°C with antibodies for anti-phospho-AMPK^{Thr172}, p38MAPK$^{Thr180/Tyr182}$, and p53^{Ser15}, as well as total protein content of AMPK, p38MAPK, GAPDH (Cell Signalling, UK), and PGC-1α (Calbiochem, Merck Chemicals, UK) all at concentrations of 1:1,000 in 1 × TBST. The next morning, membranes were washed for a further 3 × 5 min in TBST and subsequently incubated with anti-species horseradish peroxidase-conjugated secondary antibody (Bio-Rad or Dako, UK) for 1 h at room temperature. After a further 3 × 5 min washes in TBST, membranes were exposed in a chemiluminescence liquid (SuperSignal, Thermo Fisher Scientific, Rockford, IL) for 5 min. Membranes were visualized using a Bio-Rad Chemi-doc system, and band densities were determined using Quantity One image-analysis software. Comparative samples from each subject for both exercise protocols were run on the same gel, and all gels were run in duplicate to verify responses. It should be noted that all raw densitometry data were used for statistical analysis purposes so as to compare within-subject responses to both the HIT and CONT protocols. However, because it is technically incorrect to compare densitometry data between gels (and hence, between subjects), for graphical purposes each subject's preexercise value in both trials was normalized to 1 (hence no error bars are shown for this time point on Figs. 2–5) such that values at postexercise and 3 h postexercise are subsequently expressed as fold change relative to preexercise values. This approach has been used previously by us (43) and other researchers (15, 46).

Real-time RT-PCR. Total RNA was isolated from muscle biopsies (20–30 mg) using Trizol reagent (Invitrogen), according to the manufacturer's protocol. RNA quality and quantity were determined using Implen Nanophotometer (Implen, Munchen, Germany) and the RNA was stored at −80°C. cDNA was synthesized using random hexamers (Applied Biosystems) and Superscript III enzyme (Invitrogen), using manufacturer's protocol. Gene-specific expression data were obtained using probes selected from Human Universal Probe Library (Roche Diagnostics) with compatible oligonucleotide primers (MWG Eurofins). The primers and corresponding probes are shown in Table 1. One microliter of each sample was analyzed in triplicate with negative controls using AB 7500 Real-Time Quantitative PCR instrument (Applied Biosystems) and Agilent Brilliant II qPCR Master Mix with Low ROX (Agilent Technologies). One microliter of cDNA, 500 nM of primer, and 200 nM of probe were used for each 20-μl reaction (Table 1). The following cycling parameters were used: 50°C for 2 min, initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/elongation at 60°C for 1 min. Data were collected and analyzed using AB SDS 1.43 Software (Applied Biosystems, Foster City, CA). Changes in mRNA content were calculated according to the $2^{-\Delta\Delta Ct}$ method where GAPDH was used as the housekeeping gene.

Blood analyses. Blood samples were drawn pre- and postexercise from a superficial vein in the anticubital crease of the forearm using standard venepuncture techniques (Vacutainers Systems, Becton-Dickinson). Samples were collected into vacutainers containing EDTA or lithium heparin and stored on ice until centrifugation at 1,500 *g* for 15 min at 4°C. Following centrifugation, aliquots of plasma were stored at 80°C for later analysis. Samples were analyzed for plasma glucose, nonesterified fatty acids (NEFA), and glycerol concentration using commercially available kits (Randox Laboratories, Antrim, UK). Each sample was analyzed in duplicate.

Statistical analysis. Statistical analysis was conducted using the Statistical Package for Social Sciences software program (version 15). Data were analyzed using a two-way repeated-measures General Linear Model where the within factor was time and the between factor was exercise condition (HIT vs. CONT). Where there were significant main effects, Bonferroni post hoc tests were used to locate the differences. All data in text, figures, and tables are presented as mean (SE) with P values ≤ 0.05 indicating statistical significance.

RESULTS

Physiological and metabolic responses to HIT and CONT. Heart rate, blood lactate, and RPE data during exercise are shown in Table 2. In accordance with the differences in activity profiles between trials, the exercise-induced increases (P < 0.01) in heart rate, RPE, and blood lactate was significantly greater (P < 0.01) in HIT compared with CONT. Muscle glycogen decreased (P < 0.001) by ~30% in both conditions with no difference (P = 0.618) between exercise protocols (Table 3). Similarly, both exercise protocols increased plasma glucose concentration (P = 0.002) with no difference between conditions. However, the exercise-induced increases in plasma NEFA (P < 0.001) and glycerol (P < 0.001) were significantly greater in the CONT trial compared with the HIT protocol (P = 0.04 and P = 0.02, respectively) (Table 3).

Exercise-induced kinase activation. Representative Western blots are shown in Fig. 1. Phosphorylation of AMPK^{Thr172} increased 1.5-fold postexercise (P = 0.04) with no difference

Table 1. *Primer and probe sequences used for real-time PCR*

Gene	Forward Primer	Reverse Primer	Probe
GAPDH	GCTCTCTGCTCCTCCTGTTC	ACGACCAAATCCGTTGACTC	60
MnSOD	CTGGACAAACCTCAGCCCTA	TGATGGCTTCCAGCAACTC	22
HSP72	ACCAAGCAGACGCAGATCTTC	GCCCTCGTACACCTGGATCA	70
PGC-1α	CAAGCCAAACCAACAACTTTATCTCT	CACACTTAAGGTGCGTTCAATAGTC	13

Table 2. *Heart rate, RPE, and blood lactate during the HIT and CONT protocols*

	Time, min							
	0	10	13	22	25	40	43	50
Heart rate, beats/min*‡								
HIT	89 ± 7	184 ± 9	163 ± 15	188 ± 8	163 ± 13	189 ± 8	163 ± 12	178 ± 9
CONT	89 ± 7	171 ± 11	172 ± 10	177 ± 9	179 ± 8	182 ± 8	182 ± 12	186 ± 9
RPE, AU*‡								
HIT	6 ± 0	13 ± 2	12 ± 2	16 ± 2	13 ± 1	18 ± 1	15 ± 2	16 ± 2
CONT	6 ± 0	12 ± 1	13 ± 1	14 ± 1	15 ± 1	17 ± 1	17 ± 1	17 ± 1
Blood lactate, mmol/l*†‡								
HIT	1.3 ± 0.5	8.0 ± 1.74	6.4 ± 2.0	9.4 ± 2.9	7.1 ± 2.3	9.4 ± 2.6	7.3 ± 2.1	6.2 ± 2.8
CONT	1.7 ± 0.9	4.8 ± 1.94	5.0 ± 2.2	6.4 ± 3.4	5.6 ± 2.3	5.9 ± 3.2	6.4 ± 3.9	5.5 ± 2.4

Values are means ± SE. HIT, high-intensity interval training running; CONT, moderate-intensity continuous running; RPE, rate of perceived exertion. *Significant main effect of exercise, †significant main effect of condition, ‡significant interaction: $P < 0.05$.

($P = 0.44$) between conditions and returned to basal levels at 3 h postexercise (Fig. 2). Similarly, phosphorylation of p38MAPK$^{Thr180/Tyr182}$ increased 1.9- and 1.5-fold immediately following exercise for HIT and CONT, respectively ($P < 0.001$), although there was no significant difference between exercise protocols ($P = 0.47$) (Fig. 3).

PGC-1α mRNA and protein content. Muscle PGC-1α mRNA content increased fourfold at 3 h following exercise ($P = 0.01$) with no difference between conditions ($P = 0.80$; Fig. 4*A*). There were no changes in total PGC-1α protein content at any time during the HIT or CONT trials ($P = 0.20$; Fig. 4*B*).

p53 phosphorylation. p53^{Ser15} phosphorylation showed a tendency to increase immediately postexercise ($P = 0.07$) but did not reach statistical significance until 3 h following exercise. At this time point, p53 phosphorylation was increased 2.7-fold and 2.1-fold in HIT and CONT trials, respectively ($P = 0.01$). There was no difference in p53^{Ser15} phosphorylation between exercise protocols ($P = 0.91$; Fig. 5).

HSP72 and MnSOD mRNA. Although there was a 3- to 4-fold increase in HSP72 mRNA immediately postexercise, this did not reach statistical significance ($P = 0.10$). At 3 h postexercise, however, there was an approximate 4-fold increase in both trials ($P = 0.04$) with no difference ($P = 0.87$) between conditions (Fig. 6*A*). In contrast, neither exercise protocol increased MnSOD mRNA content at any time point postexercise ($P = 0.44$) (Fig. 6*B*).

DISCUSSION

The aim of the present study was to characterize the acute signaling responses of human skeletal to HIT and CONT running exercise protocols when matched for average intensity, duration, and distance run. We provide novel data by demonstrating that both HIT and CONT running induces comparable AMPK and p38MAPK phosphorylation immediately postexercise with similarly increased PGC-1α mRNA at 3 h postexercise. Additionally, this is the first report of exercise-induced p53 phosphorylation in human skeletal muscle. However, contrary to our hypothesis, we observed no differences in activation of the above signaling cascades between HIT and CONT running protocols that are known to be matched for work done (4).

Exercise-induced phosphorylation of AMPK is well documented in human skeletal muscle following cycling-based protocols (17, 22, 38, 39). To the authors' knowledge, however, this is the first report of AMPK phosphorylation in response to running exercise. Given that exercise increases AMPK phosphorylation in an intensity-dependent manner (18, 51), we hypothesized that HIT would augment AMPK signaling to a greater extent compared with CONT. Despite these reports, our data demonstrate no differences in exercise-induced AMPK phosphorylation between HIT and CONT running. Our chosen model of HIT consisted of 6 × 3-min periods at 90% $\dot{V}o_{2max}$ interspersed with 3-min active recovery periods at 50% $\dot{V}o_{2max}$, as well as a combined 14-min period of warm up and cool down at 70% $\dot{V}o_{2max}$. In this way, the majority of exercise in the HIT protocol (32 min) was performed at intensities equal to or greater than 70% $\dot{V}o_{2max}$. However, it appears that the repeated 3-min intervals at 90% $\dot{V}o_{2max}$ within this time scale (i.e., 18 min) offer no additional augmentation with regard to AMPK phosphorylation compared with that of 50 min of CONT running at 70% $\dot{V}o_{2max}$. Support for this hypothesis stems from the data of Howlett et al. (30) where it can be estimated that an important allosteric regulator of AMPK signaling, AMP concentration, only differs by ~2 μmol/kg dw when

Table 3. *Muscle glycogen and plasma glucose, NEFA, and glycerol before and after completion of the HIT and CONT protocols*

	HIT		CONT	
	Preexercise	Postexercise	Preexercise	Postexercise
Muscle glycogen, mmol/kg dry wt	307 ± 54	191 ± 62*	301 ± 59	190 ± 69*
Glucose, mmol/l	5.4 ± 0.4	6.5 ± 0.6*	5.7 ± 0.7	6.7 ± 0.7*
NEFA, mmol/l	0.37 ± 0.22	0.64 ± 0.28*	0.44 ± 0.19	1.1 ± 0.43*†
Glycerol, μmol/l	54 ± 20	179 ± 59*	49 ± 25	246 ± 60*†‡

Values are means ± SE. NEFA, nonesterified fatty acids. *Significant main effect of exercise, †significant main effect of condition, ‡significant interaction: $P < 0.05$.

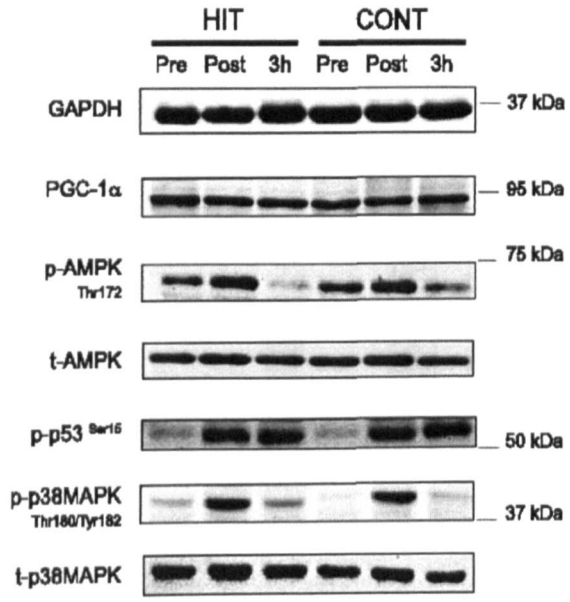

Fig. 1. Representative Western blots before (Pre), after (Post), and 3 h after (3h) the high-intensity interval training (HIT) and moderate-intensity continuous running (CONT) protocols. p-, phosphorylated; t-, total.

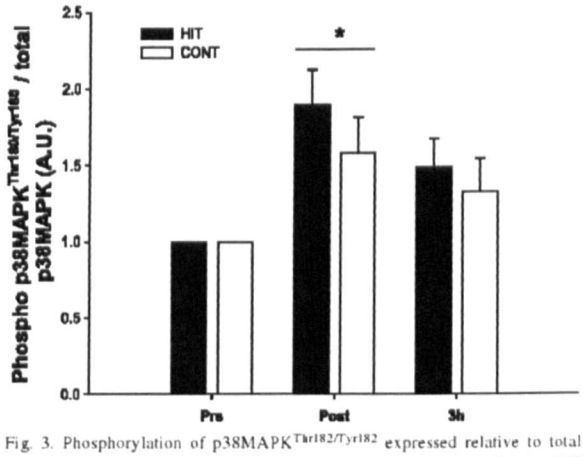

Fig. 3. Phosphorylation of p38MAPK$^{Thr182/Tyr182}$ expressed relative to total p38MAPK immediately before (Pre), after (Post), and 3 h after (3h) the HIT and CONT protocols. *Significant difference from preexercise ($P < 0.05$). Note that each subject's Pre values have been normalized to 1 (hence no error bars are shown for this time point) such that Post and 3-h values are subsequently expressed as fold change relative to Pre values.

comparing 3 min of exercise at 90% vs. 70% $\dot{V}O_{2max}$. It is possible, therefore, that a difference in relative exercise intensity of 20% $\dot{V}O_{2max}$ between exercise protocols that are already relatively intense may not be sufficient to cause further metabolic signaling responses. Furthermore, the 6 × 3-min recovery periods at 50% $\dot{V}O_{2max}$ may not offer a metabolic stress severe enough to induce AMPK signaling given that exercise at comparable intensities does not induce AMPK phosphorylation (18). In contrast, CONT running for 50 min at 70% $\dot{V}O_{2max}$ likely offers a sustained stimulus for AMPK phosphorylation as opposed to the transient periods of stress induced by the intermittent bursts of activity inherent to HIT running.

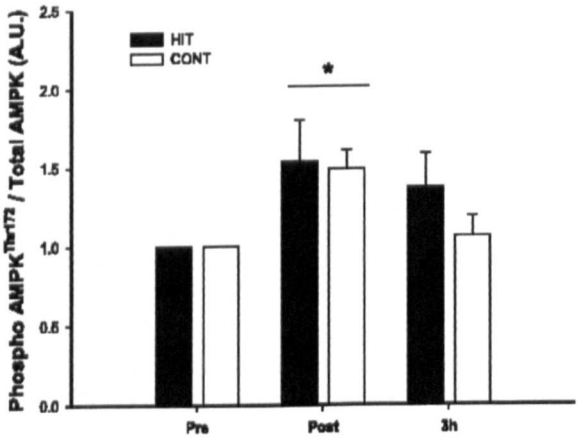

Fig. 2. Phosphorylation of AMPK^{Thr172} expressed relative to total AMPK immediately before (Pre), after (Post), and 3 h after (3h) the HIT and CONT protocols. *Significant difference from preexercise ($P < 0.05$). Note that each subject's Pre values have been normalized to 1 (hence no error bars are shown for this time point) such that Post and 3-h values are subsequently expressed as fold change relative to Pre values.

Muscle glycogen availability and utilization, another regulator of AMPK (65), were also not different between trials, which may have contributed to the similar AMPK phosphorylation observed in our study. Both resting glycogen concentration (>300 mmol/kg dw) and utilization (>100 mmol/kg dw) were similar to that observed by us (43) and others (1, 59) while using similar average exercise intensities and subject populations to that studied here. The similar decreases in muscle glycogen content may have been due to the alternating bouts of exercise in HIT between 90% $\dot{V}O_{2max}$ (thus heavily reliant on CHO oxidation) and 50% $\dot{V}O_{2max}$ (more reliant on lipid oxidation), as opposed to 50 min of CONT running at 70% $\dot{V}O_{2max}$, which likely induces sustained glycogen utilization. It is likely, however, that if our HIT protocol had incorporated intermittent periods of supramaximal exercise similar to Wingate-type HIT protocols (i.e., >100% $\dot{V}O_{2max}$), then much greater rates of glycogen utilization would have occurred, even at the expense of reduced exercise durations (24). Such rapid rates of glycogen depletion may therefore be one of the contributing mechanisms contributing to increased AMPK phosphorylation that is apparent even after just 4 × 30-s bouts of supramaximal exercise (22). Clearly, future studies examining acute signaling responses to running exercise of supramaximal exercise intensity would also be warranted.

p38MAPK is a stress-activated kinase that has repeatedly shown to be phosphorylated following acute cycling (17, 22, 38, 39) and marathon running (6, 66). Consistent with these data, we also observed increased p38MAPK$^{Thr182/Tyr180}$ phosphorylation following more practically applicable running exercise protocols, although the magnitude of phosphorylation was not different between HIT and CONT. This finding is similar to recent data demonstrating that p38MAPK phosphorylation is not affected by exercise intensity, even when a large difference between exercise protocols exists such as 80% vs. 40% $\dot{V}O_{2max}$ (18). Similar

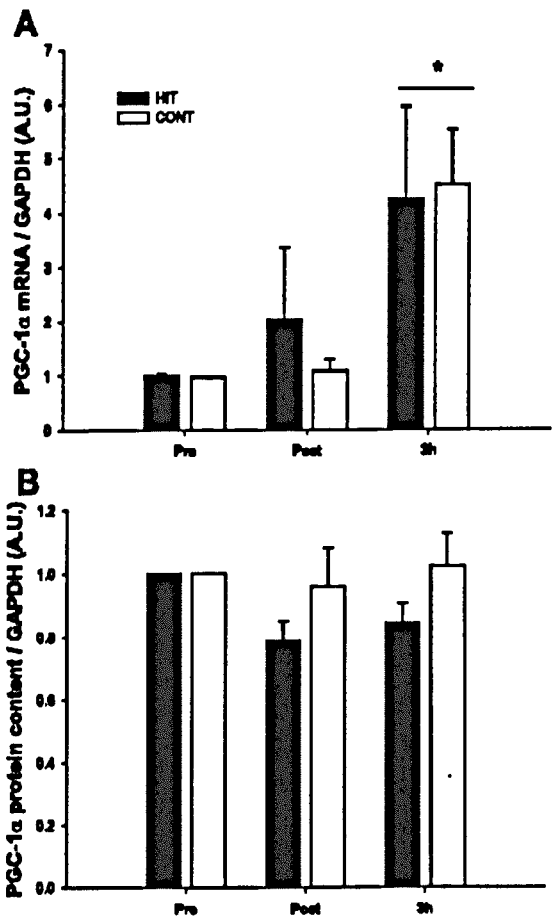

Fig. 4. PGC-1α mRNA content (*A*) and PGC-1α total protein content (*B*) expressed relative to GAPDH immediately before (Pre), after (Post), and 3 h after (3h) the HIT and CONT protocols. *Significant difference from preexercise ($P < 0.05$). Note that for *B*, each subject's Pre values have been normalized to 1 (hence no error bars are shown for this time point) such that Post and 3-h values are subsequently expressed as fold change relative to Pre values.

mRNA at 3 h postexercise in both exercise protocols with no concomitant changes in PGC-1α protein content. This time course of PGC-1α expression is well documented in human muscle following cycling protocols (22, 38, 39). In contrast, it appears that changes in PGC-1α protein are typically only observed within days of exercise (2, 46). To the authors' knowledge, only one study has measured PGC-1α mRNA changes in response to acute running (45 min of CONT running at 75% $\dot{V}O_{2max}$) where fivefold and fourfold changes (albeit not statistically significant) were observed in the soleus and vastus lateralis muscle, respectively, at 4 h postexercise (26). Our data extend these findings by confirming increased muscle PGC-1α expression in response to running exercise while also demonstrating that the magnitude of response is comparable between HIT and CONT when matched for work done.

The tumor suppressor protein, p53, has recently emerged as a regulator of mitochondrial function (35, 42, 45). p53 has been shown to modulate the activity of nuclear-encoded synthesis of cytochrome-*c* oxidase (SCO2), which is critical for the proper assembly of the cytochrome-*c* oxidase (COX) enzyme complex in the electron transport chain (42). Furthermore, PGC-1α also contains a p53 binding site in its promoter region (31) and skeletal muscles from p53 knockout mice display reduced PGC-1α protein content (54) and mitochondrial content (45, 54) compared with wild-type animals. Findings from a variety of cell types have shown that AMPK (33) and p38MAPK (56) phosphorylate p53 on serine 15, and acute contractile activity has been demonstrated to increase phosphorylation on this amino acid residue in rodent muscle (54). The present data confirm and extend these findings by demonstrating that $p53^{Ser15}$ phosphorylation also increases in human skeletal muscle at 3 h postexercise. Based on the time course of this response and similar fold changes, it is tempting to speculate that AMPK and p38MAPK signaling during exercise leads to a temporal and coordinated downstream p53 phosphorylation followed by p53-mediated transcription of PGC-1α in the hours following exercise. Future studies are required, however, to

to AMPK (27, 32, 65), p38MAPK phosphorylation is also sensitive to cytosolic calcium (64), glycogen availability (13), and ROS (25). Given the difficulties in directly assessing ROS and also due to the limited tissue obtained by our chosen biopsy technique, we attempted to indirectly ascertain the degree of redox signaling induced by our exercise protocols by examining changes in expression of two redox-sensitive genes, HSP72 (19, 34) and MnSOD (25). Although we observed no changes in MnSOD mRNA at any time point, we observed similar fourfold increases in HSP72 expression after HIT and CONT at 3 h postexercise. This magnitude and time course of adaptation is in agreement with previous literature in which similar subject populations and CONT exercise protocols have been studied (48). These are the first data, however, to observe increased expression of members of the heat shock family in response to HIT running.

In accordance with the role of AMPK and p38MAPK as upstream signaling kinases regulating PGC-1α expression (12, 64), we observed a similar fourfold increase in PGC-1α

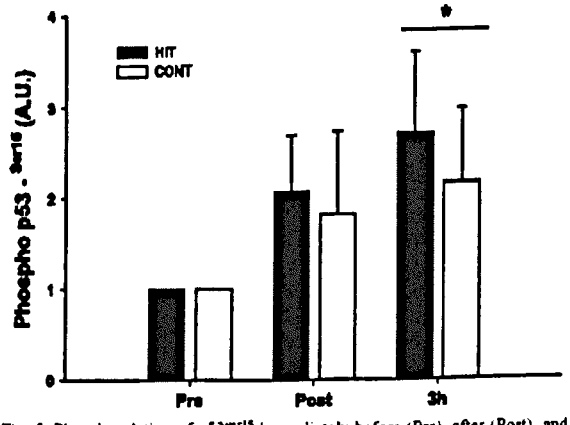

Fig. 5. Phosphorylation of $p53^{ser15}$ immediately before (Pre), after (Post), and 3 h after (3h) the HIT and CONT protocols. *Significant difference from preexercise ($P < 0.05$). Note that each subject's Pre values have been normalized to 1 (hence no error bars are shown for this time point) such that Post and 3-h values are subsequently expressed as fold change relative to Pre values.

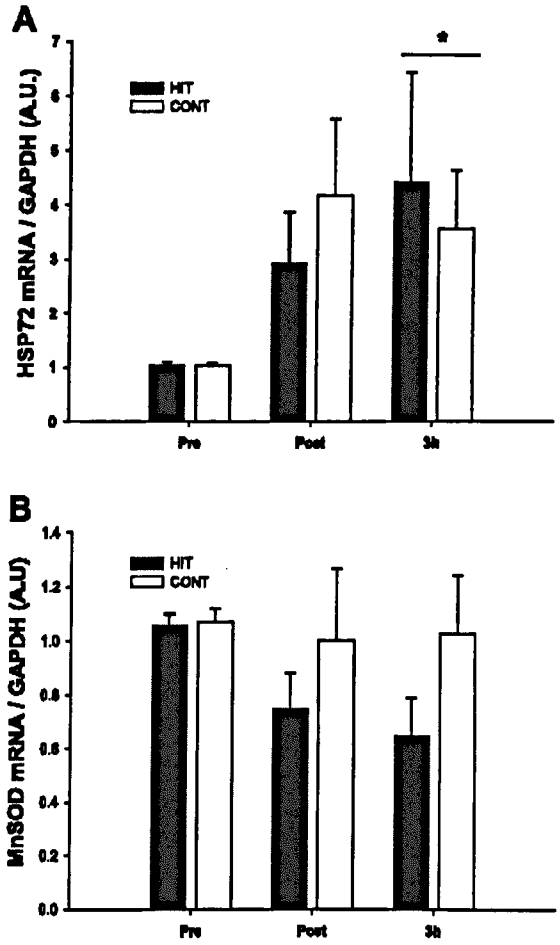

Fig. 6. HSP72 mRNA content (*A*) and MnSOD mRNA content (*B*) immediately before (Pre), after (Post), and 3 h after (3h) the HIT and CONT protocols. *Significant difference from preexercise ($P < 0.05$).

confirm the presence and physiological relevance of this signaling cascade. Furthermore, p53 has also been shown to regulate mitochondrial transcription factor A (45), and as such, the subcellular location of p53 following muscle contraction also warrants investigation.

In relation to our physiological data, it is noteworthy that CONT exercise was associated with greater elevations in plasma glycerol and NEFA compared with HIT. This finding may be due to a lactate-induced inhibition of lipolysis (40), given that lactate was consistently higher during HIT, and/or a reduction in adipose tissue blood flow during the repeated 3-min high-intensity intervals (50), thereby leading to reesterification (20). Although there have been some suggestions that NEFAs can act as signals to modify contractile-induced gene expression (14, 16), the present data confirm previous findings using either pharmacological (60) or carbohydrate-induced manipulation of NEFA availability (17) to collectively demonstrate the NEFA availability does not regulate exercise-induced PGC-1α expression.

The observation of increased lactate concentrations during HIT does not appear in accordance with similar muscle glycogen utilization between HIT and CONT running. It is important to note, however, that all muscle-related data were quantified in whole muscle homogenates as opposed to specific fiber types. Indeed, it is likely that our CONT protocol would predominantly recruit type I fiber (59) whereas our HIT protocol would stimulate both type II (during the interval periods) and type I fiber recruitment (during the active recovery periods). It is therefore possible that glycolytic flux (and hence muscle glycogen utilization) was particularly high in type II fiber during the 3-min interval periods, thereby contributing to high blood lactate levels at the completion of each interval (see Table 2, *minutes 10, 22,* and *40*). It would appear, however, that the intensity and duration of the active recovery periods were not sufficient for lactate removal. In this regard, blood lactate remains elevated during the active recovery period even though glycogenolysis and glycolytic flux would likely be reduced given the reduced relative exercise intensity of 50% $\dot{V}O_{2max}$.

In addition to muscle glycogen utilization, it is possible that the comparable signaling responses observed here may have also arisen as a function of specific responses in individual fiber types that are reflective of recruitment patterns, but which ultimately result in comparable responses in mixed muscle biopsy samples. This is especially true considering that the contractile-induced phosphorylation of AMPK (36) and p38MAPK (57) as well as training-induced increases in PGC-1α protein content (53) are particularly evident in type II fiber when the intensity of contraction is >65% $\dot{V}O_{2max}$. Future studies investigating the effects of manipulations of exercise-related variables (e.g., duration, intensity, work-rest ratio, etc.) on cellular and molecular adaptations to exercise would clearly benefit from the inclusion of histochemical techniques staining for muscle glycogen content so as to evaluate the degree of specific muscle fiber recruitment patterns.

In summary, the present study characterizes, for the first time, the responses of the acute molecular signaling pathways thought to initiate mitochondrial biogenesis in human skeletal muscle in response to two physiologically relevant and practically applicable running exercise protocols. We demonstrate that acute HIT and CONT running (when matched for average intensity, duration, and work done) induce comparable increases in AMPK and p38MAPK phosphorylation as well as PGC-1α mRNA content in human skeletal muscle. Furthermore, this is the first report of exercise-induced p53 phosphorylation in humans, consistent with the notion that this protein may be involved in regulation of contraction-induced mitochondrial biogenesis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: J.D.B., A.J.C., M.J.G., W.G., G.L.C., B.D., and J.P.M. conception and design of research; J.D.B., C.H.-J., T.-S.J., J.L., W.G., and J.P.M. performed experiments; J.D.B., C.H.-J., T.-S.J., J.L., W.G., and J.P.M. analyzed data; J.D.B., C.H.-J., T.-S.J., J.L., A.J.C., M.J.G., W.G., G.L.C., B.D., and J.P.M. interpreted results of experiments; J.D.B. and J.P.M.

prepared figures; J.D.B. and J.P.M. drafted manuscript; J.D.B., C.H.-J., T.-S.J., J.L., A.J.C., M.J.G., W.G., G.L.C., B.D., and J.P.M. edited and revised manuscript; J.D.B., C.H.-J., T.-S.J., J.L., A.J.C., M.J.G., W.G., G.L.C., B.D., and J.P.M. approved final version of manuscript.

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APPENDIX 5 – Bartlett, JD, Joo, CH, Cochran, AJ, Gibala, MJ, Gregson, W, Close, GL, Drust, B, Morton, JP. The effect of carbohydrate availability on high-intensity interval running-induced alterations in metabolic gene expression in human skeletal muscle. Presented as an oral at ECSS, 2012, Bruges, Belgium.

THE EFFECT OF CARBOHYDRATE AVAILABILITY ON HIGH-INTENSITY INTERVAL RUNNING-INDUCED ALTERATIONS IN METABOLIC GENE EXPRESSION IN HUMAN SKELETAL MUSCLE

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Introduction
Acute high-intensity interval run training (HIT) activates skeletal muscle signaling pathways associated with mitochondrial biogenesis (Bartlett et al. 2012). Performing HIT with reduced endogenous and exogenous carbohydrate (CHO) availability enhances the training-induced increase in skeletal muscle oxidative capacity (Morton et al. 2009) though the mechanisms underpinning enhanced adaptation remain unclear. We tested the hypothesis that performing acute HIT with reduced CHO availability before, during and after exercise enhances acute signaling and metabolic gene expression compared with traditional nutritional strategies advising CHO provision before, during and after exercise.

Method
Muscle biopsies (vastus lateralis) were obtained from eight active men pre-, post- and 3 h post-completion of an acute bout of HIT performed with normal (NORM) or low CHO availability (LOW). In NORM, subjects consumed 8 g/kg CHO in the day before exercise and were fed CHO 2 h before (2g/kg), during (1g/min) and hourly after exercise (1.2 g/kg/h). In LOW, subjects consumed 3 g/kg CHO the day before HIT, performed glycogen depleting exercise in the evening prior to HIT and also withheld CHO intake prior to, during and for 3 h after completion of HIT.

Results
Resting muscle glycogen was higher in NORM vs. LOW (374 ± 53 vs. 103 ± 9; p=0.002). Total CHO utilization was greater in NORM vs. LOW (177 ± 6 vs. 122 ± 9 g; p=0.001) and fat utilization was lower in NORM vs. LOW (8 ± 0.2 vs. 23 ± 3 g; p=0.004). PGC-1α mRNA increased similarly 3 h following HIT (NORM: 3.5 ± 1.0; LOW: 3.0 ± 0.5; p=0.06) with no difference between conditions (p=0.58). Resting PDK-4 mRNA increased 40-fold in LOW (p=0.03), a difference which persisted after HIT (p=0.01). There were no differences in GLUT-4, CPT-1 or SCO2 mRNA before or after exercise (p>0.05).

Discussion
Reduced CHO availability does not regulate contractile-induced PGC-1α expression, suggesting this transcriptional co-activator may not regulate the enhanced training adaptation observed following low glycogen training. Reduced CHO availability augments lipid oxidation which may be due to early regulation of the pyruvate dehydrogenase complex. Biopsy samples are currently being analyzed to examine for phosphorylation of AMPK, p38MAPK and p53.

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