EFFECT OF POST-EXERCISE COLD WATER IMMERSION ON MOLECULAR RESPONSES TO HIGH-INTENSITY INTERMITTENT EXERCISE

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ABSTRACT

The balance between the stress of training and competition and sufficient recovery is critical within the development of athletic performance. This stems from the need to recover between successive intense periods of exercise and provide sufficient time through which to adapt to the prescribed training stimulus. Cold water immersion (CWI) is now widely used by athletes to enhance the rate of recovery following training and competition. However, little information currently exists with respect to its influence on skeletal muscle adaptation. Therefore, the aim of this thesis was to investigate the impact of CWI on acute markers of adaptation in human skeletal muscle following low-damaging high-intensity intermittent exercise.

The aim of study 1 (Chapter 4) was to devise a low-damaging high-intensity intermittent running protocol which would be used as the criterion mode of exercise in future studies within the thesis. The exercise was comprised of 60-min of high-intensity intermittent exercise (8 × 3-min bouts at 90% $\dot{V}O_{2max}$ interspersed with 3-min recovery) on a motorised treadmill. No significant reduction in maximal voluntary contraction of the quadriceps was observed immediately following completion of the exercise protocol or during the subsequent 7 d period compared to pre-exercise values ($P = 0.59$). Creatine Kinase (CK) concentrations remained similar to baseline following exercise ($P = 0.96$). Myoglobin (Mb) content increased following exercise ($P = 0.01$). However, values returned to baseline after 24 h ($P = 0.32$). These results suggest the high-intensity intermittent running protocol induced changes in physiological and subjective indices consistent with the effects of low muscle damaging as opposed to those changes normally associated with exercise-induced severe muscle damage.

The purpose of the second study (Chapter 5) was to examine the effects of CWI (2 × 5-min ($8^{o}C$)) on acute markers of skeletal muscle adaptation at rest. Rectal temperature remained similar throughout the CWI protocol ($P = 0.36$). However, significant reductions in skin (thigh and calf) and muscle temperature were observed immediately post-immersion and the post-immersion period ($P < 0.05$). Noradrenaline was significantly increased 3 h (355.7 ± 181pmol/l) and 6 h (390.9 ± 131pmol/l) post-immersion compared to baseline ($P < 0.01$). Muscle PGC-1α (3 h, 1.3 ± 0.2-fold; 6 h, 1.4 ± 0.3-fold) and $VEGF_{165}$ (3 h, 1.9 ± 1.4-fold; 6 h, 2.2 ± 1.0-fold) mRNA expression were significantly increased at 3 h (PGC-1α, $P < 0.001$; $VEGF_{165}$, $P = 0.03$) and 6 h (PGC-1α, $P < 0.001$; $VEGF_{165}$, $P = 0.009$) post-immersion, respectively. These results indicate that CWI enhances the upstream signalling pathways associated with mitochondrial biogenesis and angiogenesis in human skeletal muscle at rest.

The aim of the third study (Chapter 6) was to establish whether post-exercise CWI further enhances the upstream signalling pathways associated with mitochondrial biogenesis and angiogenesis in human skeletal muscle. On each occasion, participants rested passively (Cont) or undertook 2 × 5-min of CWI ($8^{o}C$) at twenty minutes after completing the intermittent exercise protocol. Rectal temperature remained similar between CWI and Cont conditions during the 3 h post-exercise recovery period ($P > 0.05$), however, skin (thigh and calf) and muscle temperature were reduced in the CWI condition compared to Cont ($P < 0.05$). PGC-

1α mRNA expression was significantly increased 3 h post-exercise under both conditions (CWI, $P < 0.001$; Cont, $P = 0.003$) with greater expression observed in CWI (CWI, 5.9 ± 3.1-fold; Cont, 3.4 ± 2.1-fold; $P < 0.001$). $VEGF_{165}$ and $VEGF_{total}$ mRNA were greater in CWI (2.4 ± 0.6-fold, 2.3 ± 0.4-fold) compared with Cont (1.3 ± 0.5-fold, 1.0 ± 0.3-fold) at 3 h post-exercise ($P = 0.01$, $P < 0.001$). These findings demonstrate that post-exercise CWI increases the expression of upstream signalling pathways associated with mitochondrial biogenesis and angiogenesis in human skeletal muscle compared with exercise alone.

Study 4 (Chapter 7) examined the influence of the repeated post-exercise CWI on upstream signalling pathways associated with mitochondrial biogenesis and angiogenesis in human skeletal muscle. On each occasion, participants rested passively or undertook 3 × 10-min of CWI (8^{o}C) at twenty minutes after completing the intermittent exercise protocol, 1 h and 2 h post-exercise. Rectal temperature was reduced during the 3rd bout of CWI and subsequent 30-min period compared to Cont ($P < 0.05$). Skin temperature (thigh and calf) remained consistently lower during the immersion periods in CWI compared with Cont ($P < 0.05$). Muscle temperature was reduced before the 2nd bout of CWI (-5.8 ± 0.3^{o}C) compared with Cont (-1.9 ± 0.4^{o}C) and remained until 50-min after the 3rd immersion ($P < 0.05$). Noradrenaline were significantly greater at 3 h and 6 h following exercise in CWI (662 ± 139pmol/l, 518 ± 158pmol/l) compared with Cont (307 ± 162pmol/l, 245 ± 156pmol/l) ($P < 0.05$). PGC-1α mRNA expression was higher after 3 h post-exercise in the Cont (2.4 ± 1.7-fold) than CWI (1.8 ± 1.0-fold) conditions respectively ($P = 0.06$). At 6 h post-exercise, PGC-1α mRNA expression was greater in CWI (2.6 ± 1.4-fold) compared to Cont (1.7 ± 1.7-fold) ($P = 0.03$). $VEGF_{165}$ and $VEGF_{total}$ mRNA increased more than ~1.6-fold at 3 h and 6 h following exercise and were similar between conditions ($P > 0.05$). These results indicate that increasing the repeated post-exercise CWI does not further increases the expression of upstream signalling pathways associated with mitochondrial biogenesis and angiogenesis in human skeletal muscle.

This thesis provides novel findings concerning the influence of high-intensity intermittent exercise and post-exercise CWI on cellular and molecular adaptations in human skeletal muscle. These findings may offer important insights for athletes wishing to maximize training adaptations.

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LIST OF ABBREVIATIONS

α_1	α_1-adrenoceptors
α_{2A}	α_{2A}- adrenoceptors
α_{2c}	α_{2c}-adrenoceptors
ac	Actylation
AMP	Adenosine monophosphate
AMPK	5'-AMP-activated protein kinase
AP-1	Activator protein 1
ARE	Antioxidant response element
ATF2	Activation transcription factor 2
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
bFGF	basic fibroblast growth factor
β-AR	β-adrenergic receptor
β2M	β2-Microglobulin
Ca^{2+}	Calcium
CamK	Calmodulin-dependent kinase
cAMP	cycle AMP
CK	Creatine kinase
CO	Carbon monoxide
Cont	Control
COX	Cytochrome c oxidase
CREB	cAMP response element binding
CRP	C-reactive protein
CS	Citrate synthase
CWI	Cold water immersion
DAMPs	Danger-associated molecular patterns
Δ	Delta/change
DOMS	Delayed onset muscle soreness
ERK	Extracellular signal-related kinases
ERR	Oestrogen-related receptor
ERR-α	Oestrogen-related receptor alpha
Flt-1	VEGF receptor 1
Flt-4	VEGF receptor 3
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLM	General linear model
GLUT-4	Glucose transport-4
HIF-1	Hypoxia-induced factor-1
HO-1/Hmox1	Heme oxygenase-1
HR	Heart rate (beat. min^{-1})
IGF-I	Insulin-like growth factor I
IL	Interleukin
IL-1β	Interleukin 1 beta
IL-1rα	Interleukin 1 receptor alpha
IFN-γ	Interferon gamma
IκB	NF-κB inhipitor
JNK	c-Jun NH2-terminal kinases
KDR/Flk-1	VEGF receptor 2

LDH	Lactate dehydrogenase
Mb	Myoglobin
MEF2	Myocyte enhancer factor 2
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatise
mtDNA	Mitochondrial DNA
MVC	Maximal voluntary contraction
MyD88	Myeloid differentiation primary response protein
N	Newton
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nfe2l2	Nuclear factor erythroid 2-related factor 2
NO	Nitric oxide
NOS2/iNOS	Nitric oxide synthase 2
NRF	Nuclear respiratory factor
O_2^-	Superoxide
P	Phosphorylation
PAF	Platelet-activation factor
PAS	Passive recovery
PGC-1α	Peroxisome proliferator-activated receptor gamma co-activator-1 alpha
PKA	Protein kinase A
PKB/Akt	Protein kinase B
PO_4	Phosphate
PPARs	Peroxisome proliferator-activated receptors
PPARδ	Peroxisome proliferator-activated receptor delta
p38MAPK	p38 mitogen-activated protein kinases
RER	Respiratory exchange ratio
RNA	Ribose nucleic acid
ROS	Reactive oxygen species
RPE	Rating of perceived exertion
SIRT1	Silent information regulator T1
Sirt1	Sirtuin 1
TF	Transcription factor
Tfam	Transcription factor A mitochondria
T_m	Muscle temperature (^oC)
TNF-α	Tumor necrosis factor-α
T_{re}	Rectal temperature (^oC)
T_{skin}	Skin temperature (^oC)
VAS	Visual analogue scale
VEGF	Vascular endothelial growth factor
$\dot{V}O_{2max}$	Maximal oxygen uptake ($mL.kg^{-1}.min^{-1}$)
$\dot{V}O_2$	Oxygen uptake ($mL.kg^{-1}.min^{-1}$)
W	Watts
WBC	Whole-body cryotherapy

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CHAPTER 1

GENERAL INTRODUCTION

1.1. BACKGROUND

The stress associated with training and competition often temporarily impairs an athlete's performance. This impairment may be acute, lasting minutes or hours and stems from metabolic disturbances associated with high-intensity exercise (Westerblad et al., 2002). Alternatively, exercise-induced muscle injury and delayed onset muscle soreness (DOMS) that often follows training with a high eccentric component may lead to impairment lasting several days (Barnett, 2006). The balance between the stress of training and competition and sufficient recovery is therefore of significant importance since an imbalance over extended periods of time may contribute to potentially long-term debilitating effects associated with overtraining (Nimmo and Ekblom, 2007).

Significant attention in the literature has focused upon evaluating the effectiveness of a plethora of recovery strategies which serve to alleviate short-term fatigue and/or the symptoms associated with exercise-induced muscle injury (Cheung et al., 2003). In line with such modalities, more recent interest has centred upon the use of cryotherapy strategies such as CWI. Decreasing local tissue temperature via cryotherapy is thought to diminish metabolism, local blood flow, pain and inflammation in injured tissue and has therefore been widely used in the treatment of acute traumatic injury (Merrick et al., 1999). It has therefore been suggested that whole limb CWI may be effective by virtue of its impact upon deep muscle blood flow (Gregson et al., 2011, Mawhinney et al., 2013). Since the inflammatory process is integral in the aetiology of exercise-induced muscle damage (Smith, 1991), cryotherapy undertaken via CWI may therefore also serve as a pertinent recovery strategy for athletes undertaking high-intensity exercise. Such observations are supported by recent findings which indicate that CWI is effective in reducing acute fatigue induced by high-intensity exercise in both sub elite (Lane and Wenger, 2004, Minett et al., 2013) and elite athletes (Vaile et al., 2008b) and the physiological and functional deficits associated with exercise-induced muscle damage (Bailey et al., 2007, Vaile et al., 2008c, Leeder et al., 2012).

Several major signalling pathways important for chronic training adaptation arise during the first few hours of recovery, returning to baseline values within 24 h after exercise (Hildebrandt et al., 2003). Whilst the effects of CWI on the recovery of performance and DOMS have been extensively studied, little attention has centred upon its effects on specific adaptation pathways during the acute post-exercise recovery period. CWI has previously been

shown to attenuate endurance and resistance training effects on muscle performance (Yamane et al., 2006). This led the authors to postulate that the effectiveness of such training interventions may be reduced under conditions were marked changes in muscle blood flow perfusion and/or muscle temperature associated with CWI arise during the initial post-exercise recovery period (Yamane et al., 2006). However, animal studies (Oliveira et al., 2004, Kim et al., 2005) have previously shown that cooling may promote alterations in cell signalling which enhance mitochondrial biogenesis and angiogenesis. Despite observations in animals (Oliveira et al., 2004, Kim et al., 2005), limited work to date has been undertaken in humans to determine the impact of cooling on signalling pathways which mediate the long-term adaptation to training. Recent work reported increased expression of PGC-1α mRNA, a transcriptional co-activator which serves as a master regulator of mitochondrial biogenesis, following combined exercise and recovery in cold (7^{o}C) compared to moderate (20^{o}C) ambient temperatures (Slivka et al., 2012). Furthermore, recovery alone in cold (7^{o}C) air increases PGC-1α mRNA expression relative to recovery in moderate temperatures (Slivka et al., 2013). More recently, Ihsan et al. (2014) observed one leg immersion in 10^{o}C water for 15-min following a single bout of high-intensity running significantly increased PGC-1α mRNA expression compared to the control leg. These studies suggest possible beneficial effects of cold application on promoting exercise-induced muscle adaptation in human. However, the magnitude of the increase in sympathetic discharge to skeletal muscle is influenced by the size of the tissue area exposed to cooling (Seals, 1990) and the magnitude of the cooling stimulus (Kregel et al., 1992). Consequently, signalling pathways important for chronic training adaptation in humans may be influenced to different degrees by nonnoxious whole body skin surface cooling relative to localized noxious cooling promoted by CWI.

1.2. AIMS AND OBJECTIVES

The aim of this thesis is to examine the effect of CWI on resting skeletal muscle cell signalling responses which regulate mitochondrial biogenesis and angiogenesis. Subsequent aims will serve to establish whether CWI modulates these responses following high-intensity intermittent exercise. As such, it is hoped the studies contained in this thesis will help inform the application of CWI for skeletal muscle adaptation.

The aims of this thesis will be achieved by objectives: -

1. The development of a laboratory based low-damaging high-intensity intermittent exercise protocol.
2. Examine the effects of CWI ($8^{o}C$) on the acute skeletal muscle cell signalling responses at rest.
3. Examine the effects of low-damaging high-intensity intermittent exercise on the acute skeletal muscle cell signalling responses.
4. Examine the effects of CWI ($8^{o}C$) on skeletal muscle cell signalling responses to acute high-intensity intermittent exercise.
5. Examine the effects of repeated CWI ($8^{o}C$) on skeletal muscle cell signalling responses to acute high-intensity intermittent exercise.

CHAPTER 2

REVIEW OF LITERATURE

2.1. INTRODUCTION

The aim of this literature review is to provide the reader with information relating to the effects of CWI on the physiological, cellular and molecular responses to exercise. The initial section of this review focuses upon influences of CWI on the recovery of muscle function following strenuous exercise. The following section focuses on the physiological mechanisms mediating the effects of CWI on exercise recovery. The final section initially examines the cellular and molecular pathways which underpin adaptations to endurance exercise followed by a review of the research which has examined the influence of temperature per se on these pathways.

Muscle fatigue, defined as a failure to maintain the required or expected power output (Edwards, 1981), commences soon after the initiation of intense exercise. It may persist for several days and may impair an athletes' performance. Recovery plays a role in reducing fatigue and exercise-induced muscle damage and mediating adaptation to exercise training. The balance between the stress of training and competition and sufficient recovery is therefore of significant importance since an imbalance over extended periods of time may contribute to potentially long-term debilitating effects associated with overtraining (Nimmo and Ekblom, 2007). A wide variety of recovery strategies have been used by athletes, coaches and trainers to assist with recovery and to maintain optimal performance, these include rest, sleep, nutritional strategies and physical modalities such as massage, active recovery and stretching. Recent interest has centred upon the use of cryotherapy strategies such as CWI as recovery strategy (Wilcock et al., 2006).

2.2. INFLUENCE OF CWI ON THE RECOVERY OF MUSCLE FUNCTION FOLLOWING STRENUOUS EXERCISE

2.2.1. Cold water immersion

Historically, the application of localized cold or cryotherapy has been used for the treatment of soft tissue injuries since decreasing local tissue temperature reduces the inflammatory response in injured tissue, local edema, swelling and pain (Meeusen and Lievens, 1986). Consequently, in recent years CWI has been increasingly used by athletes to try and accelerate recovery following competition and training by alleviating the signs and symptoms associated with exercise-induced muscle damage.

2.2.2. High-intensity exercise

A number of sports especially containing sporting competitions may require an athlete to perform consecutive high-quality exercise bouts within a short period of time. High-intensity exercise refers to stress caused from exercise with both a large mechanical stress and a heightened metabolic cost (Thompson et al., 1999). Recovery is therefore very important for these athletes to maintain optimal performance for subsequent efforts.

Numerous studies have been undertaken to determine the effects of CWI on the recovery of muscle function following high-intensity exercise. Previous studies have provided inconsistent findings, with CWI after exercise shown to both improve high-intensity exercise performance (Yeargin et al., 2006, Vaile et al., 2008a, Vaile et al., 2008b), and to provide no beneficial effects whatsoever (Buchheit et al., 2009, King and Duffield, 2009, Rowsell et al., 2009, Peiffer et al., 2010, Stacey et al., 2010). For example, the performance time in a 2-mile race was significantly faster in the CWI group (725 seconds) compared with Cont group (769 seconds) ($P < 0.05$). This equated to high-intensity exercise performance being enhanced by 6% in the CWI group compared with the no water immersion group (Yeargin et al., 2006). The observations made by Yeargin et al. (2006) have since been confirmed by investigations into the effects of CWI on high-intensity exercise performance performed on the same day (Vaile et al., 2008a) and multiple days (Vaile et al., 2008b). All CWI protocols (10^{o}C, 15^{o}C and 20^{o}C) maintained the exercise performance during two 30-min cycling bouts (15-min at a workload equal to 75% peak power output followed by a 15-min all-out time trial) separated by one hour compared with a 4.1% reduction with an active recovery (cycling continuously at

40% $\dot{V}O_{2peak}$ for 15-min) (Vaile et al., 2008a). Sprint performance was increased by 2.2% and time trial performance was maintained and slightly improved (range of 0.1 – 1.0%) in CWI (14-min at 15^{o}C), as compared with 2.6 – 3.8% reduction with passive recovery (Vaile et al., 2008b).

In contrast with the result from the previous studies, this reported that CWI to be effective in performing high-intensity running and cycling exercise, the majority of research investigating effects of CWI on high-intensity exercise performance has indicated no beneficial. All-out cycling time-trial performance has shown to be similar between CWI and control conditions (Buchheit et al., 2009, Peiffer et al., 2010, Stacey et al., 2010). In line with the observations, King and Duffield (2009) and Rowsell et al. (2009) also observed post-exercise CWI is not effective in the improvement or maintenance of exercise performance on consecutive days. The use of CWI to aid recovery form high-intensity exercise did not result in any significant improvement in exercise performance (vertical jump, 20m sprint, 10m sprint, total circuit time) during session 2 of the consecutive days of exercise performance (King and Duffield, 2009) and there were no differences between the conditions for countermovement jump height, total repeated-sprint-ability test time and shuttle-run heart rate during a 4-day simulated soccer tournament (Rowsell et al., 2009).

More recently, a meta-analysis by Leeder et al. (2012) examined the effect of CWI following high-intensity exercise on the magnitude of muscle damage and rate of recovery of muscle function. Findings indicated that CWI was highly effective in alleviating perceived ratings of DOMS following high-intensity exercise at 24 h and 48 h. CWI was also effective in reducing post-exercise concentrations of CK. However, the effects of CWI on post-exercise muscle function were inconsistent. CWI improved the rate of recovery of muscle power (vertical jump and 5 m sprint performance) but demonstrated little effect on muscle strength (e.g. isometric/isokinetic knee extension or elbow flexion) (Leeder et al., 2012). The authors suggested that variation in the CWI condition (i.e. water temperature and duration) and the nature of the exercise tasks assessed (i.e. intensity or duration) may partly explain discrepancies in the effects of CWI on strength and muscular power. Furthermore, such discrepancies may to some extent reflect differences in training status (e.g. elite vs. non elite and untrained). In order to negate any possible influence of training status, Poppendieck et al. (2013) analysed the current literature on cold application during recovery with special reference to its effect on performance in trained athletes. The results indicated that large improvements were observed for sprint performance (2.6%), endurance parameters (2.6%),

and jump (3.0%), while improvements of strength (1.8%) were smaller. Collectively, these data suggest that the performance benefits of CWI following high-intensity exercise may to some extent be mediated by the subjects training status, exercise intensity and mode of exercise assessed.

2.2.3. Eccentric exercise

The physiological stress associated with elite-level athletic training and competition result in impairment of athlete's performance. The extent of physiological stress is considerably affected by the specific exercise type, duration, intensity and the athletes' familiarization to the exercise. Excessive physiological stress responses to the exercise can induce muscle damage characterized by a reduction in the capacity to generate force, soreness and muscle tenderness (Mathur et al., 2010). A consequence of exercise-induced muscle damage causes DOMS that first appears within 24 h after the exercise, peaks between 24 and 72 h, and lasts up to 4-7 days post-exercise (Cleak and Eston, 1992, Foure et al., 2015, Lau et al., 2015). A well-established type of exercise that leads to muscle damage is eccentric exercise associated with a large mechanical stress and relatively low metabolic cost (Lastayo et al., 1999). Recently, CWI has been widely used by athletes to improve recovery after competition or training containing eccentric exercise. However, the effect of CWI on enhancing recovery from eccentric biased exercise remains to be elucidated. Vaile et al. (2008c) investigated the efficiency of CWI on recovery from muscle damage eccentric exercise by using seven sets of ten eccentric repetitions on a leg press machine and examining muscle damage markers (squat jump, isometric squat and CK). The results showed that squat jump performance and isometric force recovery were significantly enhanced at 48 and 72 h post-exercise following CWI. Creatine Kinase activity was also shown to significantly reduce at 24 and 72 h post-exercise following CWI when compared to passive recovery. Such findings indicate that CWI is more effective in improving the physiological and functional recovery from eccentric exercise-induced muscle damage than passive recovery.

In contrast, Goodall and Howatson (2008) reported repeated CWI applied following muscle-damaging exercise did not improve recovery. Nine physically active male subjects completed five sets of twenty-drop jumps. Immediately upon completion of the eccentric exercise-induced muscle damage protocol, subjects undertook CWI (up to iliac crest) at 15^{o}C for 12-min, which was subsequently repeated every 24 h thereafter for the following 3 days. Results indicated that MVC, DOMS, thigh circumference and CK were not significantly different

between CWI and control condition at any time points. The observations made by Goodall and Howatson (2008) have since been confirmed by Howatson et al. (2009) during an investigation into the effects of repeated CWI following muscle-damaging exercise on the repeated bout effect. In this study, subjects completed two bouts of an eccentric exercise-induced muscle damage protocol, separated by 14-21 days. The study design and dependent variables were similar to Goodall and Howatson's (2008) study. All variables showed no significant difference between groups following the initial bout or the repeated bout of damaging exercise.

More recently, a systematic meta-analysis review of ten studies has previously examined the effect of CWI on the recovery from muscle damage exercise, induced by eccentric contractions (Leeder et al., 2012). Only outcome variables measured at 24 h and 48 h were used for comparison. Results showed that CWI was not effective in the alleviation of DOMS at 24 h post eccentric exercise, but had a moderate effect at 48 h post eccentric exercise. Whilst a mechanistic explanation for why CWI may be more efficacious following 48 h than 24 h eccentric exercise is presently lacking, it is likely that benefits arising from the application of CWI are limited to alleviating DOMS at 24 h post eccentric exercise since the contribution to the first phase of muscle damage occurring during the exercise bout and secondary damage affect considerably until 24 h post eccentric exercise types.

Several studies have used untrained subjects or recreational athletes to examine effect of CWI on exercise performance. However, the effect of CWI in enhancing recovery from eccentric damaging exercise is equivocal. The reasons for disparate results are not clear, but differences are likely to be related to the repeated bout effect referred to the adaptation whereby a single bout of eccentric exercise protests against muscle damage from subsequent eccentric bouts (McHugh, 2003). It is possible that similar eccentric exercise results in less muscle damage in trained subjects who are more exposed to eccentric exercise while training sessions than untrained subjects. Therefore, the magnitude of effect of CWI on recovery from eccentric exercise-induced muscle damage may be related to subjects' training status. Recently, Poppendieck et al. (2013) analysed 5 studies examining the effects of CWI following eccentric strength training protocol on exercise performance of trained athletes. Results indicated that percentage improvement for eccentric strength training was +2.4%. Such findings suggest that CWI is effective in improving the physiological and functional recovery of trained athletes from eccentric exercise-induced muscle damage.

2.3. PHYSIOLOGICAL MECHANISMS MEDIATING THE EFFECTS OF CWI ON EXERCISE RECOVERY

The application of cooling is a widely used modality for the treatment of exercise-induced muscle damage. In comparison to other recovery strategies, several possible beneficial mechanisms of cooling have been suggested, including reduced post-exercise inflammation, muscle blood flow, swelling, increase in parasympathetic re-activation and perception of recovery.

2.3.1. Thermoregulatory and cardiovascular

A high intramuscular temperature following exercise is reduced by CWI via conductive heat transfer through the muscle, which exists between the raised muscle temperature and the cooler water (Mac Auley, 2001). CWI represents a much more effective cooling medium compared to cold air due to its large conductive heat transfer capacity (Costello et al., 2014). Indeed, many studies have reported that CWI causes marked decreases in skin and both superficial and deep muscle temperature (Gregson et al., 2011, Gregson et al., 2012, Mawhinney et al., 2013). For example, the greatest declines in skin and superficial muscle temperature have previously been observed immediately post-immersion with the greatest declines in deep muscle temperature occurring 30-min after immersion (Gregson et al., 2011, Mawhinney et al., 2013). In contrast with the effects on skin and muscle temperature, the effects of post-exercise CWI on core temperature are inconclusive with some reports demonstrating reduced core temperature with CWI (Yeargin et al., 2006, Peiffer et al., 2009) whilst other have reported no effects (Peiffer et al., 2010, Gregson et al., 2012, Mawhinney et al., 2013). These discrepancies may reflect differences in the assessment of core temperature and experimental procedures (e.g. water temperature, immersion duration and timing of measurements).

Alongside changes in tissue temperature, the physiologic effects of CWI are also thought to be partially mediated through temperature-induced reductions in skeletal muscle blood flow, which in turn reduces edema, secondary hypoxic damage, and the induction of inflammatory events (Thorlacius et al., 1998, Merrick et al., 1999, Lee et al., 2005). Recent studies have evaluated the effects of CWI on limb blood flow both at rest (Gregson et al., 2011) and following exercise (Vaile et al., 2011, Mawhinney et al., 2013). In a study by Gregson et al. (2011), nine men undertook CWI for a total duration 10-min (2 × 5-min, with a 2-min

window in between) in 8°C and 22°C water respectively at rest. Thigh cutaneous blood flow (laser Doppler), and superficial femoral artery blood flow (duplex ultrasound) were measured during immersion and for 30-min after immersion. Both femoral artery blood flow and conductance were reduced immediately after immersion (~30%) and remained below baseline (~35% - ~40%) throughout the 30-min recovery period under both conditions. Similarly cutaneous blood flow and conductance were also reduced during and after the immersion period under both conditions, however, a higher cutaneous blood flow was observed under the 8°C condition compared to the 22°C condition, despite lower skin temperatures at 8°C. The authors suggested that CWI (8°C) likely activated skin thermonociceptors, which subsequently led to a reflex increase in sympathetic nerve activity and consequently reduced arterial flow (Lovallo, 1975). In contrast, cooling in 22°C water may reduce limb blood flow through nonnoxious thermoreceptors know to be operable within the skin temperatures observed in the study (Hensel and Boman, 1960). The higher cutaneous blood flower during colder water immersion may be explained via cold-induced vasodilation observed at lower temperatures (Pergola et al., 1993, Sendowski et al., 1997) and a direct inhibitory effect of local cooling on the normal vasoconstrictor response (Faber, 1988). These data suggested that colder temperatures may be associated with greater reductions in muscle blood flow at rest.

The mechanisms mediating the effects of CWI on blood flow involve both neural (initial) and non-neural (prolonged) vasoconstriction (Kellogg, 2006). Cold exposure stimulates cold sensitive afferent nerves through cold receptors located in skin (Morrison et al., 2012). These signals are received by both the hypothalamic thermostat that activates efferent sympathetic nerve signals travelling to sympathetic axon terminals, stimulating the release of norepinephrine (Thompson-Torgerson et al., 2008). Norepinephrine then vasoconstricts blood vessels through α- and β-receptors (Kellogg, 2006). Prolonged cold exposure causes maintenance vasoconstriction which is mainly dependent on non-adrenergic and non-neural mechanisms including the Rho-kinase pathway and removal of nitric oxide (NO) (Thompson-Torgerson et al., 2008, Kingma et al., 2010). Cooling-induced increase in superoxide (O_2^-) activates RhoA and Rho kinase, which can stimulate vasoconstriction through two distinct pathways: 1) translocation of α_{2c}-adrenoceptors from intracellular storage to the cell membrane, joining α_{2A}- and α_1-adrenoceptors to bind norepinephrine, which leads to increased intracellular Ca^{2+} and Ca^{2+}-dependent vasoconstriction through phosphorylation myosin light chain (MLC) by myosin light chain kinase (MLCK) and 2) inhibition of myosin

light chain phosphatise (MLCP) permits extant MLC phosphorylation to remain, thereby stimulating vasoconstriction in the absence of an increase in intracellular Ca^{2+} (Thompson-Torgerson et al., 2008).

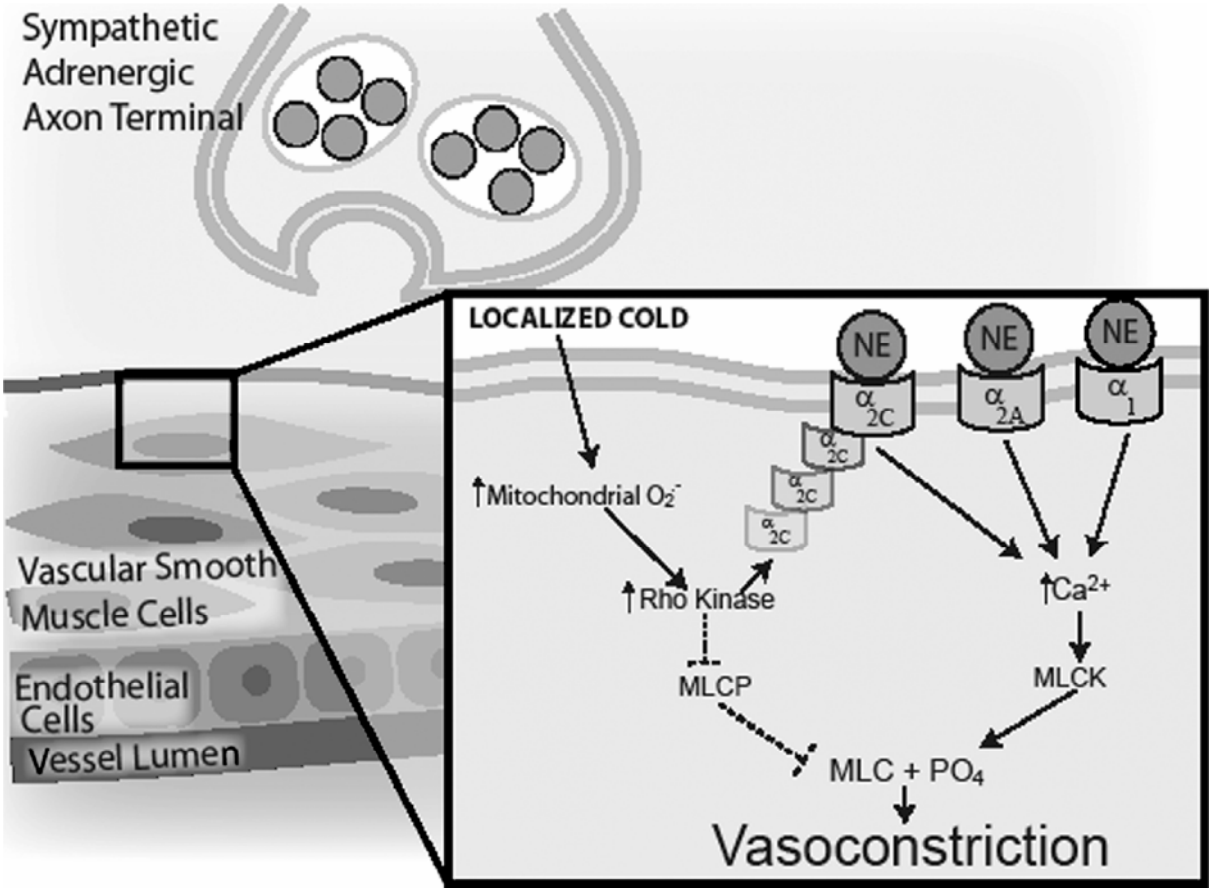

Figure 2.3.1. The role of Rho-kinase in cold-induced vasoconstriction (Thompson-Torgerson et al., 2008). α_1 α_1-adrenoceptors, α_{2A} α_{2A}- adrenoceptors, α_{2c} α_{2c}-adrenoceptors, Ca^{2+} calcium, *MLC* myosin light chain, *MLCK* myosin light chain kinase, *MLCP* myosin light chain phosphatise, O_2^- superoxide, PO_4 phosphate.

The effects of CWI on muscle blood flow following exercise may be different from those observed at rest. Vaile et al. (2011) were the first to evaluate that the effect of CWI on whole limb blood flow following exercise. Ten subjects completed an all-out 35-min cycling exercise followed by 15-min recovery interventions (CWI or active recovery). Whole limb blood flow was reduced after CWI compared with active recovery. However, the use of venous occlusion plethysmography does not distinguish between muscle and skin blood flows. In order to address such limitations, Mawhinney and colleagues (2013) examined the influence of CWI on femoral artery (duplex ultrasound) and cutaneous (laser Doppler

flowmetry) blood flows following exercise. Twelve subjects cycled at 70% peak power output until a core temperature of 38^{o}C was attained, followed by 10-min of immersion at 8^{o}C or 22^{o}C water or rest (Cont) in a randomized order. Both femoral artery and cutaneous blood flows were reduced throughout the recovery period in the cooling conditions compared to Cont, however, there was no difference between cooling conditions. The changes in cutaneous blood flow contrast with the previous study (Gregson et al., 2011), where resting cold-induced vasodilation led to higher cutaneous blood flows in 8^{o}C water compared with 22^{o}C. The authors suggested that differences in the cutaneous responses to marked cooling after exercise compared with rest may be related to the exercise-induced increase in body and local limb temperatures, which attenuate cutaneous vasoconstrictor responsiveness (Wolf and Hardy, 1941). As a result, the increase in body temperature prior to immersion may reduce the degree of vasoconstriction and prevent skin blood flow reaching low levels and the associated onset of cold-induced vasodilation, leading to sustained vasoconstriction in both 8^{o}C and 22^{o}C conditions. These finding indicate that the application of 8^{o}C and 22^{o}C water following exercise may reduce muscle blood flow to a similar extent and provide important insights into the mechanisms by which post-exercise CWI could alleviate exercise-induced muscle damage.

The magnitude of CWI-induced reduction in blood flow may be depend on both the size of the tissue area exposed to cold water and a compressive force is exerted on the body called hydrostatic pressure (Wilcock et al., 2006). The extent of tissue cooling from CWI is heavily determined by contact area due to the rate of heat transfer (Mac Auley, 2001) and decreased temperature serves to reduce blood flow through activation of cutaneous thermoreceptors (Wilson et al., 2007). Hydrostatic pressure will cause an upward and inward displacement of body fluid, aiding alterations in fluid with the intra-cellular, interstitial and intra-vascular spaces (Wilcock et al., 2006). A recent study by White and Wells (2013) examined the changes of body temperature and blood flow following partial body CWI and whole body CWI. Changes in muscle and rectal temperature and intramuscular blood flow were greater in the whole body CWI than in the partial body CWI. The reason for the difference of blood flow might be associated with hydrostatic pressure and a vasoconstriction mechanism through the release of norepinephrine. Indeed, higher plasma noreprinephrine concentrations were observed with greater reduction of body temperature in whole body cryostimulation compared to partial body cryostimulation (Hausswirth et al., 2013).

2.3.2. Metabolic and inflammatory

Exercise, especially if strenuous and including eccentric muscle contractions, leads to local muscle damage, which induces the release of various substances, such as intracellular proteins, cytokines and chemokines, ultimately resulting in an inflammatory response (Shek and Shephard, 1998). Restricting acute inflammation following muscle use has previously been seen to have a positive effect on muscle repair and adaptation (Tidball, 2005). Cryotherapy are thought to be as an effective treatment for alleviating exercise-induced muscle damage since decreasing local tissue temperature and temperature-induced reductions in blood flow are thought to diminish metabolism, pain, local edema, swelling and inflammation in injured tissue (Merrick et al., 1999). Whole-body cryotherapy (WBC) and CWI may therefore be an effective treatment modality by virtue of its effect on reducing the inflammation.

Pournet and colleagues (2011) have confirmed that WBC was effective in reducing the inflammatory process. Endurance trained male (n=11) completed a 48-min running treadmill exercise followed by passive recovery (PAS) or WBC consisting of three rooms (-10, -60 and -110^{o}C) after and at 24 h, 48 h and 72 h following exercise. During each WBC session, subjects traversed the warmer rooms and remained in the therapy room for 3-min. Interleukin 1 beta (IL-1β) and C-Reactive Protein (CRP) levels decreased and interleukin 1 receptor alpha (IL-1rα) increased following WBC when compared to PAS. In addition, Merrick et al. (1999) proposed another mechanism that the reduction in muscle tissue temperature following cryotherapy limits secondary hypoxic injury at the cellular level. The findings from such studies indicate that cryotherapy leads to decrease in cytokines activity pro-inflammatory, increase in cytokines anti-inflammatory, and reduction in the magnitude of secondary injury, ultimately resulting in reducing musculoskeletal injuries. However, a systematic review of randomised controlled trials found limited evidence that various CWI after exercise positively affect the inflammatory biomarkers (Bleakley et al., 2012). For example, recent work has demonstrated that there were no significant differences in interleukin-6 levels at various follow-ups between CWI (12^{o}C) and warm water immersion (24^{o}C) after exercise in the heat for 90-min or until volitional cessation (Lee et al., 2012).

Along with changes in the inflammatory responses to cryotherapy, accompanying immunological changes occurring may occur during cold adaptation. Jansky et al. (1996) studied the effects of CWI on the immune system. Ten subjects performed immersion in 14^{o}C

water for 1 h. CWI induced an improvement in activity of the immune system including lucocytosis. Similarly, Brenner et al. (1999) observed that exercise for 1 h at 55% of $\dot{V}O_{2peak}$ in water ($18^{o}C$) followed by cold air exposure for 2 h ($5^{o}C$) augmented the leukocyte, granulocyte, and monocyte response. However, these findings are in contrast with the result from the previous study reported that post-exercise CWI for ~20-min ($14^{o}C$) did not influence blood leukocyte counts, cytokine concentrations and neutrophil activation (Peake et al., 2008). The reasons for disparate results are likely to be related to the period of cold exposure. This is supported by Dugue and Leppanen (2000) who examined the effects of chronic whole body ice-cold water immersion on cytokines. At rest higher concentrations of plasma interleukin-6, leukocytes, and monocytes were observed in regular winter swimmers compared to inexperienced subjects. These findings suggest that CWI leads to a reduction in inflammation responses and enhancement of the immune system, which contribute to the muscle repair and adaptation from muscle damage.

2.4. MOLECULAR MECHANISMS REGULATING ADAPTATION TO ENDURANCE EXERCISE

Human skeletal muscle is known to adapt to a diverse array of contractile stimuli, such as the prolonged low frequency stimulation experienced during endurance exercise. Several major signalling pathways important for chronic training adaptation arise during the first few hours of recovery, returning to baseline values within 24 h after exercise (Hildebrandt et al., 2003). CWI is being used, therefore possible implications for mediating changes in adaptation.

2.4.1. Skeletal muscle mitochondrial biogenesis

Since the publication of Holoszy's (1967) pioneering work demonstrating that exercise training effectively stimulates mitochondrial oxygen uptake and respiratory enzyme activity in animal skeletal muscle, a number of studies (Tonkonogi et al., 2000, Nielsen et al., 2010) have also investigated the mitochondrial content and function in response to several weeks of exercise training in human muscle. Tonkonogi et al. (2000) observed four endurance-training sessions per week over a period of 6 weeks increased mitochondria content and activity of mitochondrial enzymes. Similarly, Nielsen et al. (2010) observed 10 weeks (five times per week) of aerobic cycling training increased the content of mitochondria (~40%). Considerable work over the last decade has shown that PGC-1α may coordinate the various processes which lead to exercise-induced mitochondrial adaptation and thus serve as the "master regulator of mitochondrial biogenesis" (Puigserver and Spiegelman, 2003). Lin et al. (2004) observed that mitochondrial function and mRNA levels of many mitochondrial genes are reduced in PGC-1α-deficient mice. This is supported by observations that mitochondrial number and respiratory capacity is diminished in PGC-1α-deficient mice, leading to reduced muscle performance and exercise capacity (Leone et al., 2005). These findings strongly suggest that PGC-1α is essential for expression of a large number of mitochondrial genes to meet the energy demands of exercise.

PGC-1α coactivates a number of transcription factors including peroxisome proliferator-activated receptors (PPARs), nuclear respiratory factors (NRF's), oestrogen-related receptor (ERR) and transcription factor A mitochondria (Tfam) (Olesen et al., 2010) and is mainly expressed in tissues with high energy oxidative capacity such as the heart, skeletal muscle, liver, brown adipose tissue and brain (Puigserver et al., 1998, Mootha et al., 2004). Skeletal muscle is a major tissue involved in energy expenditure response to exercise in vivo. A number of studies have investigated the PGC-1α response to acute bouts of endurance exercise using cycling (Mathai et al., 2008, Leick et al., 2010) and treadmill protocols

(Bartlett et al., 2012) in human skeletal muscle. Mathai et al. (2008) observed exhaustive cycling exercise at 65% $\dot{V}O_{2max}$ increased PGC-1α mRNA in seven untrained subjects ($\dot{V}O_{2max}$ of 48.4mL.kg^{-1}.min^{-1}) at immediately post-exercise (3-fold) and 2 h later (6.2-fold), before returning to resting levels by 24 h. PGC-1α protein increased 23% at exhaustion and remained elevated for at least 24 h. More recently, Leick et al. (2010) also observed an approximate 2.5-fold increase in PGC-1α mRNA in the vastus lateralis of 8 untrained male subjects ($\dot{V}O_{2max}$ of 46.5mL.kg^{-1}.min^{-1}) 18 h after a 90-min exercise bout at 70% $\dot{V}O_{2max}$. Unfortunately PGC-1α protein levels were not measured due to no PGC-1α specific band being detected in muscle lysates by western blotting. Consistent with previous studies, Bartlett et al. (2012) observed 50-min of treadmill exercise at 70% $\dot{V}O_{2max}$ increased PGC-1α mRNA by 4-fold at 3 h following execise in ten active male subjects ($\dot{V}O_{2max}$ of 52mL.kg^{-1}.min^{-1}). However, PGC-1α protein was unchanged compared with rest. It is possible that the reasons for the different PGC-1α protein results between studies could be related to subject training status rather then exercise protocols.

Several weeks of endurance running and cycle training increase PGC-1α mRNA and PGC-1α protein in human skeletal muscle with greater mitochondrial enzyme activities (Russell et al., 2003, Burgomaster et al., 2008). The time course of increase in PGC-1α mRNA and PGC-1α protein during exercise training has been most well documented by Perry et al. (2010). PGC-1α mRNA increased ~10-fold 4 h after the 1st high-intensity training session and returned to rest levels by 24 h. This 'saw-tooth' pattern continued until the 7th bout. However, the magnitude of the 4 h increase after each training session was progressively decreased, resulting in a 'stair-case' type response over seven training sessions. Increased PGC-1α protein expression (23%) was observed 24 h after 1st session. Moreover, unlike the reduced PGC-1α mRNA responses over seven training sessions, PGC-1α protein expression continued to increase, attaining a plateau by the 5-7th training session. These findings suggest that PGC-1α protein expression occurred later compared to PGC-1α mRNA.

There are several mechanisms that lead to the increased expression and activation of PGC-1α response to endurance exercise (Figure 2.4.1.). Two metabolic sensors, AMPK and SIRT1 (Silent Information Regulator T1) play a major role in PGC-1α activation through phosphorylation and deacetylation, respectively (Canto and Auwerx, 2009). Exercise-induced increases in calcium can improve the activities of both calcineurin and calmodulin-dependent kinase (CamK) and appears to increase PGC-1α expression (Wu et al., 2002). Other important signalling pathway that increase PGC-1α expression is p38MAPK which has been identified as a kinase that is activated by exercise (Fan et al., 2004, Little et al., 2010). Other

exercise-induced signals implicated in PGC-1α transcription include the accumulation of reactive oxygen species (ROS) (Silveira et al., 2006). In addition, PGC-1α regulates the expression of itself via an auto-regulatory loop with myocyte enhancer factor 2 (MEF2) (Handschin and Spiegelman, 2008).

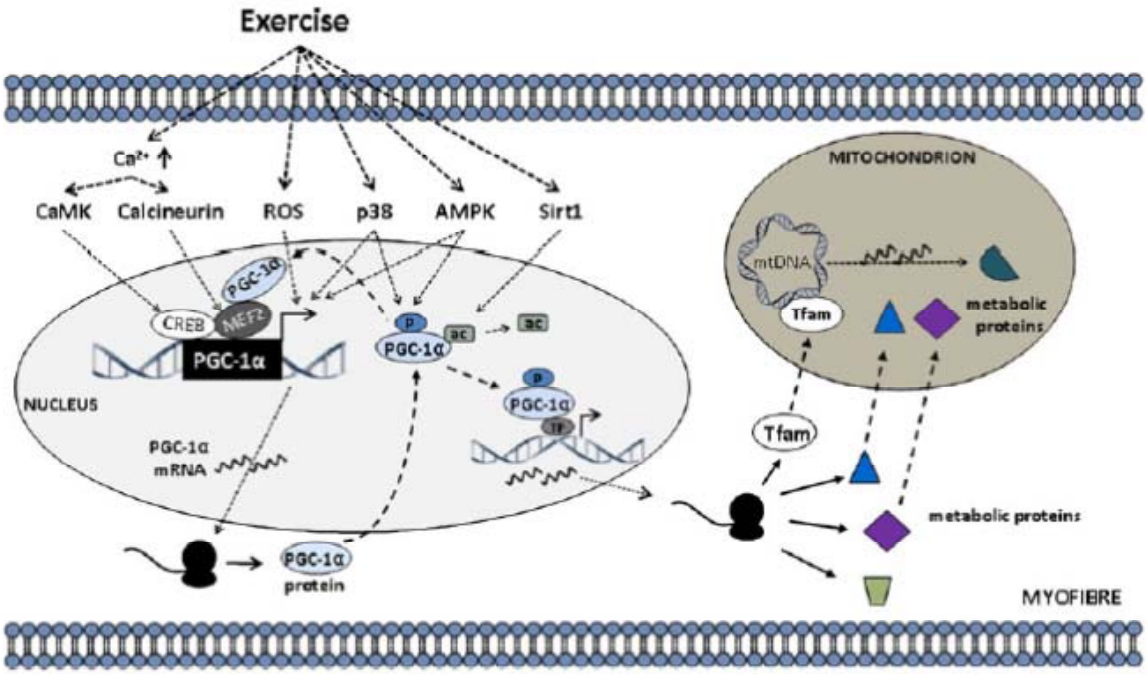

Figure 2.4.1. Exercise-induced signals involved in altering PGC-1α activity and expression (Olesen et al., 2010). *ac* actylation, *AMPK* 5'-AMP-activated protein kinase, *CamK* Ca^{2+}/calmodulin-dependent protein kinase, *CREB* cAMP response element binding, *MEF2* myocyte enhancing factor 2, *mtDNA* mitochondrial DNA, *P* phosphorylation, *p38* p38 mitogen-activated protein kinase, *ROS* reactive oxygen species, *Sirt1* sirtuin 1, *TF* transcription factor, *Tfam* transcription factor A mitochondria.

2.4.2. Skeletal muscle angiogenesis

In addition to the increase in mitochondria biogenesis, angiogenesis, which is the process of formation of new capillaries in parallel with the existing capillaries, is a well-documented adaptive response in skeletal muscle to endurance exercise (Prior et al., 2003). Exercise-induced angiogenesis can be measured as an increase in the number of capillaries surrounding fibres, the number of capillaries per unit area and the capillary-per-fibre ratio (Coggan et al., 1992). Angiogenesis is a very complex process, which is a mediated balance between angiogenic and angiostatic factors. A key role in angiogenesis is played by direct-acting angiogenic protein VEGF. VEGF is expressed in five different splice variants; $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{201}$ (Houck et al., 1991). VEGF family

stimulates cellular responses by binding to VEGF receptor 1 (Flt-1), -2 (KDR/Flk-1) and -3 (Flt-4) on the cell surface (Mustonen and Alitalo, 1995).

Morrow et al. (1990) were the first to report that motor nerve stimulation and exercising skeletal muscle increased angiogenic mediators in animals models. It is now well documented that acute exercise is accepted as a valid method by which to induce VEGF expression in animal (Breen et al., 1996, Gavin et al., 2000) and human (Gustafsson et al., 1999, Richardson et al., 1999, Gavin et al., 2004). Gustafsson et al. (1999) were the first authors to provide data regarding the response of VEGF at mRNA level to exercise in human skeletal muscle. Fifteen male subjects performed dynamic constant-load knee-extension exercise (45-min, 60 rpm). VEGF mRNA in the vastus lateralis was determined pre-exercise (1 week before) and 30-min after the exercise. Exercise increased VEGF mRNA expression by 178%. This is consistent with a report demonstrating VEGF mRNA was increased by 16-fold at 1 h after a single acute exercise bout (Richardson et al., 1999). Gavin et al. (2004) also observed that 1 h of acute cycling exercise at 50% of $\dot{V}O_{2max}$ increased VEGF mRNA by approximately 10-fold immediately after exercise. The magnitude of the change of VEGF mRNA response to acute exercise is likely to be related to the subjects' training status. This is supported by observations indicating that the significant and substantial increase in VEGF mRNA in untrained human skeletal muscle (17-fold) after acute exercise is attenuated in trained human skeletal muscle (5-fold) (Richardson et al., 2000).

The lack of data concerning VEGF protein response of human skeletal muscle to acute exercise protocols has been described. Gavin et al. (2004) were the first to report data regarding VEGF protein response following acute exercise in human skeletal muscle. In contrast to the VEGF mRNA response, 1 h exercise at 50% of $\dot{V}O_{2max}$ decreased VEGF protein immediately after exercise and VEGF protein remained below baseline value through 4 h post-exercise. This result is consistent with findings that acute exercise-induced no change in VEGF protein at 2 h post-exercise in human skeletal muscle (Gavin et al., 2005). However, the effect of chronic exercise on VEGF protein was different from those observed at acute exercise. Gustaffon et al. (2001) were the first to report an increase in VEGF at the protein level (92%) in response to knee extension exercise training. More recently, Gavin et al. (2007b) examined that difference in VEGF protein response to aerobic exercise training between young and aged men. Eight aged (mean age: 64 years) and six young (mean age: 25 years) male subjects completed 8 weeks of an aerobic exercise training. Increase in interstitial VEGF protein observed in both age groups at 1 week and 8 weeks after training. These findings suggest that several weeks of exercise training induce angiogenesis and angiogenic response in humans.

Several factors, including cytokines, growth factors, and tumour suppressor factors, seem to be associated with the regulation of VEGF production (Klagsbrun and D'Amore, 1996, Neufeld et al., 1999). Exercise-induced PGC-1α is one of the most important regulators for VEGF expression through the oestrogen-related receptor-α (ERR-α) pathway (Arany et al., 2008). In addition, a well-described candidate signal for exercise-induced VEGF production is hypoxia (Bloor, 2005). Hypoxia markedly increases a transcription factor that is termed 'hypoxia-induced factor-1' (HIF-1) that stimulates transcription of the VEGF gene (Shweiki et al., 1992, Forsythe et al., 1996). Other mechanical signals that may regulate VEGF are shear stress and wall tension within capillaries. Indeed, shear stress-dependent increase in release of NO represents an important element for the regulation of VEGF expression (Yang et al., 2008). Ouchi et al. (2005) observed AMPK and p38MAPK simulate VEGF expression in skeletal muscle. Evidence also indicates that the deacylator Sirt 1 and the transcription factors activator protein 1 (AP-1) are important for VEGF expression (Chang et al., 2006, Hoier et al., 2013). These data suggest that AMPK, p38MAPK and Sirt 1 are not only associated with the regulation of mitochondrial biogenesis but that they play an important role in the increased expression of VEGF response to exercise.

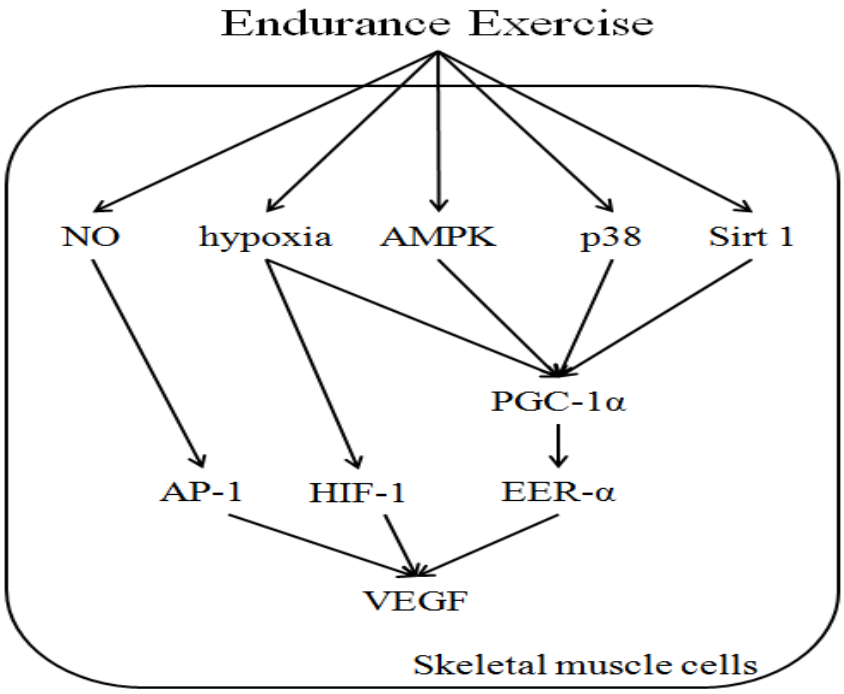

Figure 2.4.2. Exercise-induced signals involved in altering VEGF expression. *AMPK* 5'-AMP-activated protein kinase, *Ap-1* activator protein 1, *ERR-α* estrogen-related receptor-α, *HIF-1* hypoxia-inducible factor, *NO* nitric oxide, *PGC-1α* peroxisome proliferator-activated receptor gamma co-activator-1 alpha, *p38* p38 mitogen-activated protein kinase, *Sirt1* sirtuin 1, *VEGF* vascular endothelial growth factor.

2.4.3. Inflammatory response

There is evidence that acute, unaccustomed exercise, especially if done at high-intensity and for prolonged durations, can cause muscle and connective tissue damage. This exercise-induced damage induces a stereotypic inflammatory response in which neutrophils rapidly invade, followed by macrophages (Shek and Shephard, 1998). Activation of both neutrophils and macrophages increase accumulation of free radicals within the phagosomes and extracellular fluid of the injured muscle, which elicit the secretion of pro-inflammatory cytokines (Shephard, 2001). These factors then activate signalling pathways including various protein kinases, phosphatises, and deacetylases, which in turn regulate molecular machinery controlling gene expression (Peake et al., 2015). Through these signalling pathway, acute inflammation following muscle use has previously been seen to have a positive effect on muscle repair and adaptation in skeletal muscle (Tidball, 2005).

Mitochondrial biogenesis can be induced by exercise-induced acute inflammatory cell stress. The innate immune response is activated through the recognition of danger-associated molecular patterns (DAMPs) composed of microbial antigens (pathogen-associated molecular patterns) and intrinsic factors released into the circulation (alarmins) (Oppenheim and Yang, 2005). Activated cellular pattern recognition receptors by these factors increase the release of early-phase inflammatory protein mediators (tumor necrosis factor-α (TNF-α), interleukins, interferon gamma (IFN-γ), etc.), which activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), protein kinase B (PKB/Akt) or MAPK pathways (Cherry and Piantadosi, 2015). Translocation of NF-κB subunits to the nucleus after myeloid differentiation primary response protein (MYD88)-dependent or –independent phosphorylation of the NF-κB inhibitor (IκB) promotes upregulation of PGC-1α, NRF-1, NRF-2 and other components of the mitochondrial biogenesis in both CREB-dependent (Suliman et al., 2010, Sweeney et al., 2010) and CREB-independent pathways (Sweeney et al., 2011). Activated nitric oxide synthase 2 (NOS2) expression by TNF-α, IL-1β, IFN-γ and platelet-activation factor (PAF) upregulates NO, which increase mitochondria biogenesis via PGC-1α (Cherry and Piantadosi, 2015). Another group of pathwas for mitochondira biogenesis commonly associated with inflammatory factors are mitogen-activated protein kinases (MAPKs) including extracellular signal-related kinases (ERK), p38 and c-Jun NH2-terminal kinases (JNK) and PKB/Akt (Cherry and Piantadosi, 2015).

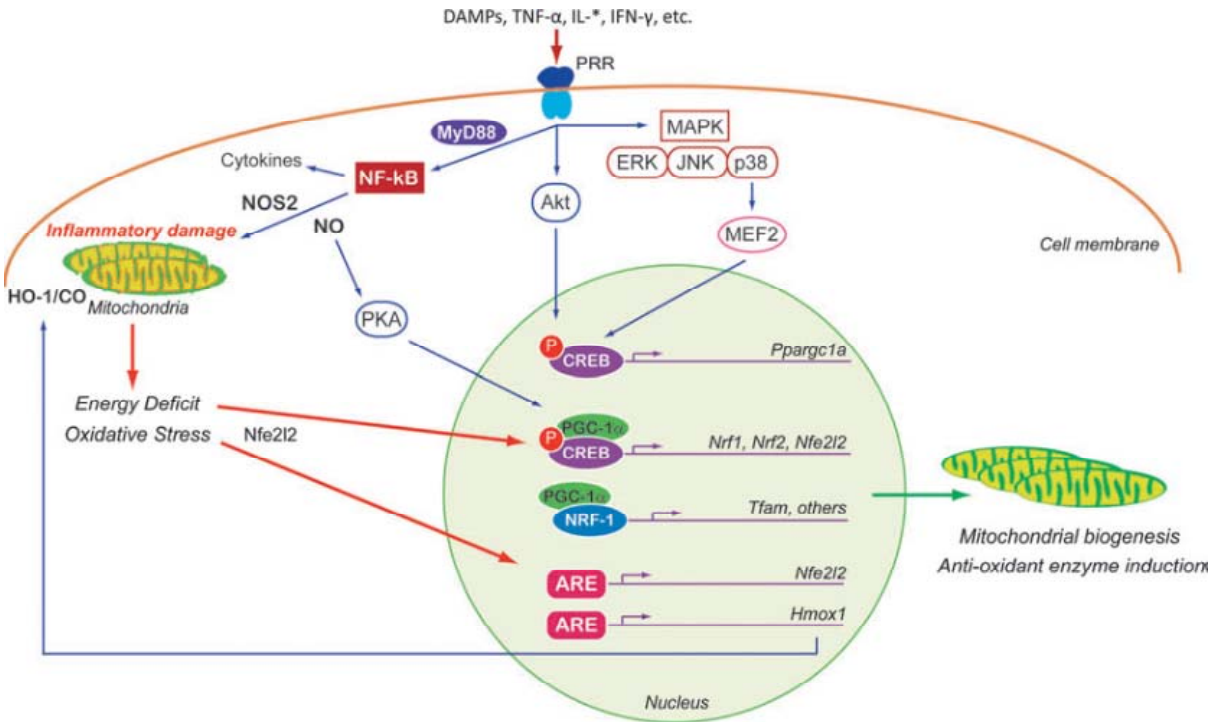

Figure 2.4.3. The pathways regulating mitochondrial biogenesis in response to inflammatory stimuli (Cherry and Piantadosi, 2015). *Akt* protein kinase B, *ARE* antioxidant response element, *CO* carbon monoxide, *CREB* cAMP response element-binding protein, *ERK* extracellular signal-related kinases, *HO-1* or *Hmox1* heme oxygenase-1, *JNK* c-Jun NH2-terminal kinases, *IL* interleukin, IFN-γ interferon gamma, *MAPK* mitogen-activated protein kinase, *MEF2* myocyte enhancer factor-2, *MyD88* myeloid differentiation primary response protein, *NF-κB* nuclear factor kappa-light-chain-enhancer of activated B cells, *Nfe2l2* nuclear factor erythroid 2-related factor 2, *PKA* protein kinase A, *NOS2* or *iNOS* nitric oxide synthase 2, *NRF* or *Nrf* nuclear respiratory factors, *PGC-1* or *Ppargc1* peroxisome proliferator-activated receptor gamma co-activator-1 alpha, *TNF-α* tumor necrosis factor-α.

2.5. INFLUENCE OF POST-EXERCISE CWI ON EXERCISE-INDUCED MITOCHONDRIAL BIOGENESIS AND ANGIOGENESIS

The numerous improvements in exercise performance are closely associated with physiological and functional adaptations to exercise training. Chronic exercise training with post-exercise CWI may further influence training adaptation in exercise performance than exercise alone. In addition to muscle adaptation response to exercise, cold stress associated with it may also have implications for cellular and molecular pathways mediating exercise adaptation.

2.5.1. Mitochondrial biogenesis

When mammals are exposed to cold, they induce physiological responses to maintain their body temperature. Adaptive thermogenesis is involved in a wide variety of biological responses. Skeletal muscle seems to be the major organ for adaptive thermogenesis. The adaptive thermogenic program in skeletal muscle involves the stimulation of mitochondria biogenesis (Liang and Ward, 2006). PGC-1α was originally found as a cold-inducible transcription coactivator of the adaptive thermogenesis response to environmental conditions such as cold exposure (Cannon et al., 1998). The importance of PGC-1α as a key transducer of physiological stimuli to the control of thermogenesis was studied by Leone et al. (2005) using PGC-1α null mice. The PGC-1α null and PGC-1$\alpha^{+/+}$ control mice were exposed at 4^{o}C for a 5 h period. Results indicated that the mean decline in core temperature was greater that 12^{o}C at the 5 h time point in PGC-1α null mice, compared to an approximately 3^{o}C decrement in PGC-1$\alpha^{+/+}$ controls. These findings suggested that PGC-1α serves a critical role in the thermogenesis.

Various investigators have confirmed that adaptive thermogenesis is likely to cause mitochondria biogenesis (Wu et al., 1999, Oliveira et al., 2004). Oliveira et al. (2004) compared the PGC-1α responses to cold exposure (4^{o}C for 4 days) and thermoneutrality exposure (23^{o}C; Cont). The results indicated that cold exposure promoted a significant increase of PGC-1α mRNA in skeletal muscle. In contrast, Seebacher and Glanvile (2010) observed that cold exposure (12^{o}C for 30 days) did not change any of mitochondrial transcription regulators including PGC-1α. These findings suggest that PGC-1α responses to cold exposure are dependent on degree of cold exposure.

As mentioned in section 2.4.1., mitochondria biogenesis in skeletal muscle occurs in response to endurance exercise. Exercise in cold exposure likely to cause further increases in mitochondrial transcription regulators than cold exposure alone. Seebacher and Glanville (2010) examined the effect of exercise in cold environment on expression of mitochondrial transcription regulators and mitochondrial enzyme activities in skeletal muscle. Rats were randomly allocated to either a cold sedentary (12^{o}C) for 30 days or exercise treatment. Rats in the exercise treatment were run in a wheel for 30-min/day on five days/week for 30 days at 60% of maximal performance. Exercise treatment induced increases in mRNA concentrations of PGC-1α, NRF-1 and PPARδ and mitochondrial enzyme activities (COX, CS and LDH), even though sedentary animals cold acclimation alone did not change any of the variables.

In human model, Slivka et al. (2012) examined the mRNA response to exercise in different environmental temperatures. Subjects completed 3 trials consisting of 1 h cycling exercise at 60% W_{max} followed by 3 h recovery in the cold (7^{o}C), room temperature (20^{o}C) and hot (33^{o}C) environments. PGC-1α mRNA was higher after 3 h post-exercise in cold trail (~8.5-fold) than the room temperature trial (~6.4-fold) or the hot trial (~4.8-fold). This study provides details regarding effect of exercise in different environmental conditions on response of PGC-1α mRNA. It was suggested that the observed increase in PGC-1α mRNA was primarily dependent upon the presence of an exercise stimulus in cold environment. Impact of recovery in different environment conditions on cellular response was unknown. Slivka et al. (2013) looked at the effect of post-exercise recovery in a cold environment on PGC-1α mRNA. Eight recreationally active male completed recovery in either 7^{o}C (cold) or 20^{o}C (room temperature) for 4 h. Recovery was performed following cycling exercise at 65% of the workload associated with $\dot{V}O_{2max}$ for 1 h at room temperature (20^{o}C). Results indicated that PGC-1α mRNA was higher after the cold trial than room temperature trial (~7.9-fold vs. ~4.0-fold, respectively). More recently, Ihsan et al. (2014) observed CWI (one leg in 10^{o}C water for 15-min) following a single bout of high-intensity running (30-min continuous running at 70% $\dot{V}O_{2max}$ followed by intermittent running at 100% $\dot{V}O_{2max}$ until exhaustion) significantly increased PGC-1α mRNA expression (~6-fold) at 3 h post-exercise compared with the control leg (~1.5-fold). However, the exercise protocol did not elicit increase in PGC-1α mRNA, consequently it remains to be established whether post-exercise CWI promotes further increase in exercise-induced expression of PGC-1α. The authors suggested that the increased PGC-1α mRNA under cooler conditions likely resulted from specific

mechanisms associated with a temperature mediated non-shivering (adaptive) thermogenesis, shivering thermogenesis and oxidative stress signalling pathways.

To gain insight into the potential adaptive thermogenesis mechanisms that might regulate this response, Puigserver et al. (1998) observed that cold stimuli increases in PGC-1α involves the sympathetic nervous system through the β-adrenergic receptor (β-AR). Indeed, recent findings have shown that β-adrenergic receptor gene expression was higher in response to cold exposure (Tabbi-Anneni et al., 2010). In particular β_3-AR is likely to play a major role in adaptive thermogenesis (Zhao et al., 1998). This has been best documented by Boss et al. (1999). Acute cold exposure (4^{o}C) for 3 h induced no change in PGC-1α mRNA in skeletal muscle of both wild type and β_3-AR null mice. However, an increase of 100% in PGC-1α mRNA was observed in β_3-AR agonist (BRL 37344) injected wild type. None of the β_3-AR agonist affected significantly PGC-1α mRNA expression in β_3-AR null mice. Activation of the β_3-AR result in enhanced cAMP signalling and the activation PKA (Fernandez-Marcos and Auwerx, 2011). The activation of β_3-AR and cAMP/PKA also stimulates p38MAPK, subsequently inducing PGC-1α transcription through activation transcription factor 2 (ATF2) (Cao et al., 2004). One possible mechanism by which cold-induced increase in PGC-1α is through shivering thermogenesis. Shivering is one method to rise body temperature after cold exposure. Shivering characterised involuntary repeated muscle contraction produces heat through the inefficiency of adenosine triphosphate (ATP) utilization (Jubrias et al., 2008). A consequence of energy expenditure to generate heat through repeated muscle contraction may enhance free cytosolic Ca^{2+} and adenosine monophosphate (AMP) level, which may increase PGC-1α via CamK and AMPK pathways (Bruton et al., 2010, Slivka et al., 2012). Cold exposure may increase the production of ROS via shivering and non-shivering thermogenesis (Wenz, 2013). An increase in metabolism is required by shivering after exposure to cold temperature to sustain core body temperature, which may increase the production of ROS (Selman et al., 2002). ROS are also produced and accumulated as a byproduct of rapid substrate oxidation with a low rate of ATP production during non-shivering thermogenesis (Bleakley and Davison, 2010). Cold-induced ROS is important stimuli for the signalling pathway for mitochondrial biogenesis (Droge, 2002) mediated by PGC-1α through p38MAPK (Kulisz et al., 2002) and/or PKB/Akt (Beeson et al., 2012).

More recently, an additional potential mechanism for increases in PGC-1α response to cold stimuli could be reduced blood flow. Norrbom et al. (2004) examined effect of restriction of

blood flow on PGC-1α expression human skeletal muscle. Nine male subjects completed cycling exercise under restricted blood flow condition. A greater exercise-induced PGC-1α mRNA increase observed in the restricted blood flow condition (8-fold) compared with the nonrestricted condition (3-fold) 2 h after exercise and the levels remained elevated 6 h after exercise. The authors suggested that the increase of the PGC-1α with restricted blood flow is associated with CamK and AMPK since enhanced the metabolic perturbation by restriction of blood flow requires a higher degree of muscle activation and higher intracellular calcium level. As previously mentioned in section 2.3.1., resting CWI and post-exercise CWI reduce blood flow and the reduced blood flow remained throughout the 30-min after immersion (Gregson et al., 2011, Mawhinney et al., 2013). These findings suggest that CWI-induced reduction of blood flow can stimulate increases in PGC-1α via CamK and AMPK signalling in human skeletal muscle.

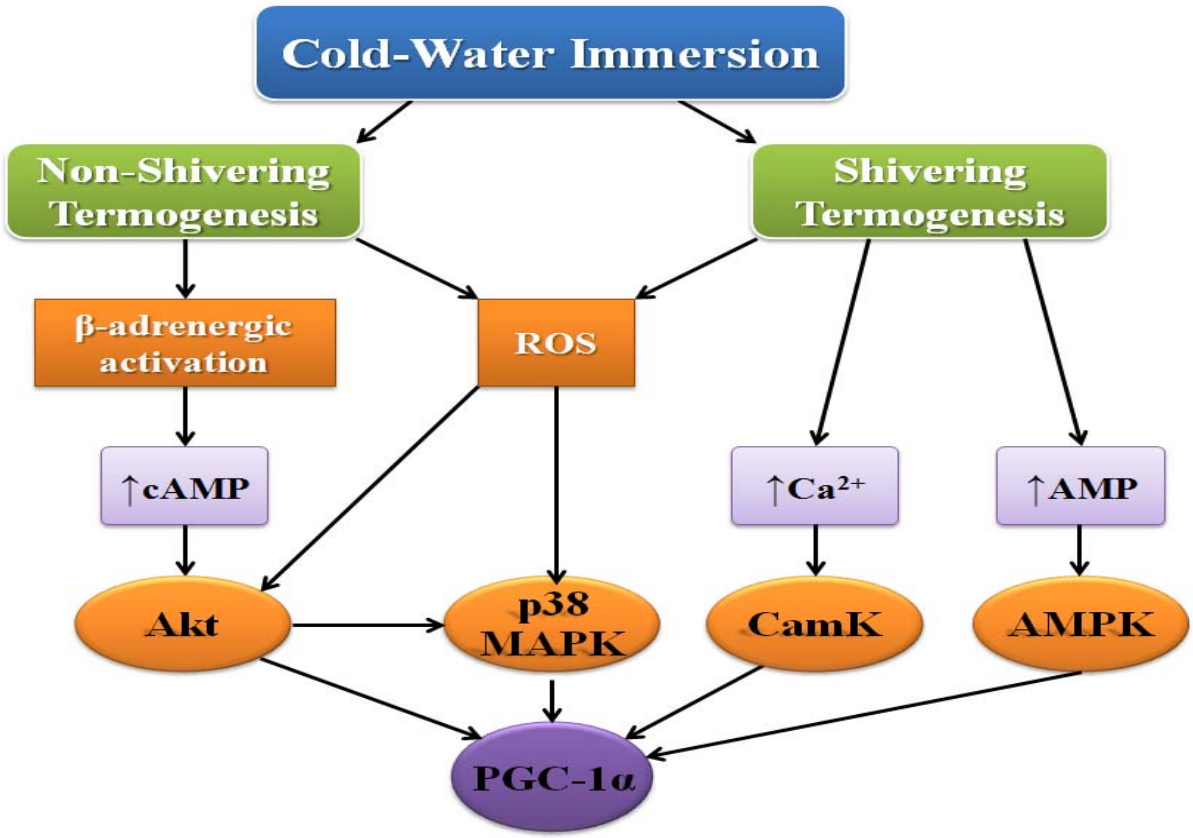

Figure 2.5.1. Post-exercise CWI induced signals involved in altering PGC-1α activity and expression. *AMP* adenosine monophosphate, *AMPK* 5'-AMP-activated protein kinase, *Akt* protein kinase B, *Ca^{2+}* calcium, *CamK* Ca^{2+}/calmodulin-dependent protein kinase, *cAMP* cycle AMP, *PGC-1α* peroxisome proliferator-activated receptor gamma co-activator-1 alpha, *p38MAPK* p38 mitogen-activated protein kinase, *ROS* reactive oxygen species.

2.5.2. Angiogenesis

In addition to the increase in mitochondria biogenesis, it is probable that one of crucial adaptive responses to physiological stimuli such as cold exposure is angiogenesis as the adaptation to an increased need for oxygen. Indeed, increase in capillarity has been observed after prolonged cold exposure in rodent skeletal muscle (Sillau et al., 1980, Suzuki et al., 1997). Bae et al. (2003) investigated effects of prolonged habitual CWI on capillarity in human skeletal muscle. Ten Korean women breath-hold divers who have been diving for more than 20 years participated in the study. The number of capillaries per fibre in different fibre types in the breath-hold divers was higher than in active women. Plausible explanation for an increased capillarity in cold exposure is change in factors controlling the angiogenesis process in skeletal muscle.

The lack of data concerning the angiogenesis factors response to cold exposure has been reported. Evidence from rodent (Asano et al., 1997, Asano et al., 1999) suggests that cold exposure ($4^{o}C$) leads to a rapid increase in VEGF and basic fibroblast growth factor (bFGF) mRNA expression in brown adipose tissue (BAT). More recently, Kim et al. (2005) observed that acute (1 h) and chronic (1 h day^{-1}, 5 days $week^{-1}$ for 20 weeks) CWI at $18^{o}C$ increased the expression of VEGF under resting conditions. In human, post-exercise CWI (one leg in $10^{o}C$ water for 15-min) significantly increased VEGF mRNA expression (~2.7-fold) at 3 h post-exercise compared with the control leg (~1.6-fold) (Ihsan et al., 2014). In addition to the increase in VEGF expression response to cold exposure, Xue et al. (2009) reported that cold exposure induced decrease in antiangiogenic protein thrombospondin. Taken together, these findings suggest that cold-induced angiogenesis is regulated by increase in proangiogenic factors and simultaneous downregulation of antiangiogenic factors.

At present, it is difficult to explain the molecular mechanism of the cold-induced expression and secretion of proangiogenic factors. Fredriksson et al. (2000) observed that hypoxia-mimicking agents and norepinephrine are the major signalling pathways regulating the expression of VEGF in cultured brown adipocytes. However, mechanisms associated with cold-induced VEGF gene expression may be different. Fredriksson et al. (2005) examine whether cold-induced VEGF gene expression in brown adipose tissue in situ is caused by adrenergic signalling pathways directly and/or indirectly by generation of hypoxic oxygen levels resulting from the cold-induced thermogenic process. Results demonstrated that cold-induced expression of VEGF is independent of thermogenic oxygen consumption but

dependent on elevated norepinephrine levels. As mentioned in section 2.3.1., cold activates sympathetic nervous activity that leads to increased level of cAMP and PGC-1α (Wu et al., 1999). Increases in PGC-1α in response to cold stimuli such as cold room temperature and CWI may be involved in VEGF gene expression. Arany et al. (2008) reported the first evidence for a role of PGC-1α in angiogenesis, by which PGC-1α in cell culture increased VEGF mRNA. These findings have been confirmed by Chinsomboon et al. (2009) who observed that PGC-1α null mice failed to increase in VEGF after treatment with clenbuterol, a longacting β2 agonist, while 2.5-fold increase in VEGF expression was shown in wild-type animals which underpin β-adrenergic signalling which is activated by exercise and cold stimuli. Together these findings provide evidence that exercise and cold stimulation induce angiogenesis through VEGF exerts PGC-1α-mediated effects.

2.5.3. Inflammatory response

As mentioned in section 2.4.3., exercise-induced increase in inflammation factors upregulate the mitochondrial biogenesis via PGC-1α. However, post-exercise CWI might attenuate the adaptation associated with inflammatory pathways due to CWI-induced reduction of inflammation. Indeed, Peake et al. (2015) indicated that cryotherapy attenuates some key inflammatory reactions and block the production and release of important growth factors and the activity of satellite cells, which are important mediators of muscle repair and adaption in skeletal muscle. For example, macrophage infiltration, insulin-like growth factor I (IGF-I) and Pax 7 are either blocked or activated more slowly after cryotherapy (Peake et al., 2015). Cryotherapy also restricted the production of reactive oxygen and nitrogen species from 1 day to 15 days after injury in rats (Carvalho et al., 2010). In addition, increase in PGC-1α responses to cold (Slivka et al., 2012, Slivka et al., 2013, Ihsan et al., 2014) is likely to also be associated with inflammatory pathways. Indeed, basal mRNA expression inflammatory factors including tumour necrosis factor (TNF) α, IL-6, suppressor of cytokine signalling 1 and 3 in skeletal muscle was higher in PGC-1α knockout mice than in wild type (Handschin et al., 2007a, Handschin et al., 2007b). Moreover, significant increases in TNFα mRNA and serum TNFα content were observed in PGC-1α knockout mice compared to wild type (Handschin et al., 2007a). These findings are supported by observation in human model, PGC-1α mRNA was negatively correlated with IL-6 and TNFα mRNA expression in skeletal muscle (Handschin et al., 2007b). Taken together, CWI might exert mitochondrial biogenesis via PKB/Akt, Camk, AMPK and p38 pathways rather than inflammatory pathways.

2.6. SUMMARY AND CONCLUSIONS

Significant attention in the literature has examined strategies (e.g. nutrition, stretching, active recovery and massage) which may serve to alleviate short-term decrements in muscle function associated with metabolic fatigue and/or exercise-induced muscle injury. In line with such modalities, more recent interest has centred upon the use of cryotherapy strategies such as CWI. Although effects of CWI on exercise performance are inconsistent, CWI appears to be an effective method in alleviating the muscle damage associated with high-intensity exercise and exercise which incorporates a high eccentric component.

Whilst attention has focused on the influence of CWI on the restoration of short-term muscle function following exercise, limited attempt to date has centred upon the influence of CWI on the mechanisms which underpin more long term adaptation. Animal studies have shown that acute and chronic whole body cooling increases PGC-1α mRNA (Puigserver et al., 1998, Oliveira et al., 2004). Similarly acute and chronic CWI promoted a significant increase of VEGF mRNA under resting conditions (Kim et al., 2005). In human model, Slivka et al. (2012, 2013) recently have shown that exercise and recovery in cold (7^{o}C) ambient temperatures increase PGC-1α mRNA expression. However, the magnitude of the increase in sympathetic discharge to skeletal muscle is influenced by both the size of the tissue area exposed to cooling (Seals, 1990) and the magnitude of the cooling stimulus (Kregel et al., 1992). Consequently, muscle adaptation may be influenced to different degrees by nonnoxious whole body skin surface cooling relative to localized noxious cooling (CWI). Future work is therefore needed to examine the influence of post-exercise CWI on muscle adaptation in human.

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 performed in the Research Institute for Sport and Exercise Science and Department of Pharmacy and Biomolecular Sciences at Liverpool John Moores University. All of the experimental protocols and related procedures were approved by the ethical committee of Liverpool John Moores University.

3.1.2. Participants

All of the participants who volunteered to participate in each study were young healthy recreationally active males. All participants gave written informed consent to participate after details and procedures of the study had been fully explained. All participants were non-smokers, no history of neurological disease or musculoskeletal abnormality and none were under any pharmacological treatment during the course of the study.

3.1.3. Anthropometry

Participant's height (cm) was measured whilst standing in the Frankfurt plane using a stadiometer (Seca, Birminghm, U.K.). Nude body mass (kg) was recorded using precision calibrated weighing scales (Seca, Birmingham, U.K.). All assessments were undertaken on their first visit to the laboratory.

3.1.4. Exercise and CWI

Each exercise study utilised the laboratory based high-intensity intermittent running protocol designed in Study 1 (Chapter 4). Exercise was conducted on a motorised treadmill (HP Cosmos, Nussdorf-Traunstein, Germany). CWI consisted of immersing the participants' legs in 8^{o}C water for 10-min period (see section 3.7.). Participants underwent familiarisation for exercise (RPE) and CWI (shivering scale) at least one week prior to participating in the studies.

3.1.5. Dietary controls

All participants recorded nutritional and fluid intake prior to the first exercise trial. This record was photocopied and returned to the participants to permit them to repeat their

preparation for the remaining trial. Participants were instructed to ingest 5 ml of water per kg 2 h before arriving at the laboratory. Participants consumed a set amount of food and drink (150 kcal) at a rate of around 21 g of carbohydrate and 11 g protein, 2 g fat in the form of sandwich plus low calorie flavoured water during post-immersion period in resting CWI (Chapter 5) and repeated post-exercise CWI study (Chapter 7).

3.2. EXPERIMENTAL PROCEDURES

Prior to the completion of the experimental trials, all participants completed pre-test assessments for determination of $\dot{V}O_{2max}$ (see section 3.4.) in order to permit calculation of the running speeds required during the intermittent exercise protocol (Chapter 4, 6 and 7). On the morning of testing, participants arrived at the laboratory following an overnight fast state having refrained from exercise, alcohol, tobacco and caffeine in the 72 h prior to the test.

Upon arrival at the laboratory nude body mass (kg; Seca, Birmingham, U.K.) was measured, a rectal probe was inserted (Chapter 5, 6 and 7) and a heart rate monitor was positioned across the chest (Polar S610i, Kempele, Finland). Participants were then laid in a semi-reclined position for 30-min for instrumentation (and to stabilize physiological status). Participants commenced the CWI (Chapter 5; see section 3.7.) and then remained seated in a semi-reclined position for 3 hours under normal laboratory temperatures (23 ± 0.5^{o}C) and rested on a bed for 3 hours with consuming a set amount of food and drink (see section 3.1.5.) after changing the wet clothes. In Chapter 4, 6 and 7 participants commenced the intermittent running protocol (see section 3.8.). Immediately after the intermittent running exercise, participants lay on a bed for twenty minutes for the completion of several measurements (Chapter 6 and 7). Each participant was then required to complete a 10-min period of immersion in 8^{o}C water (see section 3.7.) or remained seated for 10-min under normal laboratory temperatures (Chapter 6 and 7). They remained seated in a semi-reclined position for 30-min under normal laboratory temperatures (23 ± 0.5^{o}C). Participants then rested on a bed for two hours without consuming food after changing the wet clothes (Chapter 6) or required to complete 2 × 10-min period in 8^{o}C water (see section 3.7.) or remained seated for 10-min under normal laboratory temperatures at 1 h and 2 h post-exercise (Chapter 7). Participants then rested on a bed for 3 hours with consuming a set amount of food and drink (see section 3.1.5.) after changing the wet clothing (Chapter 7). The trials were conducted in a counterbalanced, randomized order, at least 1 week apart (Chapter 6 and 7).

3.3. CARDIO-RESPIRATORY MEASUREMENTS

3.3.1. Heart rate

Participants were fitted with a short-range radio telemetry system for the measurement of heart rate (beats·min^{-1}, Polar S610i, Kempele, Finland) in all CWI and exercise experiments.

3.3.2. Assessment of respiratory gases

Participants were fitted with a facemask for measurement of respiratory gases during CWI and exercise periods. Expired fractions of oxygen and carbon dioxide were analysed via breath to breath measurement using an on-line gas analysis system (Oxycon Pro, Wuerzberg, Germany) after calibration with known reference gases.

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

Each participant completed a standard incremental treadmill test on a motorised treadmill (see section 3.1.4.) for determination of $\dot{V}O_{2max}$. The protocol commenced at a treadmill speed of 10 km.h^{-1} for 4-min followed by 2-min stages at 12 km.h^{-1}, 14 km.h^{-1} and 16 km.h^{-1}, respectively. Upon completion of the 16 km.h^{-1} stage, the treadmill incline was increased by 2% every 2-min thereafter until volitional exhaustion. Volitional exhaustion was defined as the point at which the participant could no longer continue. The $\dot{V}O_{2max}$ was taken as the highest value attained in any 30 seconds average. Several criteria were used to determine the attainment of $\dot{V}O_{2max}$. These included a heart rate within 10 beats.min^{-1} of age-predicted maximum, maximal respiratory exchange ratio (RER) > 1.1 and volitional fatigue. All participants attained the specified criteria for the attainment of $\dot{V}O_{2max}$. Expired fractions of oxygen and carbon dioxide were analysed via an on-line gas analysis system (see section 3.3.2.). Heart rate (HR) was measured continuously using short-range radio telemetry (see section 3.3.1.).

3.5. MEASUREMENT OF PSYCHO-PHYSIOLOGICAL VARIABLES

3.5.1. Ratings of perceived exertion (RPE)

Participants reported ratings of perceived exertion during exercise were recorded using a 15-point Borg Scale (Borg, 1970). The category ratio scale that was used is displayed in Table 3.5.1.

Table 3.5.1. Borg scale used for participants' ratings of their perceived exertion during exercise.

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

3.5.2. Ratings of perceived shivering

Participants reported ratings of shivering during immersion and the post immersion resting period were reported using 4-point shivering rating scale (Wakabayashi et al., 2006). The category ratio scale that was used is displayed in Table 3.5.2.

Table 3.5.2. Subjective shivering used for participants' ratings of their shivering during resting period.

Rating	Description
1	No Shivering
2	Slight Shivering
3	Moderate Shivering
4	Heavy Shivering

3.6. THERMOREGULATORY VARIABLES

A rectal probe (ELAB, Rødovre, Denmark) was inserted 15 cm beyond the external anal sphincter for the assessment of rectal temperature. A skin thermistor (ELAB, Rødovre, Denmark) was securely taped to the upper thigh and calf for the assessment of skin temperature. Muscle temperature (T_m) was assessed using a needle thermistor inserted into the vastus lateralis (ELAB, Rødovre, Denmark). Thigh skinfold thickness was measured using Harpenden skinfold calipers (Baty International, Burgess Hill, U.K.) and divided by 2 to determine the thickness of the thigh subcutaneous fat layer over each participants vastus lateralis (Enwemeka et al., 2002). The needle thermistor was then placed at a depth of 3 cm plus one-half of the skinfold measurement for determination of deep muscle temperature (3 cm). The thermistor was then withdrawn at 1 and 2 cm increments for determination of muscle temperature at 2 and 1 cm below the subcutaneous fat layer. Rectal temperature, skin temperature and muscle temperature were recorded using an electronic measuring system (ELAB, Rødovre, Denmark).

3.7. COLD WATER IMMERSION (CWI)

Despite no standard CWI protocol exists (temperature, durations, timing and frequency), CWI at cooler than 15 ^{o}C and immersion of at least 10-min in duration is currently used in the field. Recent observation from our laboratory indicate that resting lower limb CWI (2 × 5-min, with a 2-min window in between) in 8^{o}C induces marked changes in whole limb blood flow (Gregson et al., 2011). In the present studies, participants completed a 10-min period of immersion in 8^{o}C water or remained seated for 10-min under normal laboratory temperatures (23 ± 0.5^{o}C). In the immersion condition, participants were positioned in a semi-reclined position. Participants were then lowered into the water until the thighs were fully submersed for a total duration of 10-min (2 × 5-min, with a 2-min intermission above the water). At the end of immersion, participants were raised from the water using the electronic hoist and remained seated in a semi-reclined position under the temperature-controlled laboratory (23 ± 0.5^{o}C). An illustration of CWI is shown in figure 3.7.1..

Figure 3.7.1. Illustration of CWI.

3.8. LOW-DAMAGING RUNNING EXERCISE PROTOCOL

In each exercise related study participants performed 60-min of high-intensity intermittent exercise on a motorised treadmill (HP Cosmos, Nussdorf-Traunstein, Germany). A regression equation of the average $\dot{V}O_2$ vs. running speed at each stage of the $\dot{V}O_{2max}$ test was used to estimate treadmill running speed corresponding to 25%, 50%, 70% and 90% of $\dot{V}O_{2max}$ for each subject. The intermittent running protocol consisted of a 10-min warm up at a running velocity corresponding to 70% of $\dot{V}O_{2max}$. This was followed by eight 3-min bouts at a running velocity corresponding to 90% of $\dot{V}O_{2max}$ interspersed with 3-min active recovery periods (1.5-min at a velocity corresponding to 25% $\dot{V}O_{2max}$ followed by 1.5-min at velocity corresponding to 50% $\dot{V}O_{2max}$). A 5-min cool-down period (50% of $\dot{V}O_{2max}$) was undertaken following completion of the final exercise bout. This protocol used in the present thesis (Chapter 4). A schematic illustration of the exercise protocol is shown in figure 3.8.1..

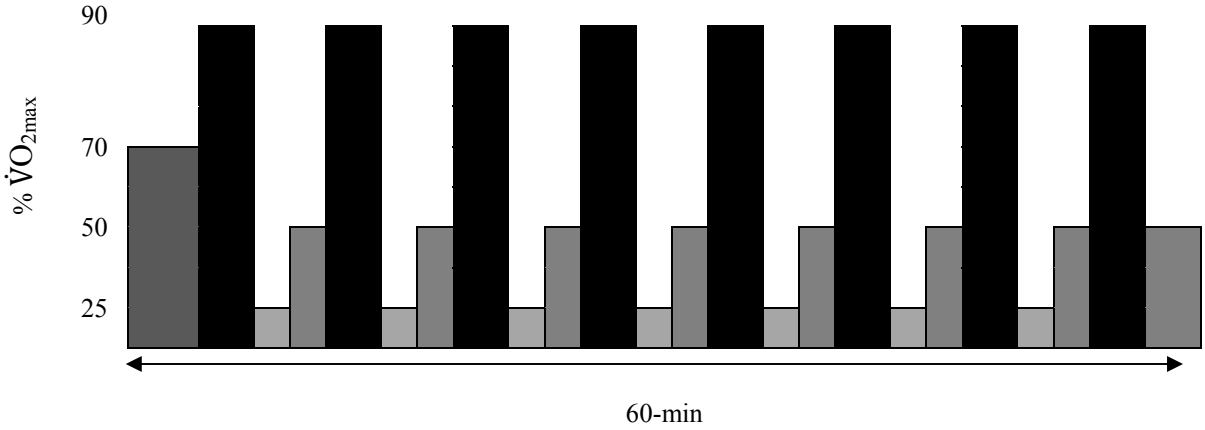

Figure 3.8.1. Schematic illustration of the exercise protocol.

3.9. PROCUREMENT AND STORAGE OF BLOOD SAMPLES

Venous blood samples were drawn from a superficial vein in the anti-cubital crease of the forearm using standard veinepuncture techniques (Becton, Dickinson, Oxford, U.K.). Samples were collected into vacutainers (Becton, Dickinson, Oxford, U.K.) containing EDTA or serum separation tubes and stored on ice or at room temperature (serum samples, ~1 h) until centrifugation at 1500 rev.min^{-1} for 15-min at 4^{o}C. Following centrifugation, aliquots of plasma and serum were stored at -80^{o}C for later analysis.

3.10. ANALYSIS OF BLOOD VARIABLES

Samples were analyzed for plasma creatine kinase (coefficient of variation ~1 %) activity and myoglobin concentration (coefficient of variation ~5 %), blood lactate using commercially available kits (Randox Laboratories, Antrim, UK). Plasma adrenaline and noradrenaline concentrations were measured using liquid chromatography-tandem mass spectrometry (Peaston et al., 2010). All samples were analysed in duplicate.

3.11. MUSCLE BIOPSIES

Muscle biopsies were obtained from separate incision sites (2~3 cm apart) from the lateral portion of the vastus lateralis muscles under local anesthesia (0.5% marcaine, without adrenaline, Astrazeneca, Wilmington, USA) using a Bard Monopty Disposable Core Biopsy Instrument 12 gauge × 10 cm length (Bard Biopsy Systems, Tempe, USA). After ensuring adequate local anesthesia, an incision was made in the lateral thigh at the biopsy site directly through the overlying skin, subcutaneous fat and fascia. Once the biopsy needle was 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. Legs for each procedure were alternated between conditions. Once obtained, samples (~60 mg of tissue) were immediately frozen in liquid nitrogen and stored at -80^{o}C for later analysis. An illustration of the muscle biopsy is shown in figure 3.11.1..

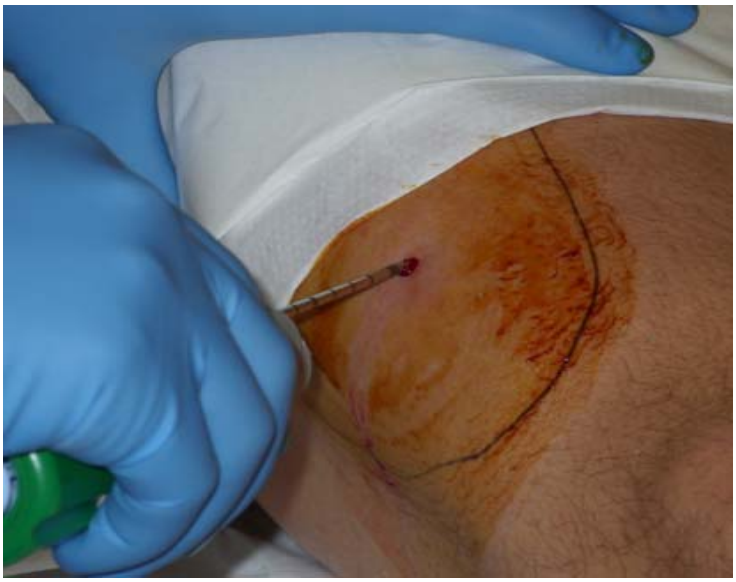

Figure 3.11.1. Illustration of the muscle biopsy.

3.12. ANALYSIS OF mRNA CONTNET OF SKELETAL MUSCLE USING QUANTITATIVE REAL TIME PCR

Total RNA was isolated from muscle biopsies (20-30 mg) using Trizol reagent (Invitrogen, Carlsbad, USA), according to the manufacturer's protocol. 1µl of RNA sample was analysed in duplicate by spectrophotometry with the use of a Nanodrop 2000 (Thermo Fisher Scientific, Loughborough, UK) to determine RNA quality and quantity. A target of A260 / A280 ratio was set at 2.0. RNA was stored at -80°C until further analysis. cDNA was synthesised using random hexamers (Applied Biosystems, Foster City, USA) and Superscript III enzyme (Invitrogen, Carlsbad, USA), using manufacturer's protocol. Gene specific expression data was obtained using probes selected from Human Universal Probe Library (Roche Applied Science, Indianapolis, USA) with compatible oligonucleotide primers (MWG Eurofins, Ebersberg, Germany), except for $VEGF_{165}$ for which Green technology was applied. One microliter of each cDNA sample was analysed in triplicate with negative controls using AB 7500 Real-Time Quantitative PCR instrument (Applied Biosystems, Foster City, USA) and Agilent Brilliant II qPCR Master Mix with Low ROX (Agilent Technologies, Columbia, USA). One microliter of cDNA, 500 nM of primer and 200 nM of probe were used for each 20-µl reaction (Table 3.12.1.). 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. For SYBR green reactions additional post-qPCR melting curve analysis was also performed for QC-purposes. 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 (coefficient of variation ~0.5 %) as a single reference gene was used due to variability in other housekeeping genes (β2M (coefficient of variation ~0.8 %) and β-actin (coefficient of variation ~0.9 %)).

Table 3.12.1. Primer and probe sequences used for real-time PCR during Chapter 5, 6 and 7.

Gene	Forward primer	Reverse primer	Probe
GAPDH	GCTCTCTGCTCCTCCTGTTC	ACGACCAAATCCGTTGACTC	60
PGC-1α	CAAGCCAAACCAACAACTTTATCTCT	CACACTTAAGGTGCGTTCAATAGTC	13
$VEGF_{total}$	CCT TGCTGCTCTACCTCCAC	CCACTTCGTGATGATTCTGC	29
$VEGF_{165}$	TGTGAATGCAGACCAAAGAAAGA	TGCTTTCTCCGCTCTGAGC	NA

3.13. 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, Burgess Hill, 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, Dorset, UK). Each sample was diluted with an equal volume of 2X Laemmli buffer (National Diagnostics, Atlanta, 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, Staffordshire, UK) using self-cast 4 % stacking and 10 % separating gels (National Diagnostics, Atlanta, USA). Gels were transferred semi-dry onto nitrocellulose membrane (Geneflow, 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 × 5-min in TBST before being incubated overnight at 4 ˚C with phosho-specific antibodies AMPK and p38MAPK (all from Cell Signalling Technology, Danvers, USA) as well as the total protein content; AMPK, p38MAPK, GAPDH (Cell Signalling Technology, Danvers, USA), VEGF (Santa Cruz Biotechnology, Heidelberg, Germany) and PGC-1α (Merck Chemicals, Awsworth, UK) all at concentrations of 1:1000 in 1 × TBST. The next morning, membranes were washed for a further 3 × 5-min in TBST and subsequently incubated with anti-species horseradish peroxidise-conjugated secondary antibody (Bio-Rad, Hemel Hempstead, 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, 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 were specific to the protein of interest, a secondary control was run on each gel. In this way, protein was loaded into the appropriate lane of the gel and the subsequent membrane was incubated in secondary antibody only.

CHAPTER 4

DEVELOPMENT OF A LOW-DAMAGING HIGH-INTENSITY INTERMITTENT RUNNING PROTOCOL

4.1. INTRODUCTION

High-intensity interval exercise is characterised by repeated bouts of high-intensity exercise interspersed with short rest periods or low intensity exercise and forms the basis of team sports such as football. High-intensity interval training is frequently used in the development of athlete training programs due to its effectiveness in promoting numerous morphological and metabolic adaptations in skeletal muscle (Morton et al., 2009). Similarly, such forms of training have recently received much attention in the literature as a potent and time efficient strategy to induce numerous physiological adaptations that resemble traditional endurance training despite a low total exercise volume (Little et al., 2010). High-intensity interval training therefore represents an increasingly popular form of activity for enhancing both health and performance.

Athletes routinely undergo a temporary drop in the capacity to produce force following high-intensity exercise (Thorlund et al., 2009). Fatigue is defined as acute impairment of exercise performance that includes both an increase in the perceived effort necessary to generate a desired force and the eventual loss of the ability to produce that force after exercise (Davis and Bailey, 1997). A short-term drop in performance is, however, normally restored within 24 h. In contrast, muscle damage characterized by a reduction in the capacity to generate force, soreness and muscle tenderness frequently occurs following intense exercise, unfamiliar exercise or eccentric contractions (Mathur et al., 2010). Symptoms generally occur within 24 h, peaking between 24 and 72 h, and last up to 4-7 days post-exercise (Cleak and Eston, 1992, Foure et al., 2015, Lau et al., 2015) and are frequently referred to as DOMS. Due to the severity of the physical demands associated with different forms of high-intensity intermittent exercise, it is important to characterize the physiological responses to such forms of exercise in specific populations so that the appropriate experimental design can be established for future studies. The degree of exercise-induced muscle damage is likely to be related to exercise type, duration, intensity and amount of eccentric contractions during exercise (Hill et al., 2014). Higher levels of exercise-induced muscle damage leads to an increase in phagocytic cell content, which affect cell signalling in many biological systems (Suzuki et al., 1999, Hancock et al., 2001). The use of a low-damaging exercise protocol therefore provides a more controlled methodological approach through which to examine the effects of exercise per se on molecular and cellular responses in human skeletal muscle (Morton et al., 2006). The aim of this study, therefore, is to determine the physiological

responses to a high-intensity intermittent running protocol in moderately trained participants frequently accustomed to performing high-intensity exercise. It was hypothesized that such a running protocol would not lead to symptoms consistent with DOMS. A low-damaging protocol will be used in future Chapters to characterize the influence of CWI on muscle adaptation to high-intensity exercise.

4.2. METHODS

4.2.1. Participants

Ten healthy active males volunteered to participate in the study [mean ± SD: age 31 ± 7.1 yr, height 174 ± 4 cm, body mass 74 ± 8 kg and the participants reached $\dot{V}O_{2max}$ (58 ± 7.1 $mL.kg^{-1}.min^{-1}$)].

4.2.2. Familiarisation

Participants underwent extensive familiarisation prior to participating in the study. During such sessions, the participants were introduced to and familiarised with the procedure of maximal voluntary contraction (MVCs) of the quadriceps (4 s duration). Approximately three to four contractions were performed in each session. Familiarization sessions were repeated until the participants MVC force demonstrated a plateau effect between subsequent sessions. This level of initial consistency was usually achieved within 3-5 sessions after which participants were then familiarised the high-intensity intermittent exercise. Participants were then considered eligible to participate in study.

4.2.3. Experimental design

Each participant was required to perform a high-intensity intermittent exercise running protocol (see section 3.8.). Maximal voluntary contraction of the quadriceps muscle was determined immediately before and after exercise and at 24 h, 48 h 72 h and 7 days following exercise. At the same time, a venous blood sample was also drawn for determination of systemic indicators of muscle damage (creatine kinase and myoglobin). A schematic illustration of the experimental design is shown in figure 4.2.1..

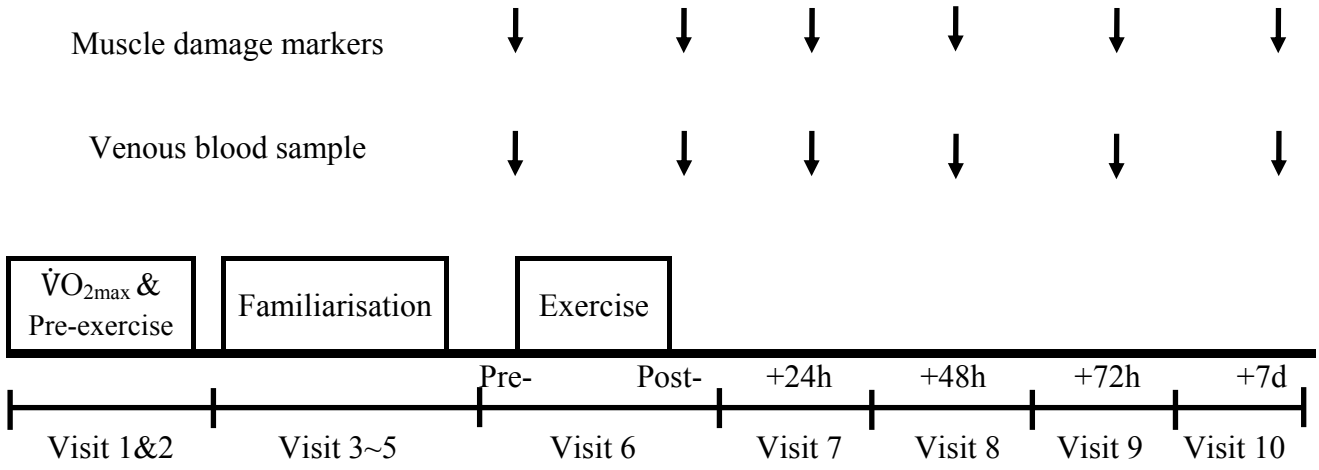

Figure 4.2.1. Schematic illustration of the experimental design.

Heart rate was continuously monitored and recorded at 5 sec intervals during the protocol (see section 3.3.1.). A finger prick blood sample for determination of blood lactate concentrations (Lactate Pro, Arkray, Japan) and the RPE (see section 3.5.1.) were obtained immediately following each exercise bout. Venous blood samples were analysed for creatine kinase activity and myoglobin concentration using clinical chemistry analyser (RX Daytona^{TM}, Randox, Crumlin, UK).

4.2.4. Maximal isometric quadriceps force

Maximal voluntary contraction of the quadriceps muscle was undertaken as previously described (Morton et al., 2005). Assessment of the maximal voluntary contraction of the quadriceps muscle has a coefficient of variation ~5 % in our laboratory (Morton et al., 2005).

4.2.5. Subjective estimation of muscle soreness

Ratings of perceived muscle soreness were assessed using a visual analog scale (Zhang et al., 2000). The scale comprised “no pain” at one end side of a 100-mm line and “extremely sore” at the other end. Muscle tenderness was assessed via a push-pull handheld dynamometer (Model FD130, Mecmesin, Italy). While participants remained in a standing position, the pressure was applied over the distal myotendinous junction (measured as 5 cm above the superior pole of the patella) (Sellwood et al., 2007), as well as the mid-belly of the rectus femoris muscle (midway between lateral condyle of the knee and head of the femur). These sites were placed along a datum line, from the mid-patella to the lateral iliac crest (Baker et al., 1997) and marked so that the same site could be tested on each occasion. If participants

formed a haematoma from the pressure applied from a previous measurement, subsequent measurements were taken right away inferior upon the datum line. All measurements were taken on each participant's right side. Pressure was applied at an even rate, at a speed of approximately 1 kg · sec^{-1} (Fischer, 1987), until the participant pointed out that the feeling had changed from 'pressure' to 'discomfort'. Measurements were taken three times at each site in sequential order with a minimum of 1-min between tests. Measurements were recorded to the nearest 0.25 kg and finally the mean score was recorded (Cleary et al., 2005).

4.2.6. Statistical analysis

All data are presented as means ± SD. Any systematic changes in MVC, VAS, creatine kinase, myoglobin, blood lactate and RPE during the intermittent running protocol and for the subsequent 7-day period were assessed using one-way within-participants general linear model (GLM). *Post-hoc* analysis by Newman-Keuls test was undertaken to examine which time points were significantly different from pre-exercise, with probability values of $P < 0.05$ assumed to indicate statistical significance.

4.3. RESULTS

4.3.1. Physiological responses to intermittent exercise

Mean heart rate significantly increased (Figure 4.3.1. A) during the intermittent protocol with values of 165 ± 11 beats·min^{-1} and 171 ± 12 beats·min^{-1} observed during the first and final bout respectively ($F = 12.722$, $P < 0.001$). These values equated to 88.5 ± 2.4% and 92 ± 4.3% of maximum HR. A similar increase in RPE was also observed with values of 15 ± 1 observed during bout 1 relative to 19 ± 1 during the final 8^{th} bout ($F = 28.664$, $P < 0.001$; Figure 4.3.1. B). Blood lactate concentrations were significantly elevated following bout 1 compared with resting values (1.2 ± 0.3 mmol·l^{-1} vs. 5.4 ± 2.4 mmol·l^{-1}; $P = 0.03$). However, blood lactate concentration did not increase further during the remaining exercise bouts ($F = 0.97$, $P = 0.42$; Figure 4.3.1. C).

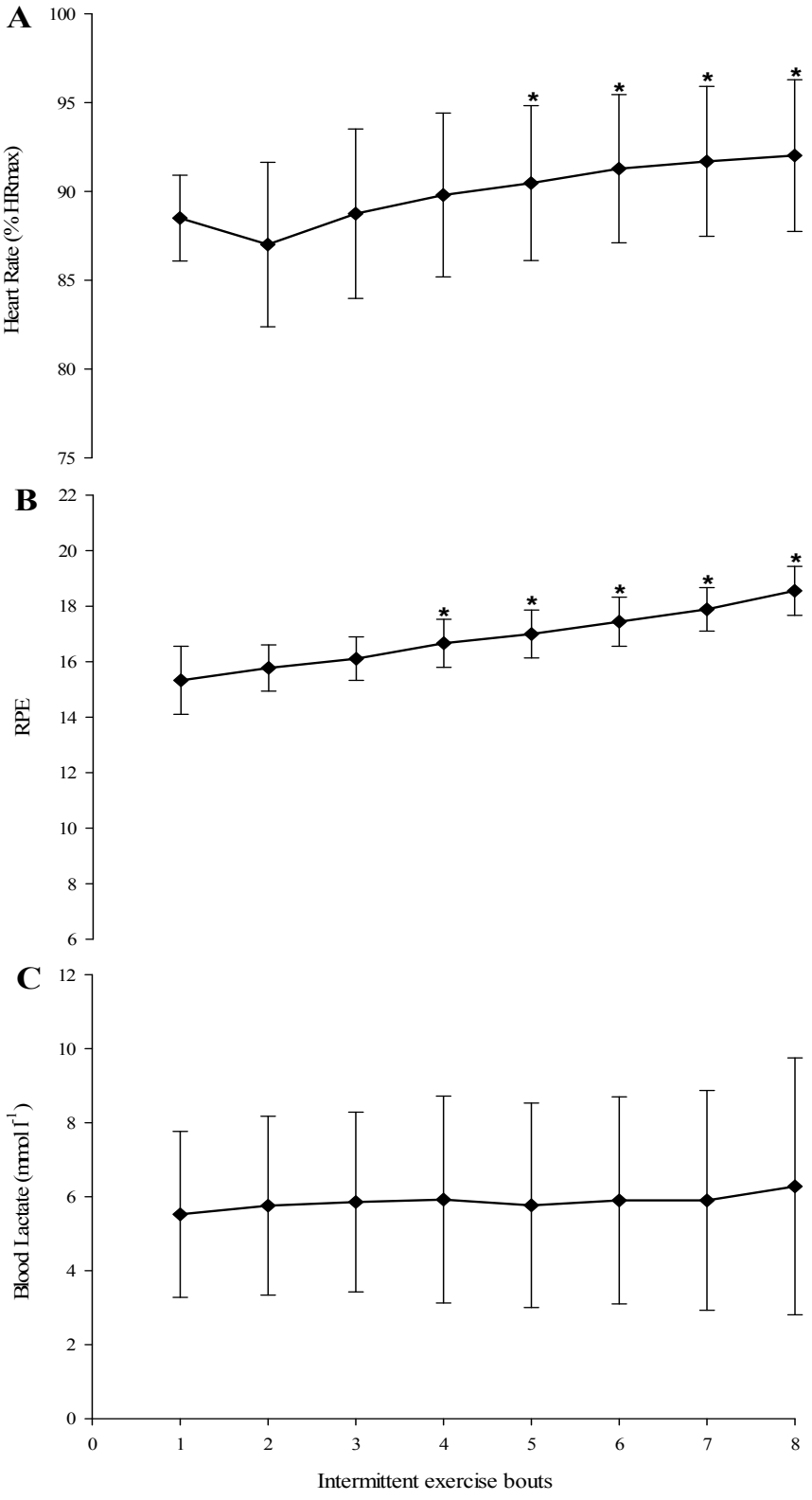

Figure 4.3.1. % HR_{max} (A), RPE (B) and blood lactate concentrations (C) during the intermittent exercise protocol (n = 10, mean ± SD). A main effect for time was found for % HR_{max} (F = 12.722, P < 0.001) and RPE (F = 28.664, P < 0.001). *P < 0.05; significantly different from the first bout.

4.3.2. Indices of muscle damage

Baseline MVC scores for the quadriceps was 648.3 ± 148.5N. No significant reduction in MVC was observed immediately following completion of the last exercise bout (623.9 ± 143.6N) or during the subsequent 7-day period compared to pre-exercise values ($F = 0.646$, $P = 0.59$).

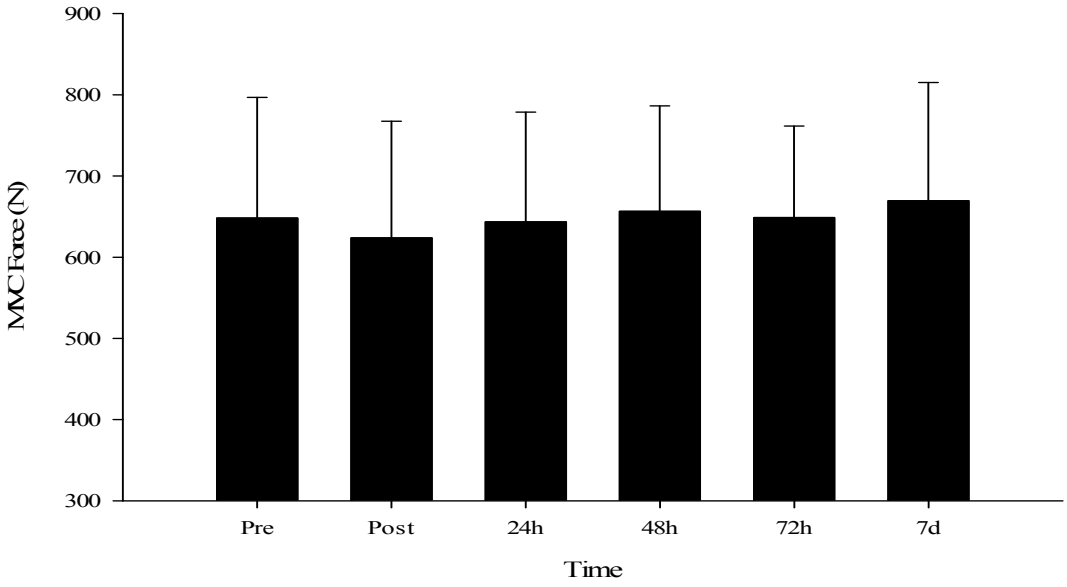

Figure 4.3.2. Maximal quadriceps isometric muscle force during the 7-day testing period (n = 10, mean ± SD). No main effect for time was observed ($F = 0.646$, $P = 0.59$).

Ratings of perceived muscle soreness were increased at 24 h ($P = 0.08$) and 48 h ($P = 0.02$) later compared to pre-exercise values (Table 4.3.1.). Ratings returned towards baseline values from 72 h onwards. Muscle tenderness (mid-belly, musculotendinous) was not increased at any stage during the 7-day testing period.

Table 4.3.1. Muscle tenderness and ratings of perceived soreness during the 7-day testing period.

Variables		Pre- exercise	Post-exercise	+24hrs	+48hrs	+72hrs	+7day
Tenderness	Mid-belly (mm)	6.4±1.5	6.1±1.3	6.4±1.4	6.5±2.7	6.5±2	7±2
	Musculotendinous (mm)	6.5±1	6.3±0.9	5.9±0.9	6.6±2.3	6.3±1.3	6.9±2
VAS (mm)		2.5±4.1	28±28.1	31.3±34.7	8.5±6.8*	1.6±2.1	0.5±0.8

Values are means ± standard deviation. *$P < 0.05$; significantly different from pre-exercise time point, VAS; visual analogue scale.

Creatine Kinase concentrations were not significantly increased immediately after exercise (P = 0.96) and at 24 h following exercise (P = 0.3; Figure 4.3.3. A). Myoglobin content was significantly increased following the intermittent exercise compared with pre-exercise rest (P = 0.01; Figure 4.3.3. B). However, values returned towards pre-exercise concentrations after 24 h (P = 0.32).

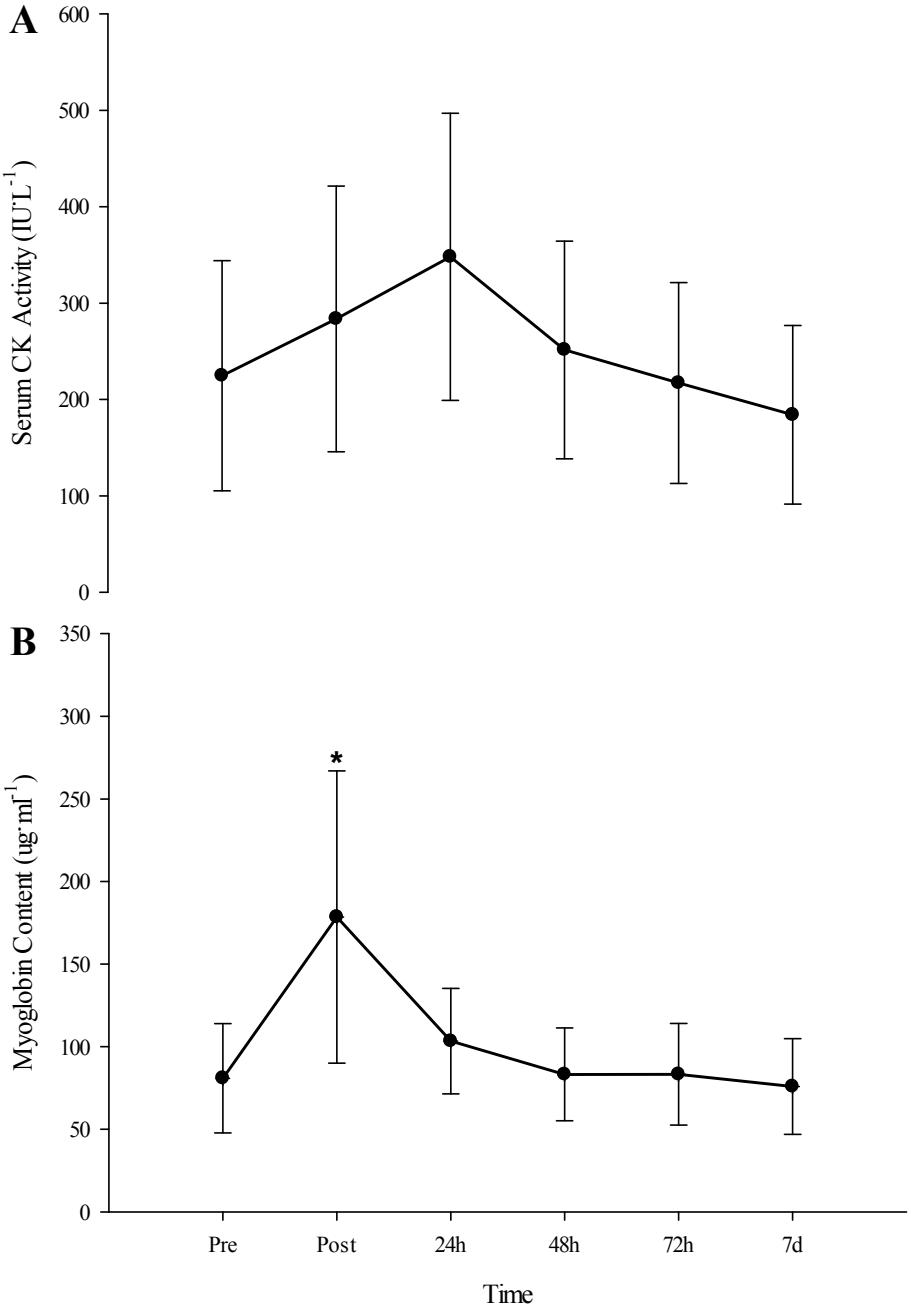

Figure 4.3.3. Plasma CK (A) and Mb (B) during the 7-day testing period (n = 10, mean ± SD). A main effect for time was found for Mb (F = 18.15, P < 0.001). *P < 0.05; significantly different from pre-exercise time point.

4.4. DISCUSSION

The findings from the present study indicate that the high-intensity intermittent running protocol did not induce symptoms normally associated with DOMS. Consequently, adaptation during the acute recovery period may be principally mediated through cell signalling within the muscle as opposed to phagocytic cells, which migrate to the muscle following damaging exercise. This protocol will therefore be used in subsequent experimental trials to determine the influence of CWI on skeletal muscle cell signalling responses in moderately trained individuals.

High-intensity intermittent exercise has recently received much interest in the scientific literature. For example, recent work has demonstrated that such modes of exercise are effective in enhancing both aerobic and anaerobic performance (Dellal et al., 2009, Tanisho and Hirakawa, 2009). Furthermore, high-intensity intermittent exercise induces oxidative adaption (Morton et al., 2009) to such an extent that it serves as an efficient and effective means of promoting increases in aerobic fitness in recreational populations relative to traditional long duration low intensity steady-rate exercise (de Souza et al., 2007, Tjonna et al., 2008). These physiological benefits are complemented by the perception that high-intensity intermittent exercise is more enjoyable compared to continuous exercise when matched for average intensity, duration, and total work done (Bartlett et al., 2011). In the present study, blood lactate values were significantly greater after the first bout of exercise (6 ± 2.5 $mmol \cdot l^{-1}$) compared to baseline (1.2 ± 0.2 $mmol \cdot l^{-1}$) and the value gradually increased by the end of exercise bout (7.3 ± 3.8 $mmol \cdot l^{-1}$). Mean HR during the exercise bouts was more than 87% of HR_{max} and the HR continued to increase to 92% of HR_{max} at the end of exercise bout. RPE score was 15 (hard) following the first bout of exercise and increased to 19 (extremely hard) following the final exercise bout. These physiological responses to the high-intensity intermittent exercise protocol compares favourably with previous studies from our laboratory (Bartlett et al., 2011, Bartlett et al., 2012).

Intense exercise causes muscle fatigue that can be recovered within a few hours with the utilization of appropriate recovery strategies (Peiffer et al., 2010). However, following unfamiliar high-intensity exercise, especially eccentric exercise, disruption of internal structures characterized by myofibrillar disorientation and damage to the cytoskeletal framework may occur in the absence of any metabolic disturbance (Green, 1997). Decrements in muscle force patterns and their time course serve as a means through which to differentiate between the symptoms of muscle fatigue and muscle damage (Jones et al., 1986, Newham et al., 1983). To observe muscle damage induced by exercise, both direct

measurements at the cellular level and indirect from changes in various indices of muscle function can be applied. One of the most appropriate and valid means to quantify muscle damage following exercise is the impairment of the ability of skeletal muscle to produce force (Faulkner et al., 1993, Warren et al., 1999). Indeed, exercise-induced muscle damage leads to significant declines in MVC by 48 h post-exercise (Bailey et al., 2007). In the current study, MVC was not significantly reduced relative to baseline either immediately following exercise or at any time point during the subsequent 7 days. This failure to observe marked reductions in muscle function suggest that muscle damage typically characterized by long-lasting decreases in force-generating ability were not evident following the current high-intensity interval protocol in the moderately trained subjects (Brown et al., 1997, Eston et al., 1996, Murayama et al., 2000, Nosaka and Sakamoto, 2001). This is supported by the failure to observe any significant changes in muscle tenderness (mid-belly, musculotendinous) during the recovery period.

Elevations in CK and myoglobin are frequently used as indicators of muscle damage with increased concentrations frequently reported for up to 5 days after damaging exercise (Chen et al., 2007). In the present study, CK concentrations showed a tendency to increase post-exercise and 24 h post-exercise with concentrations returning to pre-exercise level after 48 h. Myoglobin concentrations significantly increased following exercise with values returning to pre-exercise levels at 24 h post-exercise. Similarly, ratings of perceived muscle soreness were increased at 24 h and 48 h post-exercise with returning to pre-exercise level after 72 h. As a result, the changes in CK, myoglobin and ratings of perceived muscle soreness currently observed are representative of the symptoms of low-damaging nature of the exercise (Chen et al., 2007) rather than DOMS, which is more commonly associated with severe muscle damage (Cheung et al., 2003).

In summary, the present findings demonstrate that the current high-intensity intermittent exercise protocol does not lead to reductions in MVC or marked alterations in CK and muscle tenderness consistent with the effects of severe damaging exercise. Adaptative responses of skeletal muscle during the subsequent recovery period are therefore likely to be predominantly mediated through cell signalling within the muscle as opposed to phagocytic cells, which migrate to the muscle following damaging exercise. All indices level of muscle damage after 7 days the high-intensity intermittent are similar compared to baseline. This low-damaging exercise protocol will therefore be used in future investigations (counterbalanced randomised crossover design, at least 1 week apart) to characterize the influence of CWI on muscle adaptation responses to acute high-intensity intermittent exercise.

CHAPTER 5

THE INFLUENCE OF COLD WATER IMMERSION ON PGC-1α AND VEGF EXPRESSION IN HUMAN SKELETAL MUSCLE AT REST

5.1. INTRODUCTION

Human skeletal muscle is known to adapt to a diverse array of contractile stimuli including aerobic exercise. This stimulus has a number of health benefits, including improvements in muscle metabolism, cardiovascular function, and aerobic capacity (Dellal et al., 2009, Egan and Zierath, 2013). These benefits arise from a higher mitochondrial content within the muscle, as well as enhanced capillary density (Goodpaster et al., 2003, Robbins et al., 2009). PGC-1α, a transcriptional co-activator, serves as a master regulator of mitochondrial biogenesis with VEGF acting as the key mediator of angiogenesis in skeletal muscle (Ferrara, 2001, Puigserver and Spiegelman, 2003). In line with such observations a number of studies have frequently reported that acute cycling and running exercise increase expression of PGC-1α mRNA (Leick et al., 2010, Bartlett et al., 2012) in human skeletal muscle with increased protein expression observed after several weeks of training (Russell et al., 2003). Similarly, acute exercise is accepted as a valid method by which to induce VEGF mRNA expression in both animal (Breen et al., 1996, Gavin et al., 2000) and human (Gustafsson et al., 1999, Richardson et al., 1999, Gavin et al., 2004) skeletal muscle and exercise training increases VEGF protein (Gavin et al., 2007b).

PGC-1α and VEGF also play a role in cold-induced adaptive thermogenesis (Cannon et al., 1998, Kim et al., 2005). Marked cooling through exposure to cold air increased PGC-1α and VEGF expression in animals (Oliveira et al., 2004). Similarly in rats, acute (1 h) and chronic (1 h day^{-1}, 5 days $week^{-1}$ for 20 weeks) CWI (18^{o}C) increased VEGF mRNA and protein expression under resting conditions (Kim et al., 2005). This cold-induced elevation in mitochondrial biogenesis and angiogenesis are thought to be mediated by increased β-adrenergic activity (Himms-Hagen, 1989, Chinsomboon et al., 2009) with the decline in skin temperature provoking a profound reflex increase in sympathetic nerve activity through activation of nonnoxious thermoreceptors (Hensel and Boman, 1960). Indeed, previous findings have shown that noradrenaline concentrations and β-adrenergic receptors (β-AR) gene expression were higher in response to cold exposure (Sramek et al., 2000, Tabbi-Anneni et al., 2010). The sympathetic nervous system through β-AR enhances cyclic AMP signalling and the activation of protein kinase A (PKA), which stimulate p38MAPK (Cao et al., 2004, Fernandez-Marcos and Auwerx, 2011), resulting in increased PGC-1α and VEGF production (Ouchi et al., 2005, Wright et al., 2007).

Whole limb cooling via CWI is frequently applied immediately after exercise in an attempt to alleviate some of the physiological and functional deficits associated with exercise-induced muscle damage (Leeder et al., 2012). However, CWI may also mediate changes in skeletal muscle adaptation through the effects of increased β-adrenergic activity on PGC-1α and VEGF expression. Despite observations in animals, limited work to date has been undertaken to determine the impact of cooling on PGC-1α and VEGF expression in humans. Slivka et al. (2012) recently reported increased PGC-1α mRNA expression following combined exercise and recovery in cold (7^{o}C) compared to moderate (20^{o}C) ambient temperatures. Furthermore, recovery alone in cold (7^{o}C) air increases PGC-1α mRNA expression relative to recovery in moderate temperatures (Slivka et al., 2013). However, no study to date has determined to what extent cooling per se (e.g. without concomitant or prior exercise) increases PGC-1α and VEGF expression. An examination of this condition is important since CWI is often applied the day after training and competition with the athlete in a rested state. Furthermore, the magnitude of the increase in sympathetic discharge to skeletal muscle is influenced by the size of the tissue area exposed to cooling (Seals, 1990) and the magnitude of the cooling stimulus (Kregel et al., 1992). Consequently, PGC-1α and VEGF expression may be influenced to different degrees by nonnoxious whole body skin surface cooling relative to localized noxious cooling promoted by CWI. Therefore, the aim of the present study was to examine the influence of CWI on PGC-1α and VEGF expression in human skeletal muscle at rest.

5.2. METHODS

5.2.1. Participants

Ten healthy active men volunteered to participate in the study [mean ± SD: age 24 ± 1yr, height 175.3 ± 4cm, body mass 79.5 ± 6kg and maximal oxygen uptake ($\dot{V}O_{2max.}$) were 55.5 ± 7.3mL.kg^{-1}.min^{-1}].

5.2.2. Experimental design

Prior to the completion of an experimental trial baseline assessments (see section 3.2.), a venous blood sample (see section 3.9.), muscle temperature (see section 3.6.) and muscle biopsy (see section 3.11.) were completed and then each participant was required to complete CWI (see section 3.7.).

Rating of perceived shivering, heart rate, oxygen consumption ($\dot{V}O_2$), rectal (T_{re}) and skin temperature (thigh and calf) were measured continuously during CWI and recovery for 3 h. Muscle temperature was measured immediately post-immersion and 0.5 h, 1.5 h, 3 h, and 6 h post-immersion (see section 3.6.). Venous blood samples were also taken at 0.5 h, 1.5 h, 3 h and 6 h post-immersion (see section 3.9.) and analysed for lactate, adrenaline and noradrenaline (see section 3.10.). Muscle biopsies were obtained at 3 h and 6 h post-immersion (see section 3.11.) and analysed for PGC-1α and VEGF (see section 3.12., 3.13.). A schematic illustration of the experimental design is shown in figure 5.2.1..

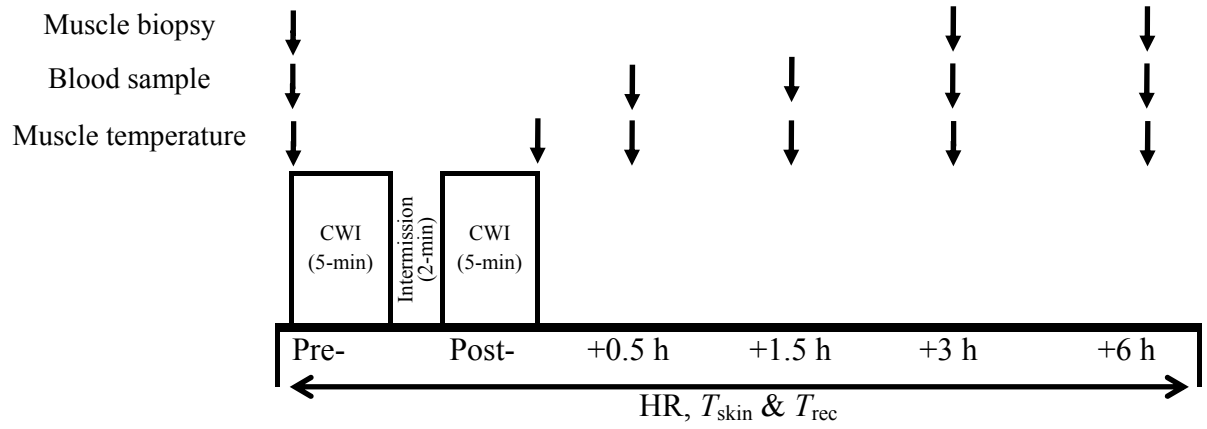

Figure 5.2.1. Schematic illustration of the experimental design.

5.2.3. Statistical analysis

All data are presented as means ± SD. Any systematic changes in thermoregulatory responses, HR, oxygen uptake, mRNAs, proteins and blood variables during resting period were assessed using one-way within-participants general linear model (GLM). A two-factor (time × depth) within-participants general linear model was undertaken to assess muscle temperature. *Post-hoc* analysis by Newman-Keuls test was undertaken to examine which trials were significantly different from pre-immersion. One sample *t* test was used to compare the immersion-induced change in shivering and changes of muscle temperature between baseline and other time points. The alpha level for evaluation of statistical significance was set at $P < 0.05$.

5.3. RESULTS

5.3.1. Thermoregulatory responses

Rectal temperature remained similar to baseline throughout immersion and the post-immersion period ($F = 1.021$, $P = 0.36$; Figure 5.3.1. A). However, thigh and calf skin temperatures decreased during the immersion period and post-immersion period with the largest reduction occurring at the end of the 10-min immersion period ($P < 0.05$; Figure 5.3.1. B, C). Skin temperature at both sites increased during the post-immersion period. At the end of post-immersion period, thigh and calf temperature remained below baseline ($P < 0.05$).

Baseline muscle temperature was 36.3 ± 0.3^{o}C, 35.6 ± 0.3^{o}C and 34.4 ± 0.8^{o}C at a probe depth of 3 cm, 2 cm and 1 cm, respectively. Muscle temperature reduced over time generally decreasing immediately post-immersion with values remaining below baseline at 3 h after immersion ($P < 0.05$; Figure 5.3.2.). These changes depended on probe depth ($F = 327.964$, $P < 0.001$). At a probe depth of 3 cm and 2 cm, greater declines in muscle temperature were detected at 1.5 h post-immersion period compared with immediately post-immersion (Figure 5.3.2. A, B). At a probe depth of 1 cm, the greatest decline in muscle temperature was observed immediately post-immersion, with a more gradual increase during the 6 h post-immersion (Figure 5.3.2. C). At the end of post-immersion period, muscle temperature reached baseline ($P > 0.05$).

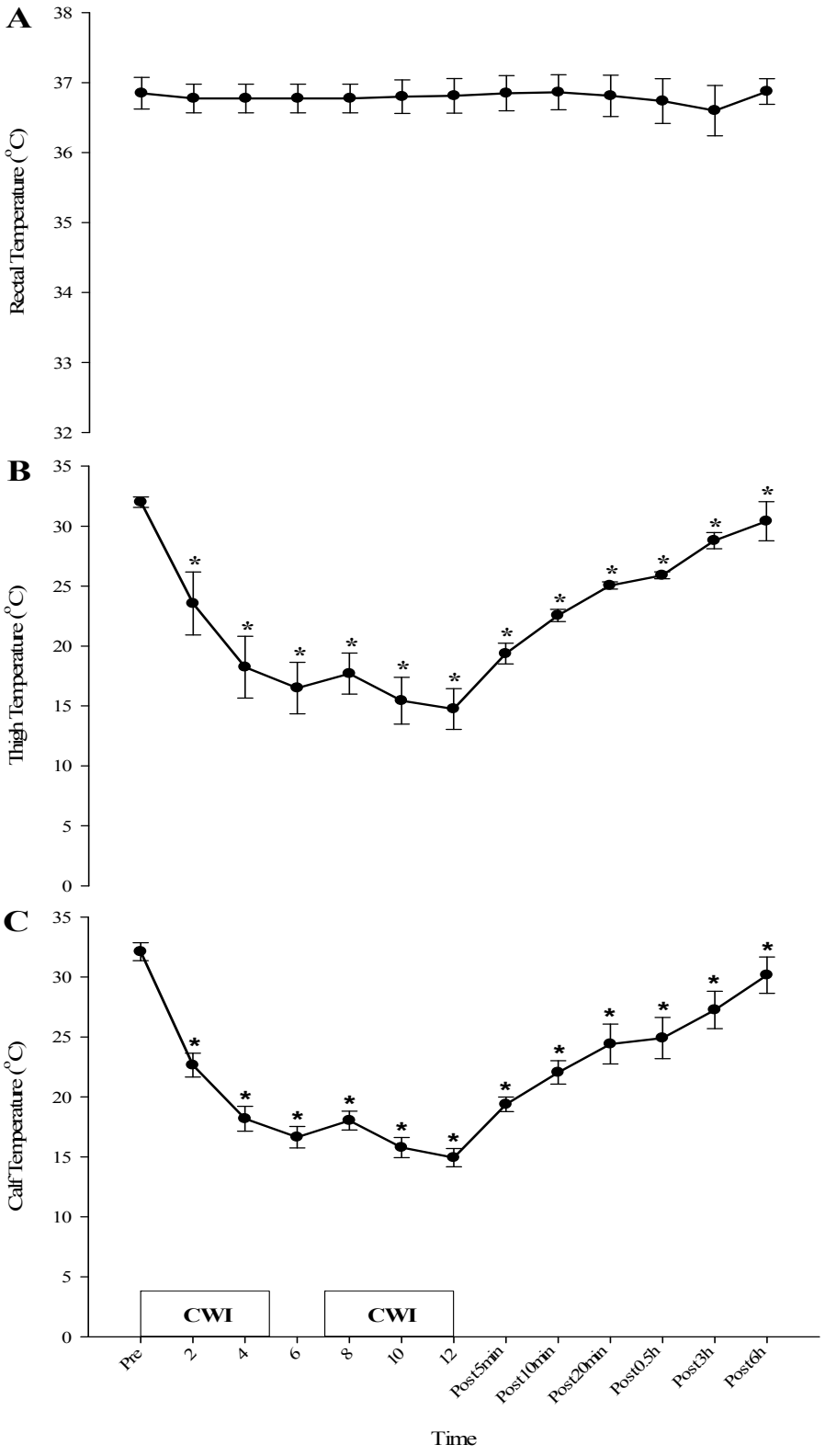

Figure 5.3.1. Rectal (A) and skin (thigh; B and calf; C) temperature during immersion and the 6 h post-immersion period (n = 10, mean ± SD). A main effect was found for time for skin temperature (thigh; F = 205.145, calf; F = 288.341, $P < 0.001$). *$P < 0.05$; significantly different from pre-immersion.

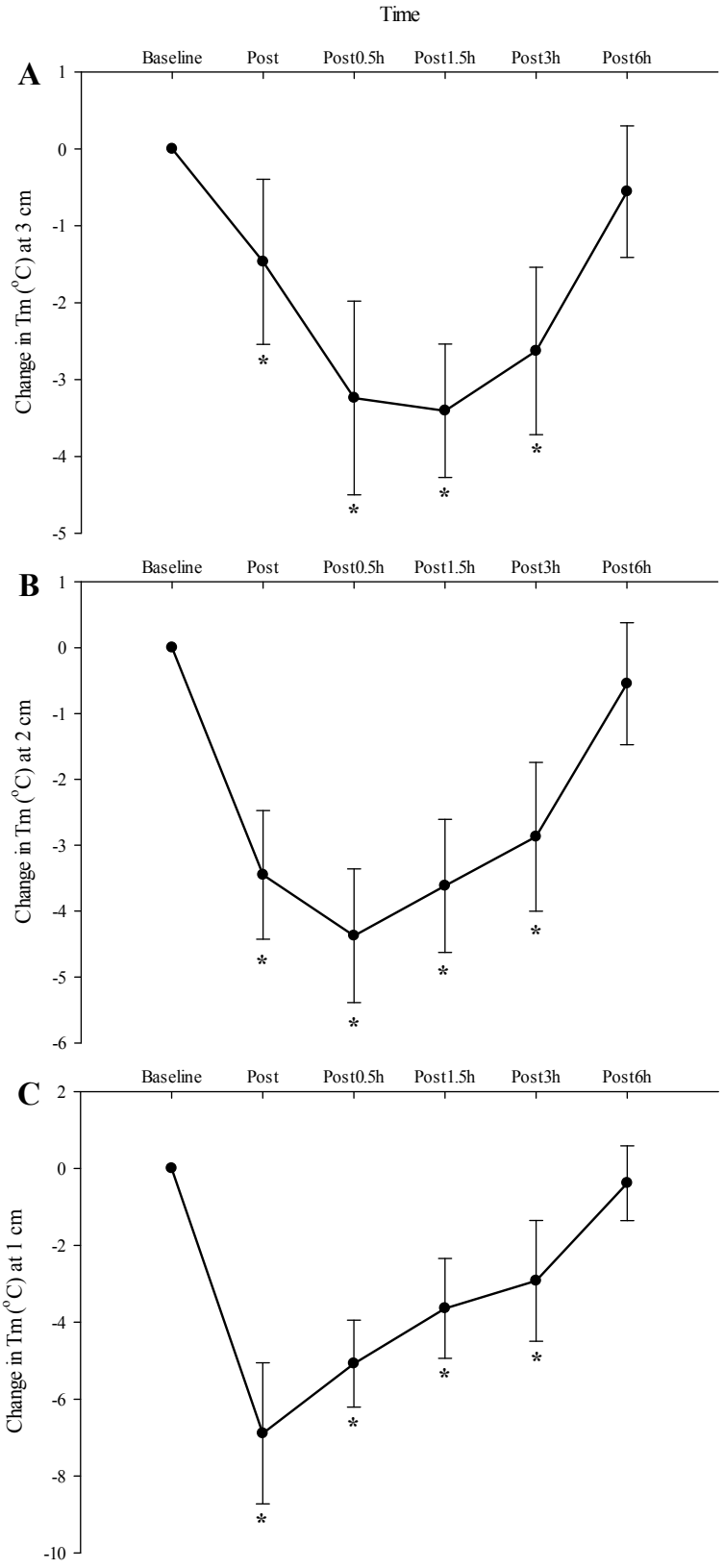

Figure 5.3.2. Changes (**Δ**) in muscle temperature immediately before and after immersion, 0.5 h, 1.5 h, 3 h and 6 h after immersion at temperature probe depths of 3 cm (A), 2 cm (B) and 1 cm (C) (n = 10, mean ± SD). *$P < 0.05$; significantly different from pre-immersion.

5.3.2. Metabolic responses

Heart rate increased (Δ 13 beats·min^{-1}) during the initial period of CWI ($P < 0.001$; Figure 5.3.3. A). Heart rate returned to baseline after 4-min of immersion and gradually decreased during the remaining immersion period and post-immersion. At the end of post-immersion period, heart rate remained below baseline ($P = 0.02$). Oxygen uptake was elevated at 6-min (Δ 1.9mL.kg^{-1}.min^{-1}; $P = 0.03$) and 8-min (Δ 1.5mL.kg^{-1}.min^{-1}; $P = 0.04$) of immersion (Figure 5.3.3. B). Oxygen uptake decreased following immersion period and remained below pre-immersion throughout the 30-min recovery period, with significant difference at 20-min post-immersion (Δ -1.5 mL.kg^{-1}.min^{-1}; $P = 0.05$).

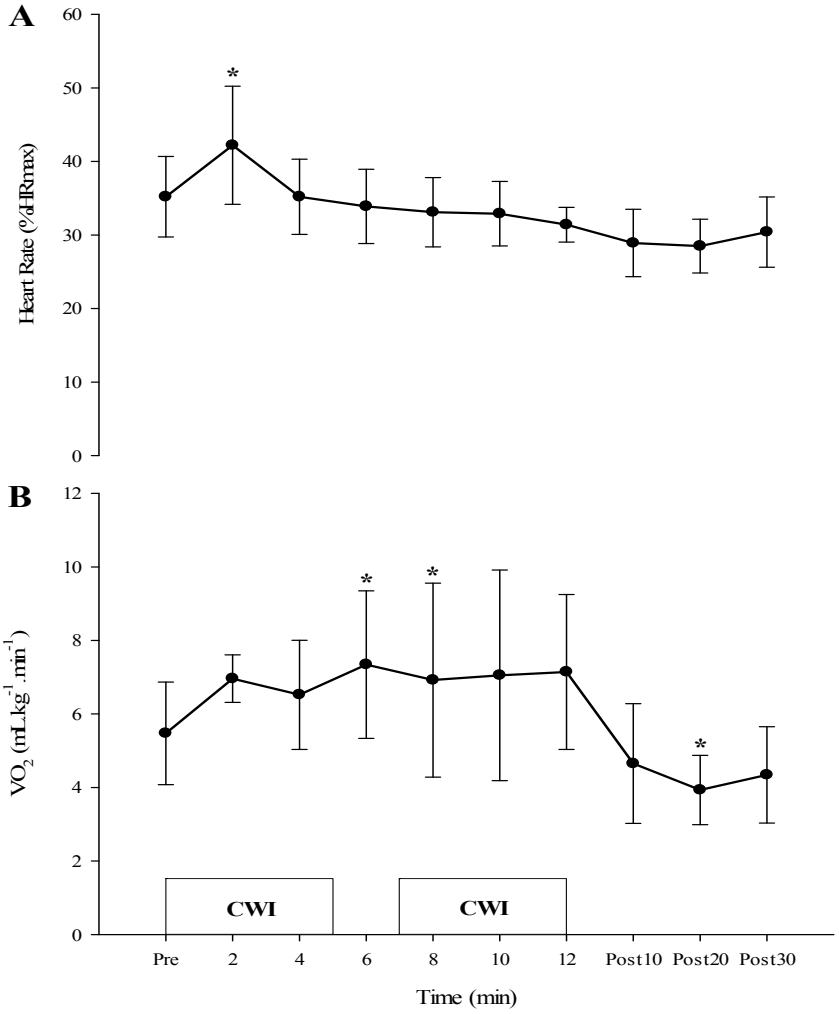

Figure 5.3.3. % HR_{max} (A) and oxygen consumption (B) during immersion and post-immersion period (n = 10, mean ± SD). A main effect for time was found for heart rate ($F = 13.113$, $P < 0.001$), oxygen consumption ($F = 10.22$, $P < 0.001$). *$P < 0.05$; significantly different from pre-immersion.

5.3.3. Subjective shivering response

Subjective rating of shivering increased immediately following immersion (P = 0.01), a difference that remained throughout the immersion period (P < 0.05; Figure 5.3.4.). It returned to baseline at 20-min post-immersion and remained by the end of post-immersion.

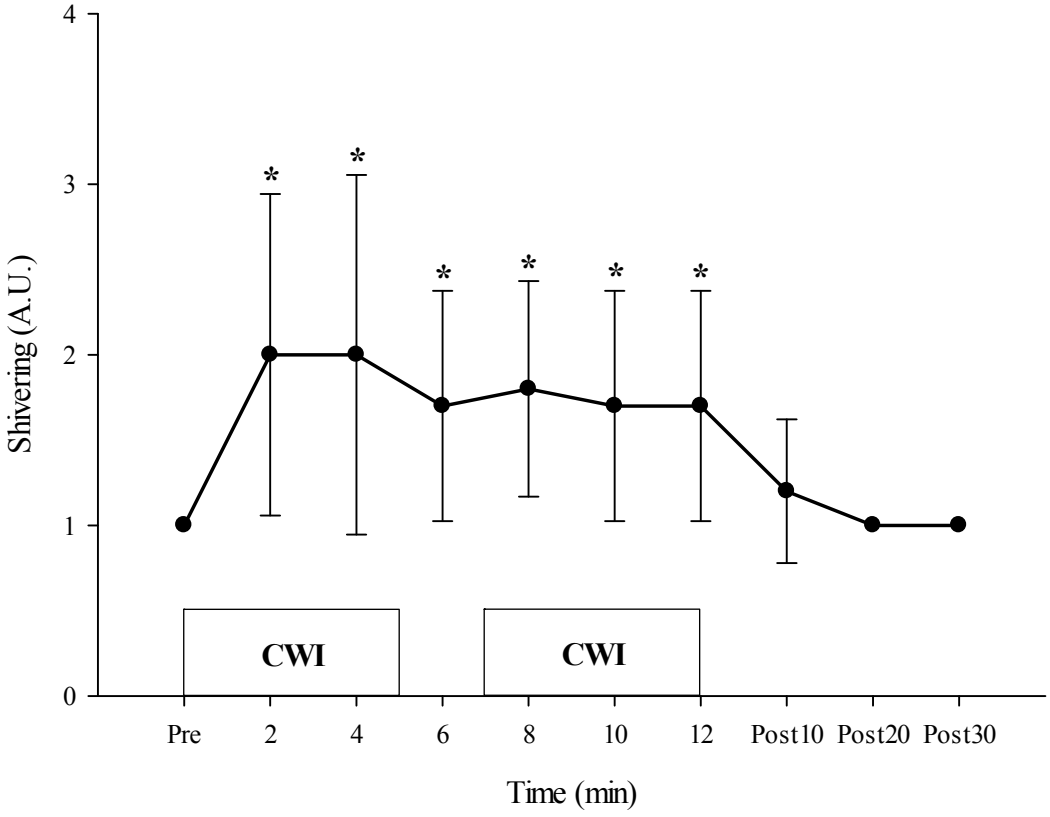

Figure 5.3.4. Shivering during immersion and post-immersion period (n = 10, mean ± SD). *P < 0.05; significantly different from pre-immersion.

5.3.4. Blood variables

There was no difference in adrenaline before or after the immersion period ($F = 1.589$, $P = 0.22$; Figure 5.3.5. A). In contrast, noradrenaline was significantly increased 3 h post-immersion and concentrations continued to increase 6 h post-immersion period ($P < 0.01$; Figure 5.3.5. B).

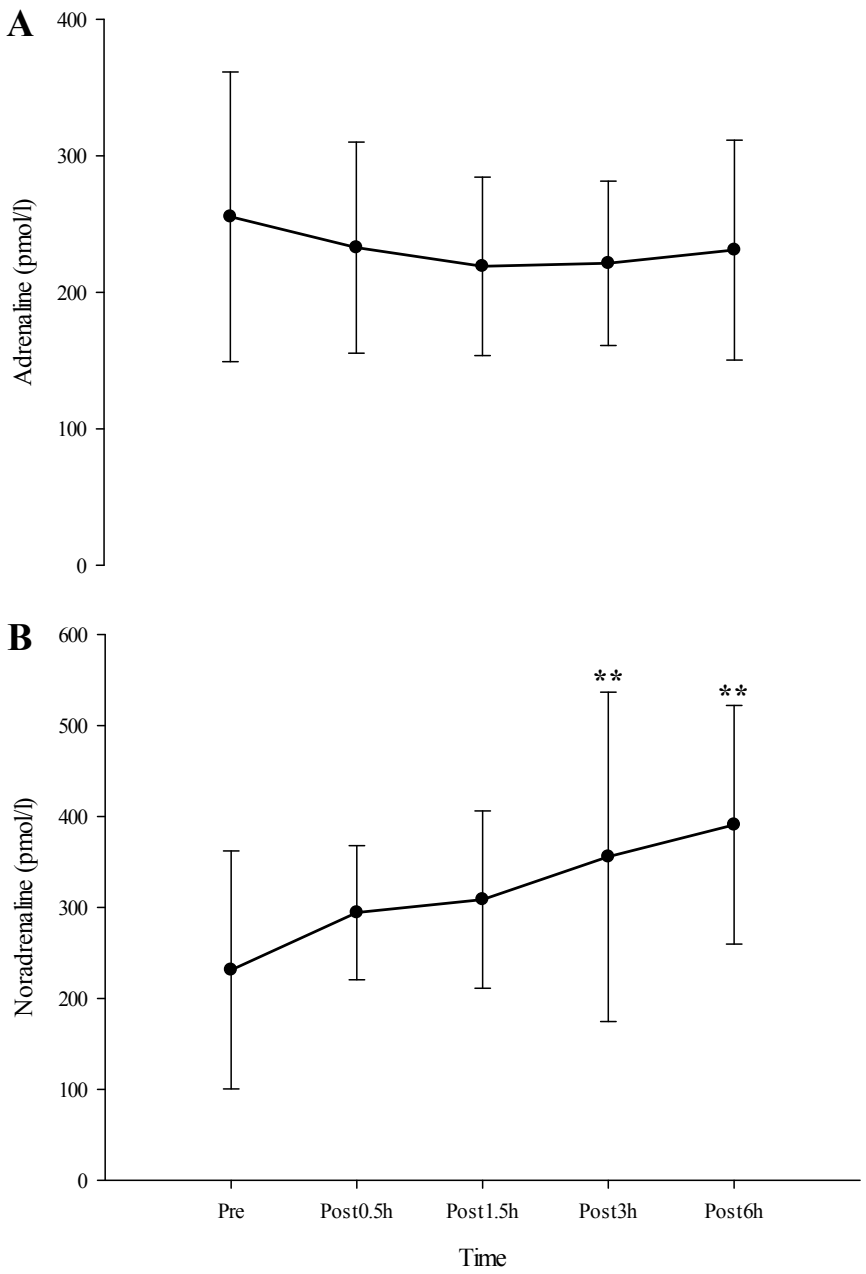

Figure 5.3.5. Adrenaline (A) and noradrenaline (B) pre-immersion and post-immersion period (n = 10, mean ± SD). A main effect for time was found for noradrenaline ($F = 5.904$, $P = 0.02$). **$P < 0.01$; significantly different from pre-immersion.

5.3.5. PGC-1α mRNA and protein content

Muscle PGC-1α mRNA content was significantly increased ~1.3-fold and 1.4-fold at 3 h (P = 0.001) and 6 h post-immersion, respectively ($P < 0.001$; Figure 5.3.6. A). No change in PGC-1α protein was observed any stage post-immersion ($F = 1.545$, $P = 0.25$; Figure 5.3.6. B).

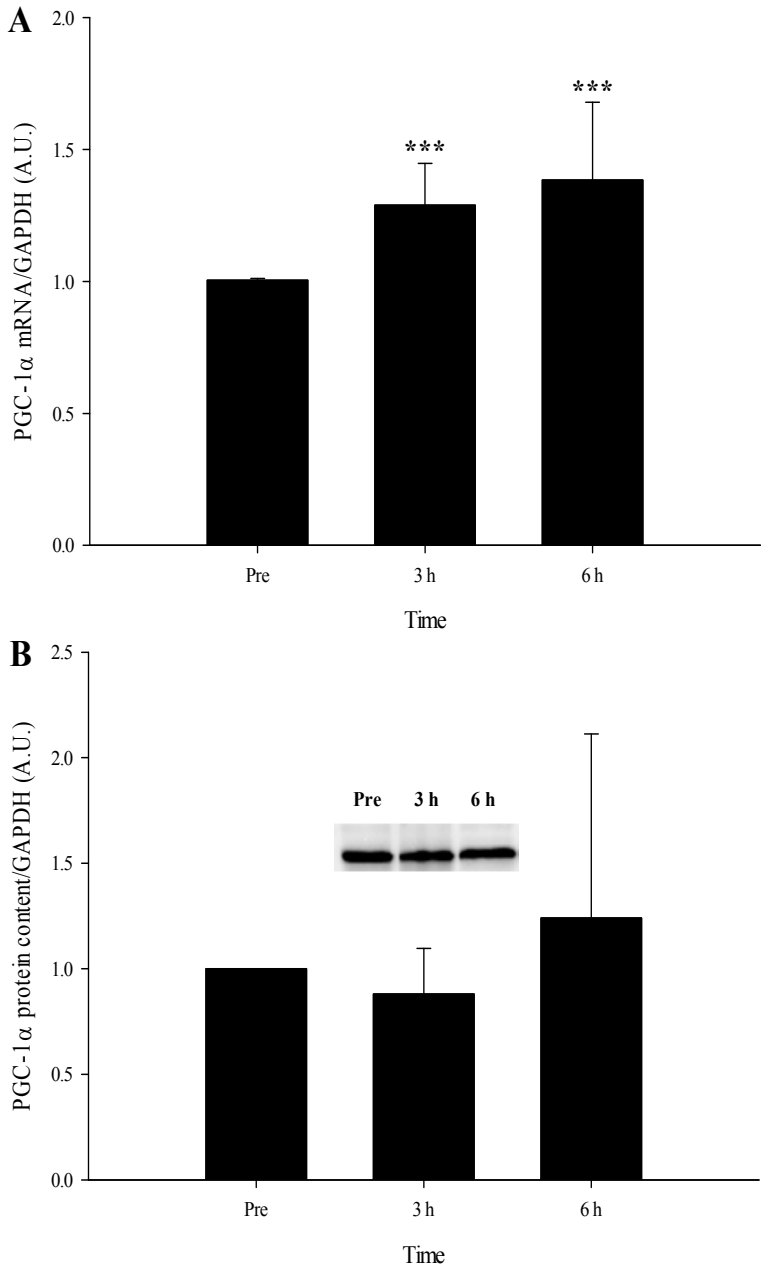

Figure 5.3.6. PGC-1α mRNA (A) and PGC-1α protein (B) pre-immersion and post-immersion period (n = 10, mean ± SD). A main effect for time was found for PGC-1α mRNA ($F = 13.932$, $P = 0.01$). ***$P \leq 0.001$; significantly different from pre-immersion.

5.3.6. VEGF mRNA and protein content

$VEGF_{165}$ mRNA was significantly increased ~1.9-fold and ~2.2-fold at 3 h ($P = 0.03$) and 6 h post-immersion, respectively ($P = 0.009$; Figure 5.3.7. A). However, there were no change in $VEGF_{total}$ mRNA ($F = 1.155$, $P = 0.33$; Figure 5.3.7. B) and VEGF protein ($F = 0.008$, $P = 0.97$; Figure 5.3.7. C) content during post-immersion period.

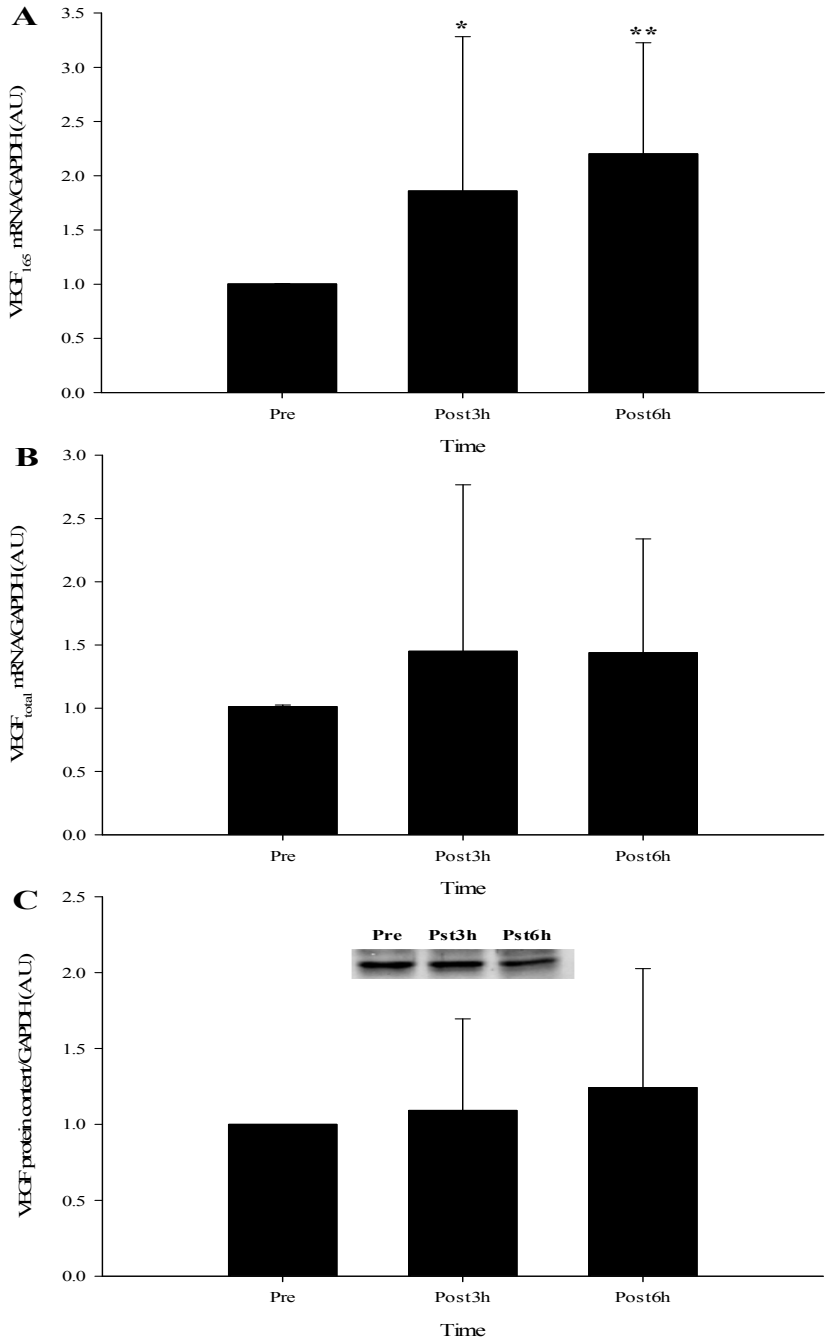

Figure 5.3.7. $VEGF_{165}$ mRNA (A), $VEGF_{total}$ mRNA (B) and VEGF protein (C) pre-immersion and post-immersion period (n = 10, mean ± SD). A main effect for time was found for $VEGF_{165}$ mRNA ($F = 6.004$, $P = 0.02$). *$P < 0.05$, **$P < 0.01$; significantly different from pre-immersion.

5.4. DISCUSSION

In the present investigation CWI at rest induced a significant increase in PGC-1α and $VEGF_{165}$ mRNA expression at 3 h post-immersion in human skeletal muscle which remained up-regulated for at least 6 h post-immersion. To the authors knowledge this is the first study to date to demonstrate that acute, noxious, lower limb CWI as typically by athletes during training and competition enhance signalling pathways important in mitochondria biogenesis and angiogenesis at rest.

Cryotheraphy has long been established as an effective treatment for alleviating exercise-induced muscle damage since decreasing local tissue temperature and temperature-induced reductions in blood flow are thought to diminish metabolism, pain, local edema, swelling and inflammation in injured tissue (Merrick et al., 1999). In the present study, resting CWI decreased superficial and deep muscle temperatures for at least 3 h post-immersion. Greater reductions in superficial temperature were observed immediately after immersion with deep muscle temperature declining to a greater extent 1.5 h after immersion. These findings are consistent with recent observations which indicated that post-exercise CWI reduced superficial and deep muscle temperatures immediately after immersion with further decreases in deep temperatures observed 30-min after immersion (Gregson et al., 2011). This phenomenon can be explained by removal of the cooling source per se, subsequent exposure of the limbs to room air temperature. Furthermore, the hemodynamic exchange between the cooler surface and the warmer deeper tissue leads to a gradual increase in superficial tissue temperature and a corresponding decrease in deep tissue temperature over time (Enwemeka et al., 2002). Skin temperature dropped by ~15^{o}C following CWI gradually increasing until 6 h post-immersion. This reduction is similar with the previous study reported that 10-min CWI in 8^{o}C water reduced skin temperature by approximately 15^{o}C (Gregson et al., 2011). Unlike muscle and skin temperature, there was no difference in rectal temperature in the present study. This is in disagreement with recent study, as it has been reported that rectal temperature declines significantly when subjects completed CWI in 8^{o}C water (Gregson et al., 2011). The discrepancy maybe explained by differences in baseline between present (36.9^{o}C) and previous study (approximately 37.3^{o}C) at rest. Due to the greater temperature difference between the body and the modality leads to the transfer of heat more quickly from the warmer object to the cooler (Palmer and Knight, 1996), rectal temperature might significantly reduce throughout the post-immersion period in the previous study. Indeed, rectal

temperature at 30-min post-immersion in the previous study (approximately 37.1^{o}C) was still higher compared with baseline of present study (36.9^{o}C).

PGC-1α was originally found as a cold-inducible transcription coactivator of the adaptive thermogenesis response to environmental conditions such as cold exposure (Cannon et al., 1998). Indeed, animal studies (Puigserver et al., 1998, Oliveira et al., 2004) have shown that chronic whole body cooling increases PGC-1α mRNA. More recently, Slivka et al. (2012) observed that PGC-1α mRNA increased to a greater degree after 1 h cycling exercise and recovery under the cold laboratory temperatures (7^{o}C) than in the room temperature trial (20^{o}C) in human skeletal muscle. However, whilst these whole body cooling studies focused on exercise in difference environmental conditions, no study to date, has looked at whether cold per se (e.g. cooling without prior exercise) increases PGC-1α and VEGF expression. Furthermore, the magnitude of the increase in sympathetic discharge to skeletal muscle is influenced by the size of the tissue area exposed to cooling (Seals, 1990) and the magnitude of the cooling stimulus (Kregel et al., 1992). Consequently, PGC-1α and VEGF expression may be influenced to different degrees by nonnoxious whole body skin surface cooling relative to localized noxious cooling (CWI). We presently observed a 1.3-fold increase in PGC-1α mRNA 3 h after immersion which remained elevated 6 h (1.4-fold) after immersion. These findings are consistent with previous research demonstrating that cold exposure to 4^{o}C for 4 days promoted a significant increase of 5.3-fold in PGC-1α mRNA content in animal skeletal muscle (Oliveira et al., 2004). The quantitative and temporal patterns of change in PGC-1α mRNA did not reflect the changes in PGC-1α protein. These finding is in contrast with the result from the previous study reported that PGC-1α protein content was increased in rodent muscle (Oliveira et al., 2004). The reasons for the disparate result by Oliveira et al. (2004) and the present study are not clear but may be related to differences in the subject (animal vs. human) and/or the cold exposure condition (4 days at 4^{o}C air vs. 10-min at 8^{o}C water). However, exercise studies would suggest that increase in PGC-1α protein is typically only observed within days of exercise (Baar et al., 2002, Perry et al., 2010).

The mechanism by which resting CWI increases PGC-1α involves the sympathetic nervous system through the β_3-AR (Boss et al., 1999), leading to enhanced cAMP signalling and the activation of protein kinase A (Fernandez-Marcos and Auwerx, 2011). In the present study, resting CWI elicited a marked increase in noradrenaline at 3 h (~54%) and 6 h (~69%) after immersion. This result is supported by Sramek et al. (2000) who reported that immersion in

water at 14°C for 1 h induced a stimulation of thermogenesis and activation of the sympathetic nervous system, as evident from the increased noradrenaline concentrations (~530%) throughout immersion and post-immersion period. Differences in the magnitude of increase in noradrenaline between the studies likely reflect the magnitude of the cold stimulus applied (e.g. period of CWI; 10-min vs. 1 h). Recent observation from our laboratory indicate that increase in noradrenaline concentrations following CWI is dependent on reduced skin temperature rather than core temperature (Gregson et al., 2012). The decline in skin temperature provokes a profound reflex increase in sympathetic nerve activity through activation of nonnoxious thermoreceptors (Hensel and Boman, 1960). The other possible mechanism responsible for the increase in PGC-1α mRNA with CWI is associated with greater increases in energy expenditure (Slivka et al., 2013). Indeed, in the current study, oxygen uptake was immediately elevated after immersion, which persisted throughout the immersion period. Subjective rating of shivering significantly increased immediately post-immersion, a difference that remained throughout the immersion period. These findings support previous study by Stocks et al. (2004) that showed involuntary contraction of the quadriceps associated with shivering increase metabolic heat production 5-6 times above resting values.

Although a number of genes are involved complex process of angiogenesis, VEGF is the key pro-angiogenic factor in skeletal muscle (Ferrara, 2001). Exercise is accepted as a valid method by which to induce VEGF expression in animal (Gavin et al., 2000) and human (Richardson et al., 1999, Gavin et al., 2004). Prolonged thermogenesis in a cold environment had also caused an increase in a capillarity density in animal (Sillau et al., 1980, Suzuki et al., 1997) and human skeletal muscle (Bae et al., 2003). To our knowledge, only one previous study has examined the effect of CWI on VEGF expression in animals, which demonstrated that the expression of $VEGF_{165}$ mRNA and protein after prolonged CWI were markedly higher than these of Cont (Kim et al., 2005). Consistent with previous study, we observed that resting CWI increased $VEGF_{165}$ mRNA during post-immersion period. However, there was no change in $VEGF_{total}$ mRNA content in the present study. These findings would seem to suggest that $VEGF_{165}$ isoform acting as survival factor for vascular endothelial cells (Ferrara et al., 2003) is more responsive to thermogenesis in cold environment among encoding VEGF isoforms (121, 165, 189 and 206) in humans. In the present study, VEGF protein concentration was unchanged during post-immersion period. This result is in contrast with results from the previous study that reported that CWI increased VEGF protein content

in animal skeletal muscle under resting conditions (Kim et al., 2005). The reason for the difference between studies is not readily apparent but the acute CWI (10-min at 8^{o}C) in the present study may not be enough to stimulate an increase in VEGF protein content compared with the chronic condition (1 h day^{-1}, 5 days week^{-1} and for 20 weeks) in the previous study.

One limitation of the current study surrounds the failure to use a control condition with baseline measures serving as the control. However, the increase in PGC-1α mRNA and $VEGF_{165}$ mRNA may predominately reflect cooling per se rather than any increased mediated sympathetic stress from repeated biopsies. These results are supported by previous findings which indicated that muscle biopsy sampling at sites separated by 3 cm and 2 h does not increase VEGF mRNA expression in resting biopsy samples (Gavin et al., 2004). Similarly Cartoni et al. (2005) reported that repeat biopsies at pre, post (46.2 ± 7.4-min), 2 h and 24 h post-biopsy did not induce changes in mRNA level for transcriptional regulators that control mitochondrial biogenesis and functions, including PGC-1α mRNA.

In summary, the present data demonstrate for the first time that acute, noxious, CWI at rest augments the expression of PGC-1α mRNA and $VEGF_{165}$ mRNA in human skeletal muscle. CWI strategies frequently undertaken by athletes during training and competition may therefore potentially influence the upstream signalling pathways associated with mitochondrial biogenesis and angiogenesis. This protocol will therefore be used in future studies which evaluate the effects of acute post-exercise CWI on PGC-1α and VEGF expression in human skeletal muscle.

CHAPTER 6

COLD WATER IMMERSION AUGMENTS THE EXERCISE-INDUCED EXPRESSION OF PGC-1α IN HUMAN SKELETAL MUSCLE

This study was presented orally at the Annual Congress European College of Sport Science (ECSS) (see Appendix 1), Liverpool July 2011.

6.1. INTRODUCTION

The stress associated with training and competition often temporarily impairs an athlete's performance. Significant attention in the literature has therefore focused upon the development of recovery strategies which serve to alleviate short-term fatigue and/or the symptoms associated with exercise-induced muscle injury (Cheung et al., 2003). Recent interest has centred upon the use of cryotherapy strategies such as CWI (Wilcock et al., 2006). Decreases in muscle temperature and whole limb blood flow associated with CWI (Gregson et al., 2011, Mawhinney et al., 2013) are thought to reduce pain and inflammation in injured tissue and has therefore been widely used in the treatment of acute traumatic injury (Merrick et al., 1999). Since the inflammatory process is integral in the aetiology of exercise-induced muscle damage (Smith, 1991), cryotherapy undertaken via CWI has therefore been frequently used in an attempt to reduce the physiological and functional deficits associated with exercise-induced muscle damage (Leeder et al., 2012).

Alongside the physiological changes which underpin the recovery of muscle function, signalling pathways important for chronic training adaptation are also activated during the first few hours of recovery from exercise (Hildebrandt et al., 2003). In this regard, it is well established that regular endurance exercise mediates an increase in skeletal muscle mitochondrial density and angiogenesis in response to the increased metabolic requirements during exercise (Holloszy, 1967, Prior et al., 2003). Considerable work over the last decade has shown that PGC-1α, a transcriptional co-activator, coordinates cell signalling pathways which mediates exercise-induced mitochondrial adaptation and thus serve as the "master regulator of mitochondrial biogenesis" (Puigserver and Spiegelman, 2003). This is supported by numerous observations which indicate that both acute bouts of exercise (Perry et al., 2010, Bartlett et al., 2012, Bartlett et al., 2013) and extended periods of endurance training (Perry et al., 2010) increase the expression of PGC-1α. In addition to its role in mediating mitochondrial biogenesis, PGC-1α also plays an important role in exercise-induced angiogenesis through its effects on VEGF (Chinsomboon et al., 2009). Indeed, acute aerobic and resistance exercise induce an increase in the expression of VEGF mRNA and protein (Ryan et al., 2006, Gavin et al., 2007a).

In addition to its role in exercise-induced mitochondrial biogenesis and angiogenesis, PGC-1 also plays an important role in linking nuclear receptors to the transcriptional program of adaptive thermogenesis (Puigserver et al., 1998). These effects of cool exposure are thought to be mediated by increased β-adrenergic activity (Himms-Hagen, 1989, Chinsomboon et al., 2009) with the decline in body temperature provoking a profound reflex increase in sympathetic nerve activity through activation of nonnoxious thermoreceptors (Hensel and Boman, 1960). Both acute (Puigserver et al., 1998) and chronic (Oliveira et al., 2004) whole body cold exposure increase PGC-1α mRNA, VEGF mRNA and their protein expression in animals at rest (Kim et al., 2005). Similarly, in humans we have previously shown that lower body CWI undertaken at rest increases the expression of PGC-1α and VEGF mRNA in skeletal muscle (Chapter 5) whilst Slivka et al. (2013) observed increased PGC-1α expression during recovery in cold (7^{o}C) ambient temperatures than during moderate (20^{o}C) ambient temperature following exercise. It is expected that lower limb CWI following exercise may further increase in the exercise-induced expression of PGC-1α and VEGF.

Limited work to date has examined the influence of post-exercise cooling on PGC-1α and VEGF expression in humans. Slivka et al. (2012) recently reported increased expression of PGC-1α mRNA following 1 h cycling exercise (60% W_{max}) and 3 h of whole body recovery in cold (7^{o}C) ambient temperatures. Similarly, 3 h of whole body cooling per se (7^{o}C) also increased PGC-1α mRNA expression relative to recovery in moderate temperatures after 1 h cycling exercise (Slivka et al., 2013). However, the magnitude of the increase in sympathetic discharge to skeletal muscle is influenced by both the size of the tissue area exposed to cooling (Seals, 1990) and the magnitude of the cooling stimulus (Kregel et al., 1992). Consequently, more intense localized noxious cooling via CWI as utilised by athletes may influence signalling pathways associated with mitochondrial biogenesis and angiogenesis to a different extent compared with nonnoxious whole body skin surface cooling (Slivka et al., 2013). Therefore, the aim of the present study was to examine the influence of post-exercise CWI on PGC-1α and VEGF expression in human skeletal muscle following an acute bout of high-intensity intermittent exercise.

6.2. METHODS

6.2.1. Participants

Nine healthy active male volunteered to participate in the study [mean ± SD: age 25 ± 4yr, height 174.7 ± 4.7cm, body mass 78.3 ± 9.2kg and the participants reached $\dot{V}O_{2max}$ (58.1 ± 7.2mL.kg^{-1}.min^{-1})]. One of the participants took part in both studies (Chapter 5 and 6).

6.2.2. Experimental design

Prior to the completion of the experimental trials baseline assessments (see section 3.2.), a venous blood sample (see section 3.9.) and muscle biopsy (see section 3.11.) were completed.

Rating of perceived exertion (RPE) was obtained immediately following each exercise bout (see section 3.5.1.). Heart rate (see section 3.3.1.), oxygen consumption ($\dot{V}O_2$; see section 3.3.2.), shivering (see section 3.5.2.), rectal (T_{re}) and skin temperature (thigh and calf; see section 3.6.) were measured continuously during CWI and recovery for 30-min. Muscle temperature was measured post-exercise and CWI and 1 h and 3 h post-exercise. Muscle biopsies were obtained immediately post-exercise and 3 h post-exercise. Venous blood samples were also taken immediately post-exercise and 1 h and 3 h post-exercise. A schematic illustration of the experimental design is shown in figure 6.2.1..

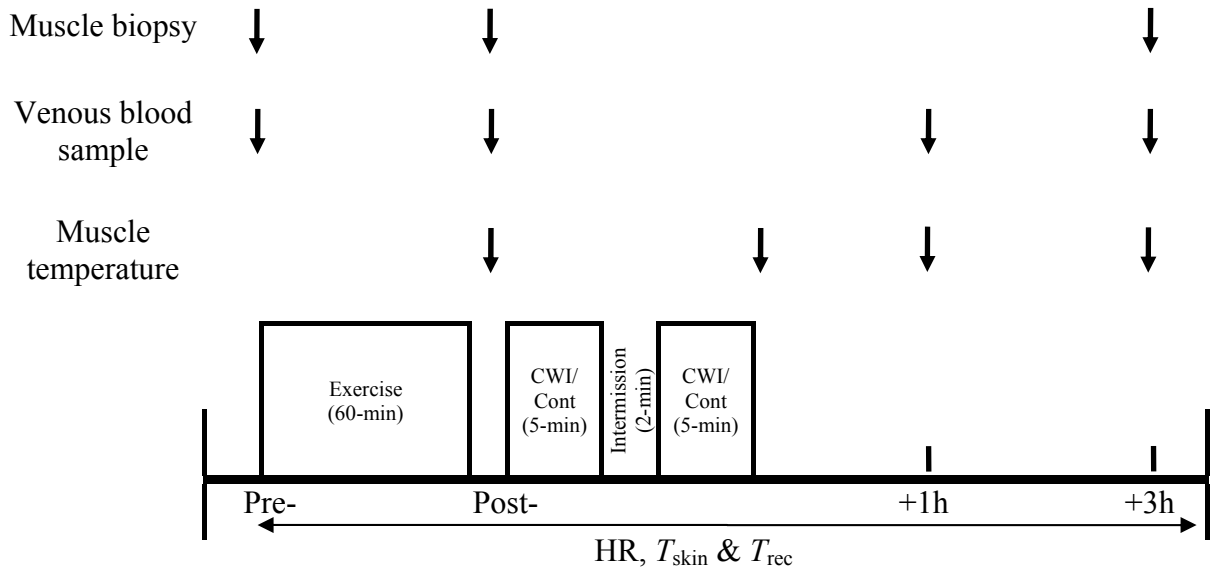

Figure 6.2.1. Schematic illustration of the experimental design.

6.2.3. Statistical analysis

All data are presented as means ± SD. A two-factor (condition × time) within-participants general linear model was undertaken to determine any treatment differences between the Cont and CWI conditions. The assumption of sphericity (homogeneity of covariance) was assessed and corrected for using the Huynh-Feldt epsilon (Atkinson and Nevill, 2001). Because there were only 2 levels in the main effect of condition, follow-up multiple comparisons were not necessary. A significant effect of time was followed up with planned multiple contrasts in line with the a priori hypotheses. Therefore, data at the specific time points were compared with the baseline (first) time point using Newman-Keuls multiple contrasts. Where a significant interaction between condition and time was observed, differences between conditions were examined at each time point using Newman-Keuls multiple contrasts. Baseline values were compared using a paired samples *t* test. One sample *t* test was used to compare the immersion-induced change in shivering and changes of muscle temperature between baseline and other time points. The alpha level for evaluation of statistical significance was set at $P < 0.05$.

6.3. RESULTS

6.3.1. Exercise response

Exercise HR ($P > 0.05$) and RPE ($P > 0.05$) were similar between Cont and CWI conditions. Heart rate significantly increased from the first to final 8th bout under both conditions (Cont, 176±8 ~ 183±9 beats·min^{-1}; CWI, 179±6 ~ 183±7 beats·min^{-1}; $F = 41.428$, $P < 0.001$). Heart rate during the final exercise bout equated to 94% and 95% of HR_{max} respectively in the Cont and CWI conditions. Rating of perceived exertion following the final 8th bout was 19 ± 1 and 20 ±1 in the Cont and CWI conditions respectively.

6.3.2. Thermoregulatory responses

Rectal temperature was similar prior to and throughout immersion and the post-immersion period under both conditions ($P > 0.05$; Figure 6.3.1. A). Rectal temperature declined throughout immersion and the post-immersion recovery period in both conditions with the greatest decrements observed at 3 h post-exercise ($P < 0.05$; Figure 6.3.1. A). Thigh and calf skin temperature were similar prior to immersion under both conditions (Figure 6.3.1. B, C). There was a greater rate of decrease in thigh and calf temperature in CWI compared to the Cont condition ($P < 0.05$) during the immersion period, with the largest difference between conditions occurring at the end of the 10-min immersion period (Figure 6.3.1. B, C). Thigh and calf temperature increased following 10-min of immersion in CWI with values remaining relatively stable in the Cont condition, however, thigh and calf temperature remained lower in CWI compared to Cont at 3 h after exercise ($P < 0.05$; Figure 6.3.1. B, C).

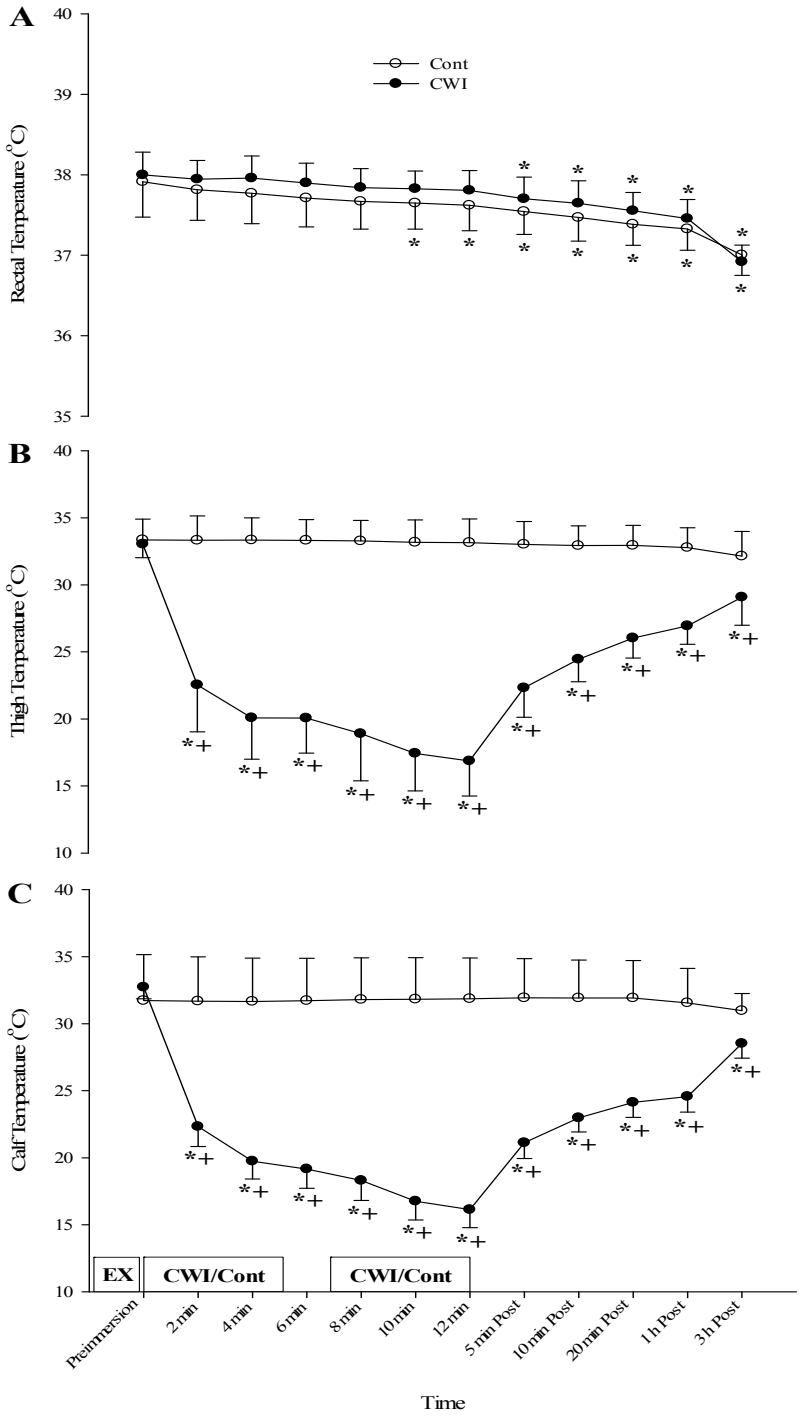

Figure 6.3.1. Rectal (A) and skin (thigh; B and calf; C) temperature during recovery in the CWI and Cont conditions (n = 10, mean ± SD). A main effect for time was found for rectal temperature (F = 115.187, P < 0.001). Main effects for condition (F = 18.093, P = 0.002; F = 4.864, P = 0.05) and time (F = 119.979, F = 109.56, P < 0.001) were found, along with a significant interaction between condition and time for skin (thigh and calf) temperature (F = 92.806, F = 146.166, P < 0.001). *P < 0.05; significant difference from pre-immersion. ^{+}P < 0.05; significant difference between conditions.

Pre-immersion muscle temperature was similar between CWI and Cont conditions at a probe depth of 3 cm (Cont, 39.4 ± 0.6°C; CWI, 39.4 ± 0.6°C; $P > 0.05$) and 2 cm (Cont, 39.0 ± 0.6°C; CWI, 38.7 ± 0.6°C; $P > 0.05$). Muscle temperature was reduced over time ($F = 95.891$, $P < 0.001$; Figure 6.3.2.), generally decreasing immediately post-immersion ($P < 0.05$) with further decreases observed 1 h and 3 h post-exercise ($P < 0.05$). These changes depended on probe depth ($F = 10.779$, $P < 0.001$). At the depth of 3 cm, greater declines in muscle temperature were detected at 1 h post-exercise compared with immediately post-immersion (Figure 6.3.2. A). At a probe depth of 2 cm (Figure 6.3.2. B), the greatest decline in muscle temperature was observed immediately post-immersion, with a more gradual decline during the 1 h post-exercise period.

The changes in muscle temperature over time also depended upon conditions ($F = 29.317$, $P < 0.001$). Immediately after immersion, changes in muscle temperature were greater in CWI compared with Cont (Cont, 2.0 ± 0.6°C; CWI, 3.7 ± 2.2°C) and the greater difference was continued until 3 h after exercise. These differences were in turn dependent on probe depth ($F = 50.208$, $P < 0.001$). Immediately after immersion, changes in muscle temperature at 3 cm were generally similar between conditions (Cont, 1.8 ± 0.4°C; CWI, 2.5 ± 1.0°C; $P > 0.05$). However, the changes in muscle temperature 1 h (Cont, 2.5 ± 0.6°C; CWI, 4.7 ± 1.3°C; $P < 0.05$) and 3 h after exercise (Cont, 3.0 ± 0.9°C; CWI, 4.6 ± 1.0°C; $P < 0.05$) were greater in CWI compared with Cont (Figure 6.3.2. A). At a depth of 2 cm (Figure 6.3.2. B), the reduction in muscle temperature was greater in CWI immediately after immersion, 1 h and 3 h after exercise ($P < 0.05$).

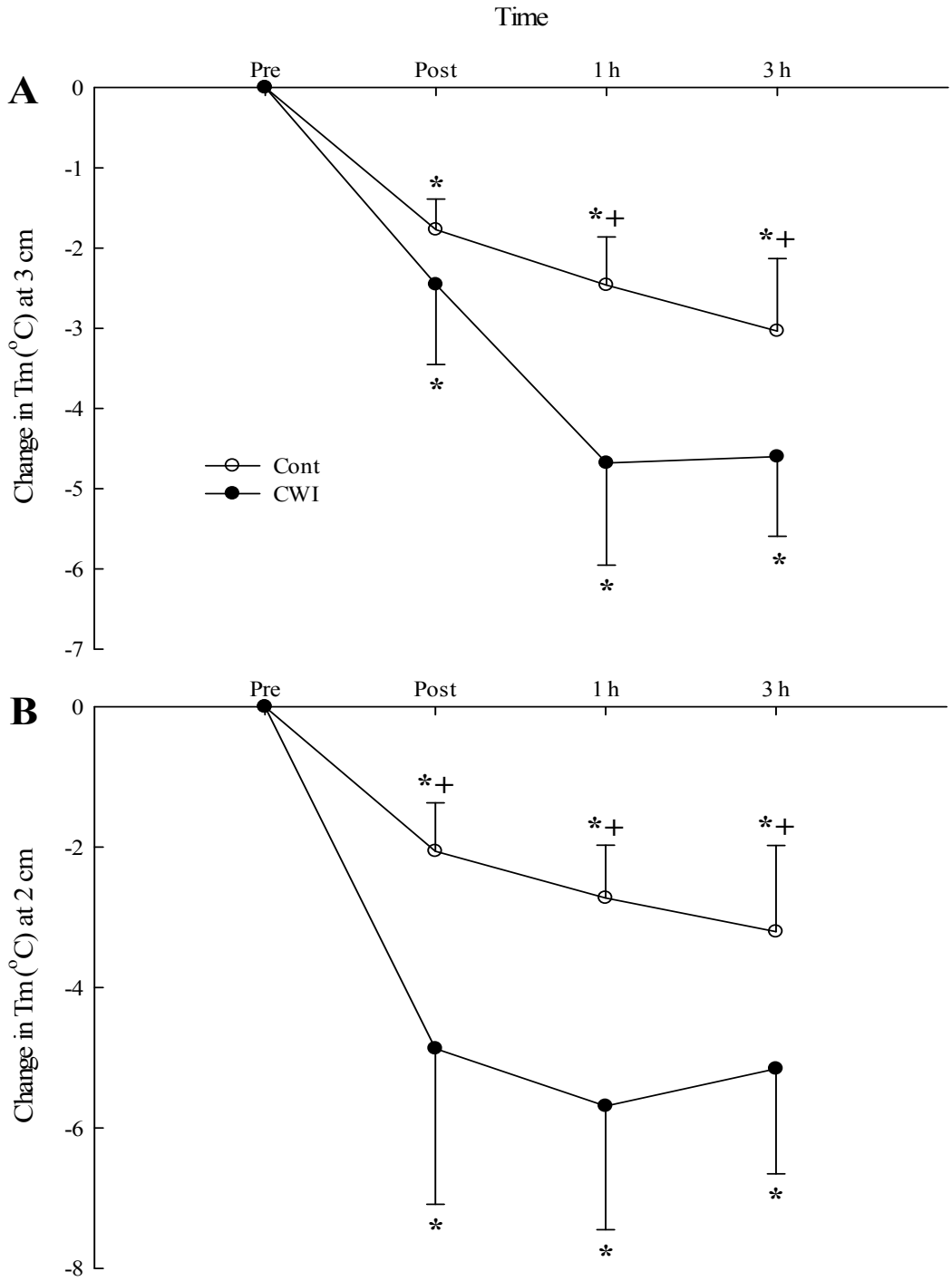

Figure 6.3.2. Changes (**Δ**) in muscle temperature immediately before and after immersion, 1 h and 3 h after exercise, at temperature probe depths of 3 cm (A) and 2 cm (B). (n = 10, mean ± SD). Main effects for condition (F = 29.317, P < 0.001) and time (F = 95.891, P < 0.001) were found, along with a significant interaction among condition, time and probe depth (F = 8.225, P < 0.001). *P < 0.05; significant difference from pre-immersion. ^{+}P < 0.05; significant difference between conditions.

6.3.3. Metabolic responses

Pre-immersion HR (Cont, 89 ± 10 beats·min^{-1} (45 ± 5% HR_{max}); CWI, 90 ± 8 beats·min^{-1} (46 ± 4% HR_{max}); $P > 0.05$) and $\dot{V}O_2$ (Cont, 6.7 ± 1.1mL.kg^{-1}.min^{-1}; CWI, 7.4 ± 1.1mL.kg^{-1}.min^{-1}; $P > 0.05$) was similar between conditions. HR was higher during the initial period of CWI compared to Cont, with the largest difference occurring after 2-min of immersion (Cont, 87 ± 10; CWI, 102 ± 6; $P < 0.05$) (Figure 6.3.3. A). HR during the 30-min post-immersion period was similar between conditions. Oxygen uptake was generally elevated throughout immersion in CWI compared to the Cont ($P < 0.05$) (Figure 6.3.3. B), however, values remained similar during the post immersion period.

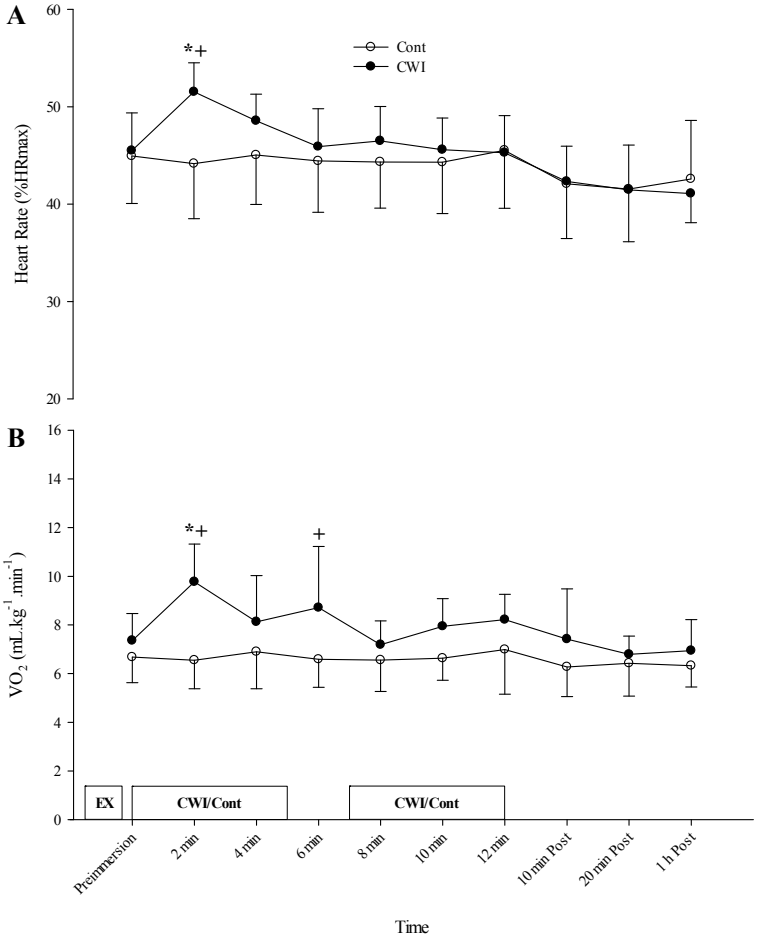

Figure 6.3.3. % HR_{max} (A) and oxygen consumption (B) during recovery in the CWI and Cont conditions (n = 9, mean ± SD). A main effect for time ($F = 17.457$, $P < 0.001$; $F = 5.076$, $P = 0.01$) was found with a significant interaction between condition and time for both heart rate and $\dot{V}O_2$ ($F = 10.115$, $P < 0.001$; $F = 2.082$, $P = 0.05$). *$P < 0.05$; significant difference from pre-immersion. $^{+}P < 0.05$; significant difference between conditions.

6.3.4. Subjective shivering response

Subjective rating of shivering was significantly elevated in CWI (slight shivering) compared to Cont (no shivering) during the 2-min and 8-min of immersion ($P < 0.05$; Figure 6.3.4.).

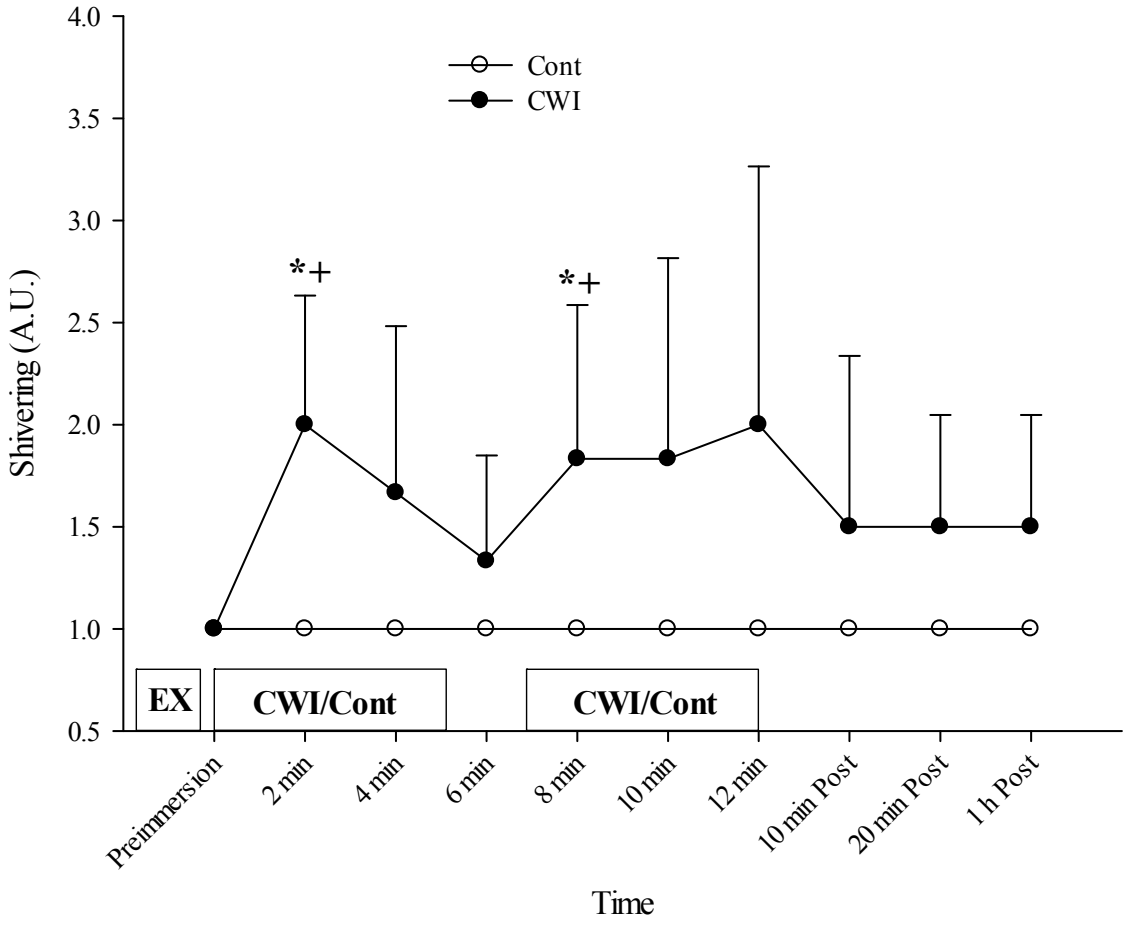

Figure 6.3.4. Shivering during recovery in the CWI and Cont conditions (n = 9, mean ± SD). *$P < 0.05$; significant difference from pre-immersion. $^{+}P < 0.05$; significant difference between conditions.

6.3.5. Blood variables

Significant higher blood lactate concentration were observed immediately after exercise compared with pre-exercise in both conditions (Cont, 7.4 ± 3.3 mmol·l^{-1}; CWI, 7.7 ± 3.0 mmol·l^{-1}; $P < 0.05$), with no significant interaction between condition ($P = 0.7$). Blood lactate were similar prior to immersion between Cont (2.2 ± 0.6 mmol·l^{-1}) and CWI (2.4 ± 0.5 mmol·l^{-1}), respectively ($P = 0.8$). Blood lactate was reduced 1 h post-exercise in both condition (Cont, 3.3 ± 1.2 mmol·l^{-1}; CWI, 3.4 ± 1.5 mmol·l^{-1}; $P > 0.05$) and remained unchanged by the end of post-exercise (Cont, 2.7 ± 0.7 mmol·l^{-1}; CWI, 3.0 ± 0.6 mmol·l^{-1}; $P > 0.05$).

Table 6.3.1. Plasma lactate before, immediately after exercise, 1 h and 3 h post-exercise in the CWI and Cont conditions ($n = 9$, mean ± SD).

	Cont				CWI			
	Pre	Post	1h	3h	Pre	Post	1h	3h
Lactate, mmol·l^{-1}	2.2±0.6	7.4±3.3*	3.3±1.2	2.7±0.7	2.4±0.5	7.7±3.0*	3.4±1.5	3.0±0.6

A main effect for time ($F = 24.856$, $P < 0.001$) was found, along with no interaction between condition and time for plasma lactate ($F = 0.076$, $P = 0.96$). *$P < 0.05$; significant difference from pre-exercise.

6.3.6. PGC-1α mRNA and protein content

Post-exercise PGC-1α mRNA remained similar to pre-exercise under both conditions ($P > 0.05$). However, PGC-1α mRNA increased in both Cont (~3.4-fold; $P = 0.003$) and CWI (~5.9-fold; $P < 0.001$) at 3 h post-exercise with a greater increase observed in CWI ($P < 0.001$; Figure 6.3.5. A). No change in total protein content of PGC-1α when expressed relative to GAPDH was observed at any time point in Cont or CWI conditions ($P > 0.05$; Figure 6.3.5. B). Representative western blots are shown in figure 6.3.5. B.

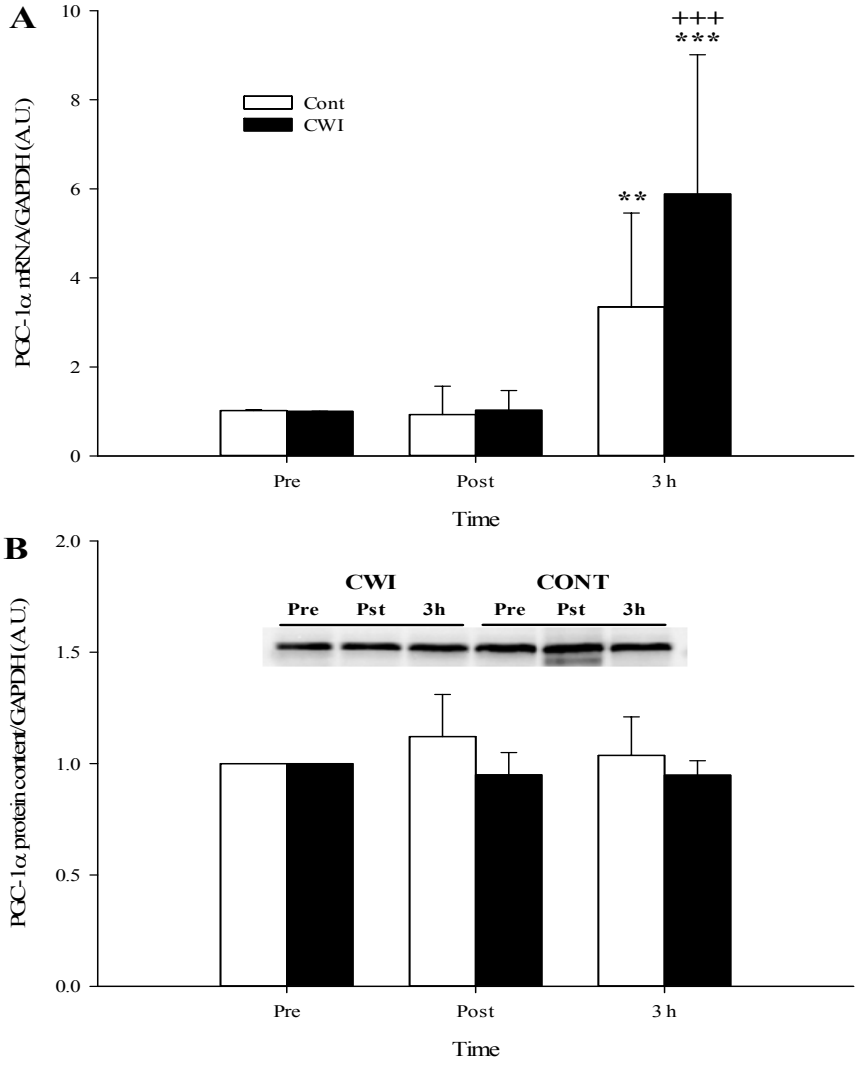

Figure 6.3.5. PGC-1α mRNA (A) and PGC-1α protein (B) before, immediately after exercise and 3 h post-exercise in the CWI and Cont conditions ($n = 9$, mean ± SD). A main effect for time ($F = 19.103$, $P = 0.002$) was found, along with a significant interaction between condition and time for PGC-1α mRNA ($F = 20.922$, $P = 0.001$). $^{**}P < 0.01$, $^{***}P < 0.001$; significant difference from pre-exercise. $^{+++}P < 0.001$; significant difference between conditions.

6.3.7. VEGF mRNA and protein content

$VEGF_{165}$ and $VEGF_{total}$ mRNA did not change immediately after exercise in both conditions ($P > 0.05$). However, $VEGF_{165}$ and $VEGF_{total}$ mRNA increased in CWI only (~2.4-fold, ~2.3-fold) compared with Cont (~1.3-fold, ~1.0-fold) at 3 h post-exercise ($P = 0.01$, $P < 0.001$; Figure 6.3.6. A, B). No change in total protein content of VEGF when expressed relative to GAPDH was observed at any time point in Cont or CWI conditions ($P > 0.05$). Representative western blots are shown in figure 6.3.6. C.

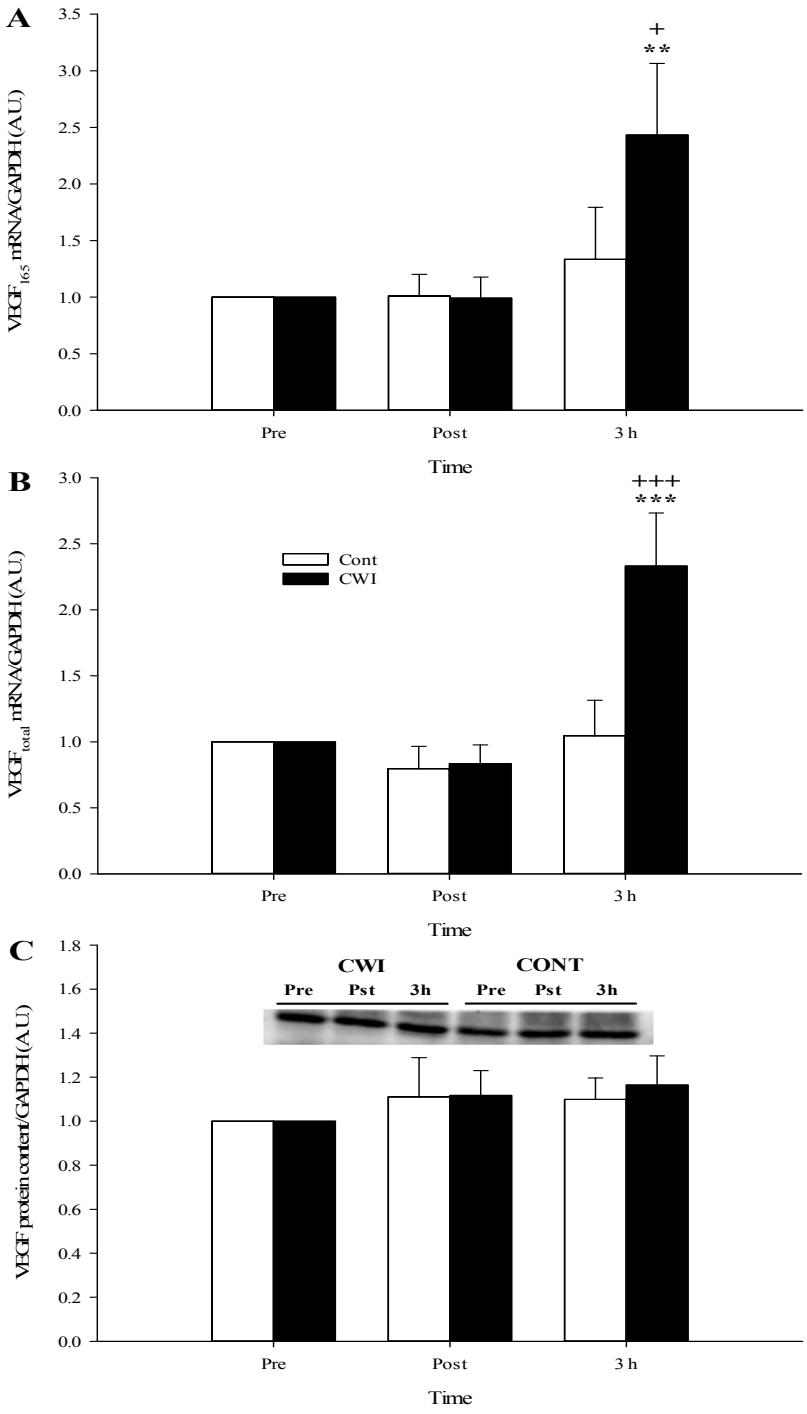

Figure 6.3.6. $VEGF_{165}$ mRNA (A), $VEGF_{total}$ mRNA (B) and VEGF protein (C) before, immediately after exercise and 3 h post-exercise in the CWI and Cont conditions (n = 9, mean ± SD). There was a trend for an interaction between condition and time for $VEGF_{165}$ mRNA (F = 4.307, P = 0.06). A main effect for time (F = 6.434, P = 0.02) was found, along with a significant interaction between condition and time for $VEGF_{total}$ mRNA (F = 23.23, P < 0.001). **P < 0.01, ***P < 0.001; significant difference from pre-exercise. ^{+}P < 0.05, ^{+++}P < 0.001; significant difference between conditions.

6.4. DISCUSSION

Data from the present investigation demonstrate that acute post-exercise CWI increases the expression of PGC-1α, $VEGF_{165}$ and $VEGF_{total}$ mRNA 3 h post-exercise compared to exercise alone. These findings suggest that post-exercise CWI strategies undertaken by athletes in training and competition may impact on cell signalling pathways which influence training adaptation.

Endurance exercise is a powerful stimulus for structural and functional adaptations of mitochondria, including mitochondria biogenesis. PGC-1α coordinates cell signalling pathways which lead to exercise-induced mitochondrial adaptation and thus serve as the "master regulatory of mitochondrial biogenesis" (Puigserver and Spiegelman, 2003). In the present study, PGC-1α mRNA expression remained unchanged immediately following eight 3-min high-intensity intermittent exercise bouts (90% of $\dot{V}O_{2max}$). These findings confirm previous observations in response to six 3-min running bouts at 90% of $\dot{V}O_{2max}$ (Bartlett et al., 2012). In contrast, Mathai et al. (2008) demonstrated that continuous running to exhaustion at 65% $\dot{V}O_{2max}$ increased PGC-1α mRNA in human skeletal muscle by ~3-fold immediately after exercise. The reason for the discrepancy in the study by Mathai et al. (2008) and the present study are unclear, but could be explained by differences in exercise type (continuous cycling exhaustion vs. treadmill intermittent exercise) and duration (~2 h vs. 1 h). Although no change immediately after exercise, a ~3.5-fold increase in PGC-1α mRNA was observed 3 h after exercise in the present study. The magnitude of these changes compare favourably with previous observations (~3-fold to ~8-fold) following high-intensity interval exercise (Cochran et al., 2010, Bartlett et al., 2012, Bartlett et al., 2013).

Exercise is a well-established as an important stimulus in angiogenesis (Andersen and Henriksson, 1977, Coggan et al., 1992, Hepple et al., 1997). VEGF is a predominantly endothelial cell-specific, 46 kDa, heparin-binding glycoprotein. The single VEGF gives rise, by differential splicing, to four different protein products, these are named $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ in humans (Hudlicka et al., 1992, Neufeld et al., 1999). In the present study, $VEGF_{165}$ mRNA and $VEGF_{total}$ mRNA expression was unaltered both immediately following exercise and 3 h post-exercise. These findings contrast with previous observations which reported that acute exercise increased VEGF mRNA ~3.5-fold to ~4.5-fold at 4 h post-exercise in human skeletal muscle (Gavin et al., 2004, Ryan et al., 2006).

Several possibilities may explain this differential findings following acute exercise in human skeletal muscle. It is possible that VEGF expression in response to exercise is dependent on subjects' training status. This is supported by Richardson et al. (2000) who showed a greater increase in VEGF mRNA in untrained human skeletal muscle after acute exercise compared to trained muscle. Indeed, our moderately trained subjects demonstrated higher $\dot{V}O_{2max}$ values ($58mL.kg^{-1}.min^{-1}$) compared to those ($41mL.kg^{-1}.min^{-1}$, $40mL.kg^{-1}.min^{-1}$) used in previous studies (Gavin et al., 2004, Ryan et al., 2006).

Cold water immersion is widely used by athletes after competition and training to enhance recovery efficiently. Furthermore, cooling associated with post-exercise CWI may have implications for adaptations as a consequence of its effects on cell signalling pathways which mediate mitochondrial biogenesis and angiogenesis (Chapter 5). However, no study to date has determined whether post-exercise CWI induces further increase in the exercise-induced expression of PGC-1α and VEGF. In the present study, CWI led to a ~6-fold increase in PGC-1α mRNA expression at 3 h post-exercise compared to exercise alone. These observations are largely consistent with recent work which reported that exercise and recovery in cold ambient temperatures ($7^{o}C$; Slivka et al., 2012) and recovery alone (Slivka et al., 2013) increases human skeletal muscle PGC-1α mRNA by ~8-fold compared to exercise and recovery under moderate temperature ($20^{o}C$). Interestingly, the relative increase in PGC-1α mRNA expression reported in the present study is slightly less than that reported by Slivka and colleagues (2012, 2013). This may reflect the different type of cooling stimulus used (whole body air vs. water) since the magnitude of the increase in sympathetic discharge to skeletal muscle is influenced by the size of the tissue area exposed to cooling (Seals, 1990) and the magnitude of the cooling stimulus (Kregel et al., 1992). Indeed, the magnitude of changes in rectal between post-exercise and 3 h post-exercise in the study of exercise and recovery in cold ambient temperatures (Slivka et al., 2012) were greater than in the present study (approximately $1.7^{o}C$ vs. $1.1^{o}C$).

The potential mechanism associated with an increase in PGC-1α when CWI is applied post-exercise, may be explained via cold temperatures increasing the activity of the sympathetic nervous system (Puigserver et al., 1998, Wu et al., 1999). Unfortunately, catecholamine concentrations were not measured in the present study due to contamination of the blood samples during the transportation of the samples for analysis. However, resting CWI increases noradrenaline at 3 h (~54%) and 6 h (~69%) post-immersion (Chapter 5).

Furthermore, we previously reported marked increases noradrenaline concentrations at 2 h (~258% vs. ~86%) and 4 h (~145% vs. ~101.9%) post-exercise compared to exercise alone (Gregson et al., 2012). In addition, cold-induced increases in energy expenditure are also closely associated with the increase in PGC-1α mRNA (Slivka et al., 2013). In the current study, low level shivering and an increase in oxygen uptake and heart rate were observed during the early stages of immersion, however, no elevation in these responses was observed during the subsequent 3 h period when PGC-1α expression was increased with CWI. This suggests the increased expression of PGC-1α mRNA may to a large extent have been driven by temperature per se. Several pathways also may lead to the increased expression of PGC-1α in response to post-exercise CWI. For example, two metabolic sensors, AMPK and SIRT1 play a major role in PGC-1α activation through phosphorylation and deacetylation, respectively (Canto and Auwerx, 2009). Another important signalling pathway that increase PGC-1α expression is p38MAPK (Fan et al., 2004, Little et al., 2010). Future studies are required, however, to confirm the relative importance of these pathways.

Increases in PGC-1α expression through β-adrenergic signalling also influences VEGF expression (Chinsomboon et al., 2009). Alongside the observed changes in PGC-1α expression in the present study, $VEGF_{165}$ (~2.5-fold) and $VEGF_{total}$ (~2.4-fold) mRNA increased 3 h post-exercise following CWI, but were unaffected by exercise alone. These findings support previous observations which indicated that resting CWI increased in $VEGF_{165}$ mRNA (approximately ~1.6-fold) in animal (Kim et al., 2005) and human skeletal muscle (~2.2-fold; Chapter 5). Similar fold changes in $VEGF_{165}$ mRNA in both the present study and our previous study (Chapter 5) confirm cold-induced changes in the activity of the sympathetic nervous system and metabolism enhance angiogenic adaptations in human skeletal muscle. However, there were no changes in VEGF mRNA with exercise in moderately trained subjects. It is therefore reasonable to speculate that endurance exercise training with CWI may cause further increase in angiogenesis compared to training alone. Further research is needed to examine whether repeated CWI can influence adaptation following exercise.

Repeated bouts of exercise lead to repeated increases in expression mRNA leading to changes in protein content (Perry et al., 2010). However, in the present study PGC-1α and VEGF protein content remained unchanged under both conditions at 3 h post-exercise. This is supported by observations indicating that matched work high-intensity intermittent and

continuous running induce no change in PGC-1α protein content at 3 h post-exercise (Bartlett et al., 2012). Similarly, Gavin et al. (2004) observed that VEGF protein response to acute cycling exercise were no changed at 4 h post-exercise. It might be possible increased mRNAs at 3 h post-exercise could cause a subsequent increase in the proteins after 3 h post-exercise. In addition, Mathai et al. (2008) reported that PGC-1α protein content was 16% greater than pre-exercise at 24 h post-exercise in human skeletal muscle. In the present study, three muscle biopsies (pre-exercise, immediately post-exercise and 3 h post-exercise) were obtained for examination of effects of exercise per se on molecular and cellular responses. However, unfortunately, we could not obtain further muscle biopsies to characterize the proteins after 3 h exercise because of ethical limitations. Further research is needed to investigate the relationship between mRNAs and proteins during a longer timeline.

It has been suggested that the drop in muscle temperature per se is important in mediating the therapeutic effects of cooling in the treatment of acute muscle injury (Merrick et al., 1999). In the present study, muscle temperature was similar immediately following CWI under both conditions with the largest differences between conditions occurring at 30-min post-immersion. In agreement, other researchers found that decreasing muscle temperature following cooling was maintained below baseline until 100-min post-cooling (Dykstra et al., 2009). Prolonged decreases in muscle temperature (150-min post-immersion) can be explained by continued conductive cooling following immersion (Romet, 1988). Changes in muscle temperature were greater in superficial (2 cm depth) muscle temperature compared with deeper (3 cm depth) muscle temperature immediately after immersion and the increase in skin and superficial muscle temperature during the 150-min after water immersion (3 h post-exercise) partly reflects removal of the cooling source per se and subsequent exposure of the limbs to room air temperature. In addition, hemodynamic exchange between the cooler surface and the warmer deeper tissue is likely to have promoted a net flow of heat leading to a gradual increase in superficial tissue temperature and a corresponding decrease in deep tissue temperature over time (Enwemeka et al., 2002).

The decline tendency in T_m currently observed is similar to previous studies which have indicated that 10-min of CWI (14^{o}C) following an endurance capacity test on a cycle ergometer (250 ± 47 W) promotes significant decreases in muscle temperature at 10-min post-CWI (cold, 33.2 ± 1.2^{o}C; Cont, 36.4 ± 0.7^{o}C) (Peiffer et al., 2009). However, the amount of changes in T_m following immersion was different between two studies. Whilst

immediate post-exercise muscle temperatures were found to be similar, the change in T_m between post-exercise and post-immersion were greater in the study of Peiffer and colleagues (2009) (approximately 5^{o}C) compared with that of the present study (2.5^{o}C). This difference is most likely due to the higher exercise intensity (eight 3-min interval at 90% of $\dot{V}O_{2max}$ vs. endurance at 57% of $\dot{V}O_{2max}$) and the longer exercise period (60-min vs. 28.9 ± 8.6-min) compared with Peiffer and colleagues (2009) study. Skin (thigh, calf) temperatures dropped significantly by 18.5 ± 2.6^{o}C (thigh), 17.2 ± 1.6^{o}C (calf) following CWI and gradually increased until 3 h post-exercise. Indeed, it has previously been demonstrated that when the skin temperature falls below 35^{o}C, vasoconstriction in the superficial tissues increases progressively and it reaches the optimal at the temperature of approximately 32^{o}C (Veicsteinas et al., 1982). Therefore, these findings suggest that the introduction of the CWI technique following exercise induces a marked reduction in skin blood flow. Interestingly, there was no significant difference in rectal temperatures between the two conditions in this study. This is in disagreement with recent studies, as it has been reported that rectal temperature declines significantly when subjects are exposed to CWI (Yeargin et al., 2006, Vaile et al., 2008b, Peiffer et al., 2009). The discrepancy maybe explained by differences in assessment technique of temperature and experimental procedures (e.g. water temperature, immersion timing point).

In summary, the present data provide the first report that post-exercise CWI augments the exercise-induced expression of PGC-1α mRNA and promote post-exercise increases in VEGF mRNA. These data suggest that post-exercise CWI may enhance the upstream signalling pathways associated mitochondrial biogenesis and angiogenesis. Further study is now required to explore the influence of CWI using different time-course of sampling and a period of high-intensity intermittent exercise training on performance and markers of muscle adaptation.

CHAPTER 7

THE EFFECTS OF REPEATED COLD WATER IMMERSION FOLLOWING HIGH-INTENSITY INTERMITTENT EXERCISE ON MUSCLE ADAPTATION

7.1. INTRODUCTION

Cold water immersion is widely used by athletes after competition and training to enhance recovery through reducing exercise-induced muscle damage (Leeder et al., 2012). However, cooling associated with post-exercise CWI may have implications for adaptations as a consequence of its effects on cell signalling pathways which mediate mitochondrial biogenesis and angiogenesis. In Chapter 5 we demonstrated that CWI at rest increases the expression of PGC-1α and VEGF in human skeletal muscle. The effect of post-exercise CWI on muscle adaption in human skeletal muscle was demonstrated in Chapter 6. Results indicated that post-exercise CWI leads to augment VEGF mRNA and the exercise-induced expression of PGC-1α mRNA at 3 h post-exercise. It is therefore possible that post-exercise CWI may cause further increase the exercise-induced expression of PGC-1α and VEGF in human skeletal muscle.

Accumulated cold stimulus by repeated CWI after exercise could regulate muscle adaptation differently with a further decrease in body temperature and increase in catecholamine. Despite increases in mRNA, changes in protein at 3 h post-exercise were not observed in Chapter 6. It is possible that repeated CWI following exercise with a longer timeline may increase exercise-induced mRNA and protein content. No study to date, however, has determined the efficacy of repeated CWI on muscle adaptation following high-intensity intermittent running exercise. Furthermore, the magnitude of the increase in sympathetic discharge to skeletal muscle is influenced by the size of the tissue area exposed to cooling (Seals, 1990) and the magnitude of the cooling stimulus (Kregel et al., 1992). Consequently, increasing the repeated CWI following high-intensity intermittent exercise may augment the effects of post-exercise CWI-induced PGC-1α and VEGF mRNA and protein contents. Therefore, the aim of the current investigation was to firstly determine whether increasing the repeated CWI following exercise further enhances cell signalling and secondly whether these alterations induce any changes in protein 6 h post-exercise in human skeletal muscle.

7.2. METHODS

7.2.1. Participants

Ten healthy active male volunteered to participate in the study [mean ± SD: age 24 ± 1yr, height 175.3 ± 4cm, body mass 79.5 ± 6kg and the participants reached $\dot{V}O_{2max}$ (55.5 ± 7.3mL.kg^{-1}.min^{-1})]. The same participants in the previous study (Chapter 5) took part in the study.

7.2.2. Experimental design

Prior to the completion of the experimental trials baseline assessments (see section 3.2.), a venous blood sample (see section 3.9.) and muscle biopsy (see section 3.11.) were completed.

Heart rate, oxygen consumption ($\dot{V}O_2$; see section 3.3.2.), shivering (see section 3.5.2.), rectal (T_{re}) and skin temperature (thigh and calf; see section 3.6.) were measured continuously during 3 × CWI and recovery for 3 h. During the recovery period, muscle biopsies (see section 3.11.) were obtained at 3 h and 6 h post-exercise. Venous blood samples (see section 3.9.) and muscle temperatures (see section 3.6.) were completed immediately post-exercise and at 1 h, 2 h, 3 h and 6 h post-exercise. A schematic illustration of the experimental design is shown in Figure 7.2.1..

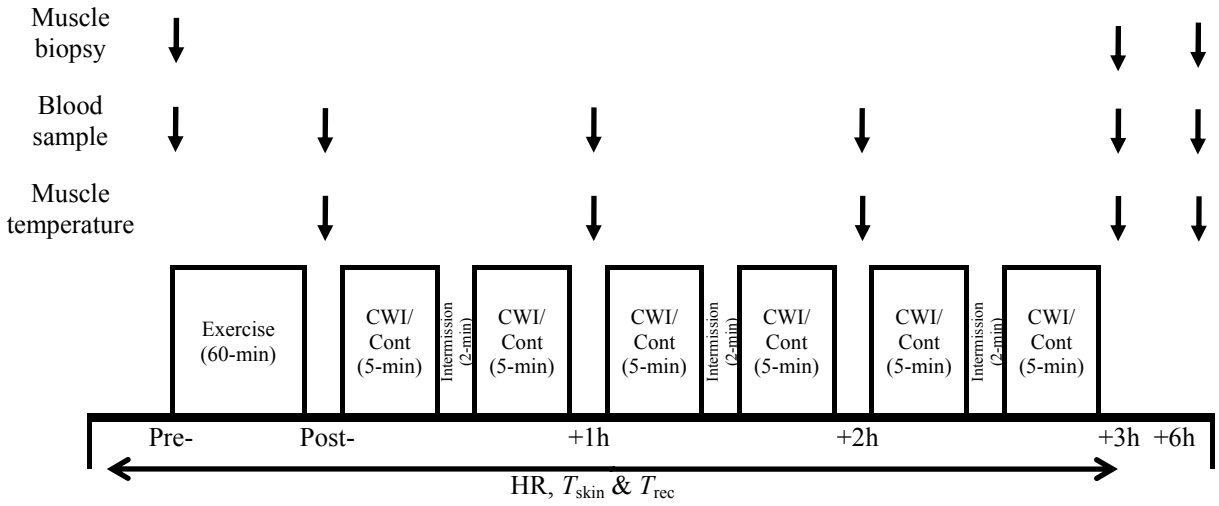

Figure 7.2.1. Schematic illustration of the experimental design.

7.2.3. Statistical analysis

All data are presented as means ± SD. A two-factor (condition × time) within-participants general linear model was undertaken to determine any treatment differences between the

Cont and CWI conditions. The assumption of sphericity (homogeneity of covariance) was assessed and corrected for using the Huynh-Feldt epsilon (Atkinson and Nevill, 2001). Because there were only 2 levels in the main effect of condition, follow-up multiple comparisons were not necessary. A significant effect of time was followed up with planned multiple contrasts in line with the a priori hypotheses. Therefore, data at the specific time points were compared with the baseline (first) time point using Newman-Keuls multiple contrasts. Where a significant interaction between condition and time was observed, differences between conditions were examined at each time point using Newman-Keuls multiple contrasts. Baseline values were compared using a paired samples *t* test. One sample *t* test was used to compare the immersion-induced change in shivering and changes of muscle temperature between baseline and other time points. The alpha level for evaluation of statistical significance was set at $P < 0.05$.

7.3. RESULTS

7.3.1. Exercise responses

Exercise HR and RPE were similar between Cont and CWI conditions ($P > 0.05$). Heart rate significantly increased from the first to the final exercise bout under both conditions (Cont, 176 ± 9 ~ 184 ± 8 beats·min^{-1}; CWI, 178 ± 8 ~ 183 ± 8 beats·min^{-1}; $P < 0.001$). Heart rate during the final exercise bout equated to 95% and 96% of HR_{max} in the Cont and CWI conditions respectively. Rating of perceived exertion following the final exercise bout was 19 ± 1 and 20 ± 1 in the Cont and CWI conditions respectively.

7.3.2. Thermoregulatory responses

Rectal temperature (Cont, 37.7 ± 0.3^{o}C; CWI, 37.8 ± 0.1^{o}C; $P = 0.29$) prior to immersion was similar between conditions. Rectal temperature significantly declined throughout the recovery period in both conditions ($P < 0.05$; Figure 7.3.1. A). A greater rate of decrease in rectal temperature was only observed during the third bout of CWI compared to the Cont condition ($P < 0.05$). Thigh (Cont, 31.7 ± 2.4^{o}C; CWI, 31.5 ± 6.6^{o}C; $P = 0.93$) and calf temperature (Cont, 31.9 ± 2.9^{o}C; CWI, 31.4 ± 6.1^{o}C; $P = 0.83$) prior to immersion were similar between conditions. There was a greater rate of decrease in thigh and calf temperature in CWI compared to the Cont condition ($P < 0.05$) during the three immersion periods with the largest difference between conditions occurring during the third bout of CWI ($P < 0.05$; Figure 7.3.1. B, C). Thigh and calf temperature increased following every 10-min of immersion in CWI with values remaining stable in the Cont condition.

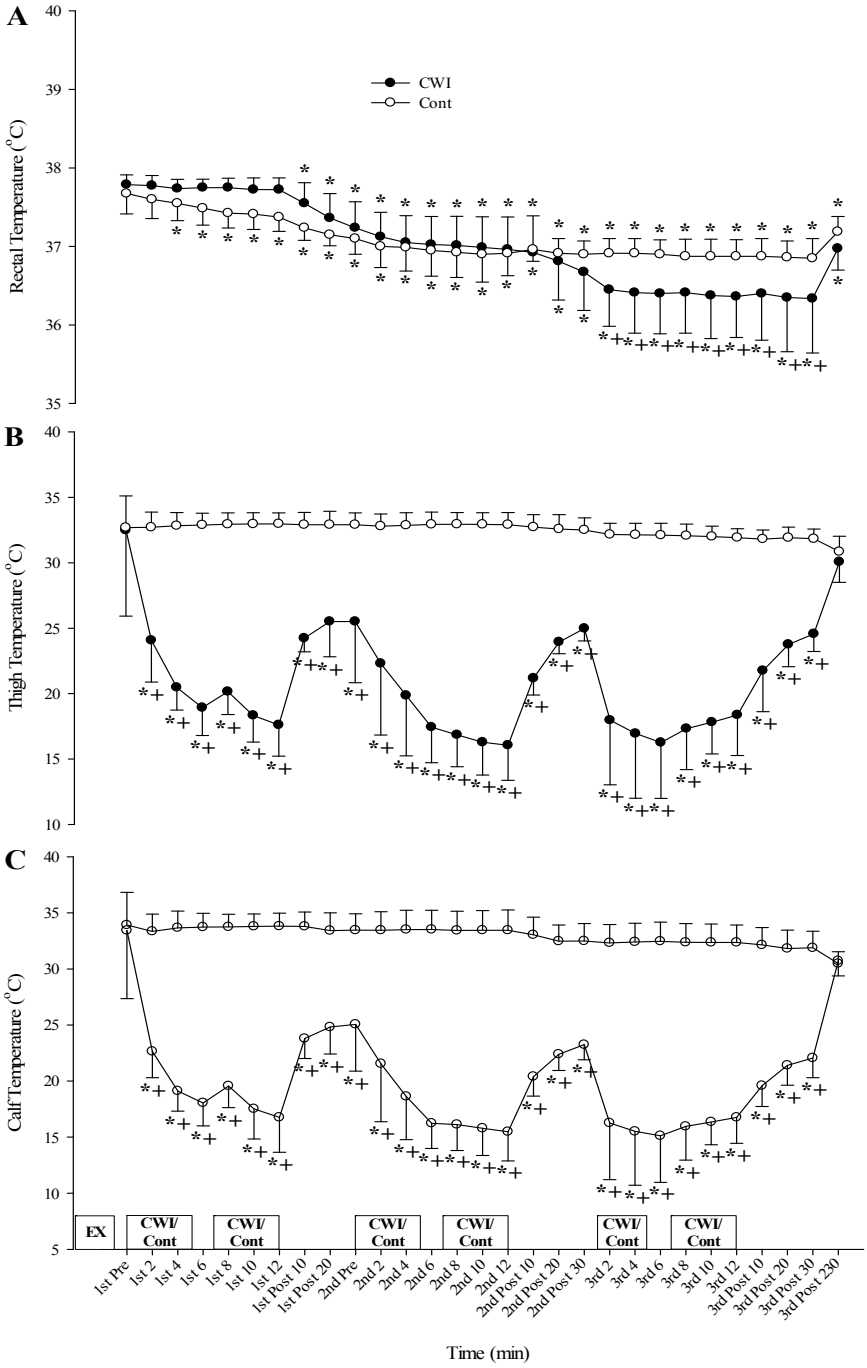

Figure 7.3.1. Rectal (A) and skin (thigh; B and calf; C) temperature during recovery in the CWI and Cont conditions (n = 10, mean ± SD). A main effect for time (F = 41.294, P < 0.001) was found with a significant interaction between condition and time for rectal temperature (F = 23.017, P < 0.001). Main effects for condition (F = 542.898, F = 491.688, P < 0.001) and time (F = 14.586, F = 17.912, P < 0.001) were found, along with a significant interaction between condition and time for skin (thigh and calf) temperature (F = 18.507, F = 24.656, P < 0.001). *P < 0.05; significant difference from baseline. ^{+}P < 0.05; significant difference between conditions.

Muscle temperature prior to immersion was similar between conditions at a probe depth of 3 cm (Cont, 38.9 ± 0.2°C; CWI, 38.9 ± 0.2°C; $P = 0.94$), 2 cm (Cont, 38.4 ± 0.2°C; CWI, 38.4 ± 0.2°C; $P = 0.86$) and 1 cm (Cont, 37.4 ± 0.3°C; CWI, 37.2 ± 0.3°C; $P = 0.54$). Muscle temperature was reduced over time ($F = 30.34$, $P < 0.001$; Figure 7.3.2.), generally decreasing 1 h post-exercise ($P < 0.05$) with a further decrease observed at 2 h post-exercise ($P < 0.05$). These changes depended upon probe depth ($F = 6.528$, $P = 0.02$). At the depth of 3 cm, greater declines in muscle temperature were detected at 2 h post-exercise compared with 1 h post-exercise (Figure 7.3.2. A). At a probe depth of 2 and 1 cm (Figure 7.3.2. B, C), the greatest decline in muscle temperature was observed at 1 h post-exercise, with a more gradual decline during the 2 h post-exercise.

The changes in muscle temperature over time also depended on conditions ($F = 93.897$, $P < 0.001$). At 1 h post-exercise, changes in muscle temperature were greater in CWI compared with Cont (Cont, 1.9 ± 0.4°C; CWI, 5.8 ± 0.3°C; $P < 0.05$) and the greater difference continued until 3 h post-exercise. These differences were in turn dependent on probe depth ($F = 28.963$, $P < 0.001$). At the deepest depths, differences between conditions were least. However, there was still a difference between conditions 3 h after exercise at 2 cm ($P < 0.05$; Figure 7.3.2. B) and 3 cm ($P < 0.05$; Figure 7.3.2. A). At a depth of 1 cm (Figure 7.3.2. C), the reduction in muscle temperature was greater in CWI at 1 h, 2 h and 3 h post-exercise ($P < 0.05$).

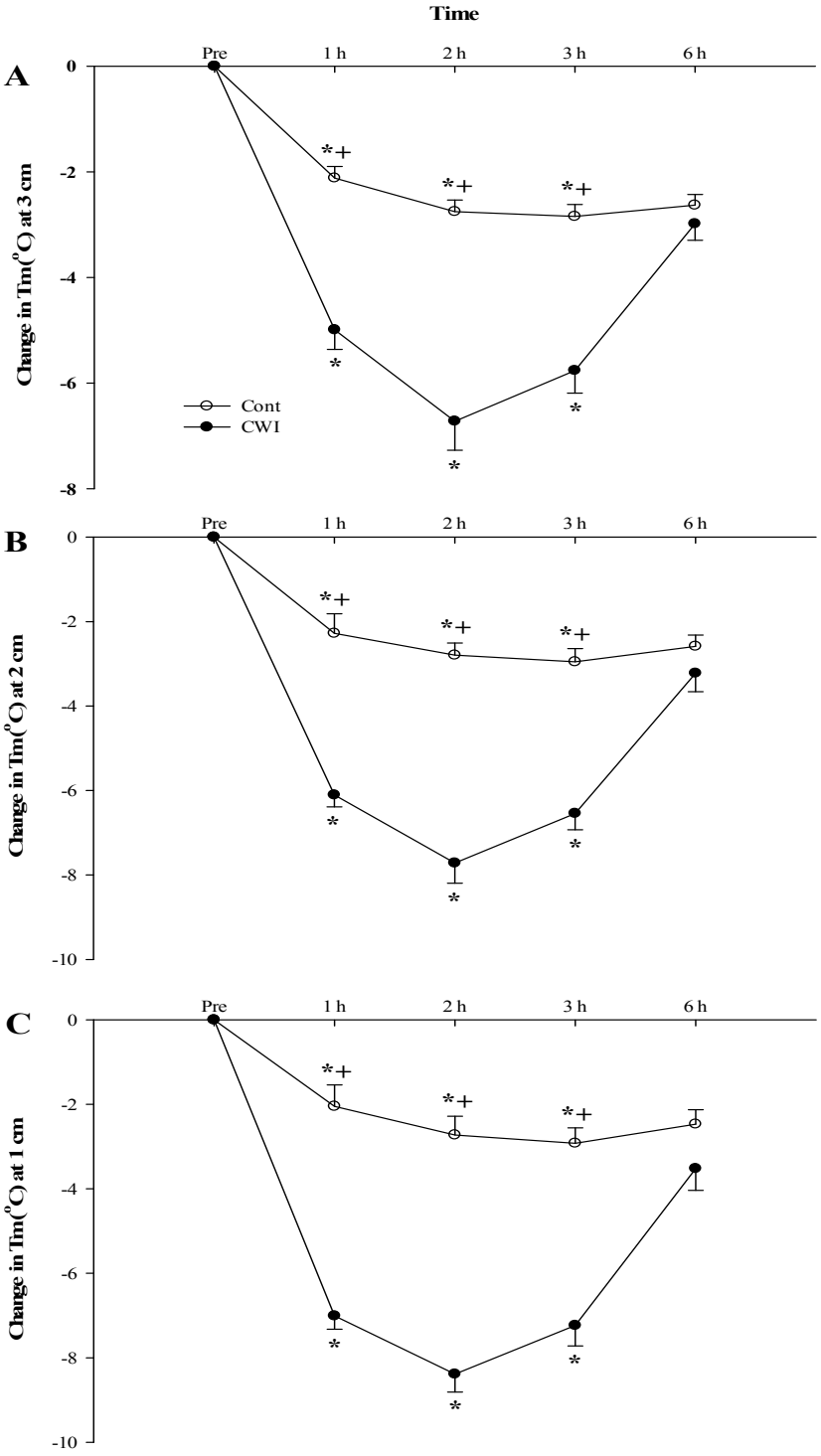

Figure 7.3.2. Changes (**Δ**) in muscle temperature immediately before immersion and 1 h, 2 h, 3 h and 6 h post-exercise, at temperature probe depths of 3 cm (A), 2 cm (B) and 1 cm (C) (n = 10, mean ± SD). Main effects for condition (F = 93.897, P < 0.001) and time (F = 30.34, P < 0.001) were found, along with a significant interaction among condition, time and probe depth (F = 3.388, P = 0.01). *P < 0.05; significant difference from baseline. ^{+}P < 0.05; significant difference between conditions.

7.3.3. Metabolic responses

Pre-immersion HR (Cont, 91 ± 11 beats·min^{-1} (48 ± 6% HR_{max}); CWI, 91 ± 8 beats·min^{-1}(48 ± 4% HR_{max}); P = 0.87) along with HR during the first and second immersion periods was similar between conditions. However, a greater rate of increase in HR was observed during 3rd immersion in CWI compared to Cont condition (P < 0.05), with the largest difference observed at the start of the 10-min immersion period (Figure 7.3.3. A). Pre-immersion $\dot{V}O_2$ (Cont, 4.8 ± 0.3mL.kg^{-1}.min^{-1}; CWI, 5.6 ± 0.3mL.kg^{-1}.min^{-1}; P = 0.64) was similar between conditions. However, $\dot{V}O_2$ increased significantly during 1st immersion in CWI compared to Cont condition (P < 0.05; Figure 7.3.3. B). There was a tendency for $\dot{V}O_2$ to increase two-fold according to repeated CWI, with the largest difference occurring during 3rd immersion period between conditions (P < 0.05).

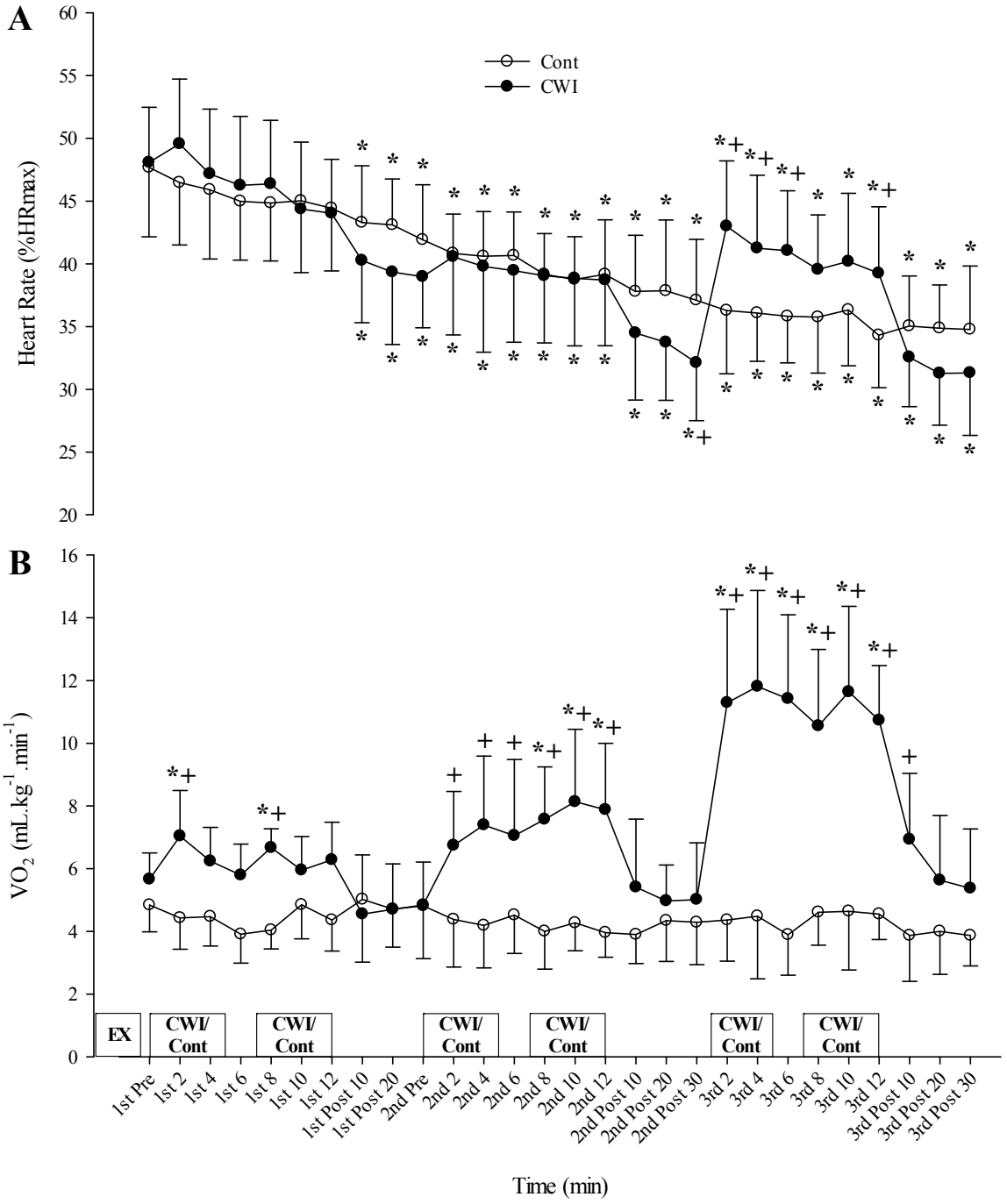

Figure 7.3.3. % HR_{max} (A) and oxygen consumption (B) during recovery in the CWI and Cont conditions (n = 10, mean ± SD). A main effect for time (F = 49.953, P < 0.001) was found with a significant interaction between condition and time for heart rate (F = 6.835, P < 0.001). Main effects for condition (F = 157.072, P < 0.001) and time (F = 15.613, P < 0.001) were found, along with a significant interaction between condition and time for $\dot{V}O_2$ (F = 17.677, P < 0.001). *P < 0.05; significant difference from baseline. ^{+}P < 0.05; significant difference between conditions.

7.3.4. Subjective shivering response

Subjective rating of shivering was only elevated in CWI compared to Cont during each immersion period ($P < 0.05$; Figure 7.3.4.). Shivering was greatest during the third bout of immersion.

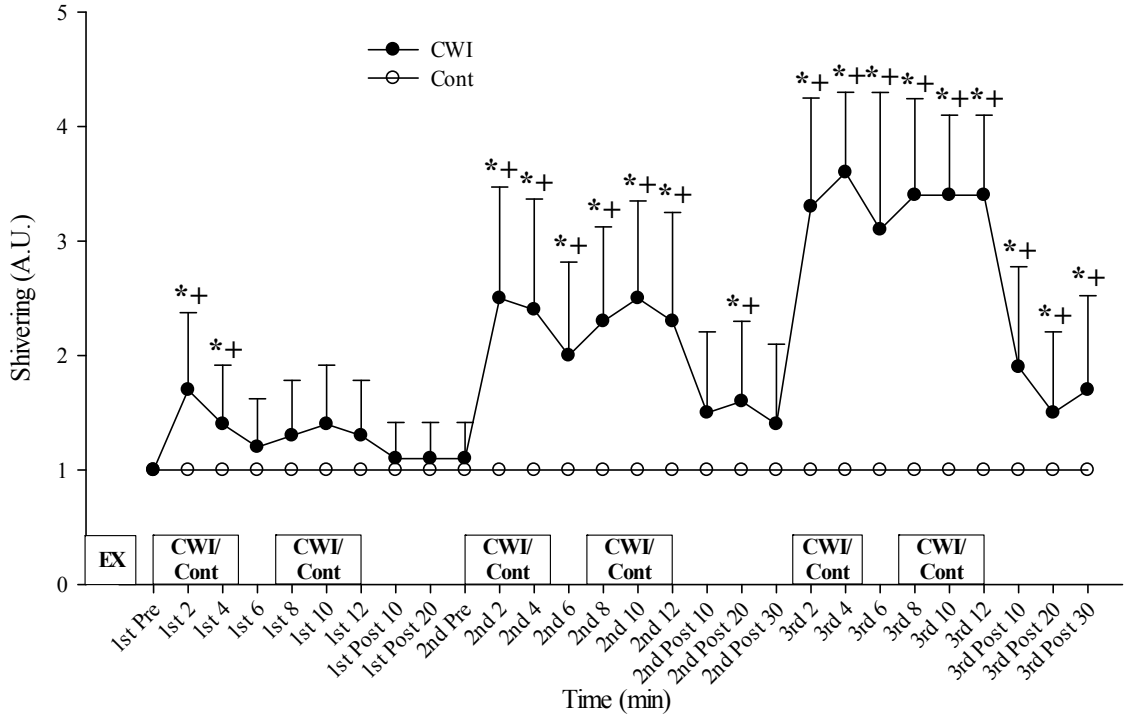

Figure 7.3.4. Shivering during recovery in the CWI and Cont conditions ($n = 10$, mean ± SD). *$P < 0.05$; significant difference from baseline. $^{+}P < 0.05$; significant difference between conditions.

7.3.5. Blood variables

Blood lactate were similar prior to exercise between Cont (0.9 ± 0.2 mmol·l^{-1}) and CWI (1.0 ± 0.2 mmol·l^{-1}), respectively (P = 0.5). Significant higher blood lactate concentration were observed immediately after exercise compared with base-line in both conditions (Cont, 3.8 ± 1.1 mmol·l^{-1}; CWI, 4.2 ± 1.8 mmol·l^{-1}; $P < 0.05$), with no significant interaction between conditions (P = 0.4). Blood lactate was reduced 1 h after exercise in both conditions (Cont, 1.5 ± 0.8 mmol·l^{-1}; CWI, 1.3 ± 0.4 mmol·l^{-1}; $P > 0.05$) and remained unchanged throughout immersions and the post-immersion period (average of 1.1 ~ 1.4 mmol·l^{-1} in Cont and 0.9 ~ 1.2 mmol·l^{-1} in CWI; $P > 0.05$).

Table 7.3.1. Plasma lactate before, immediately after exercise, 1 h, 2 h, 3 h and 6 h post-exercise in the CWI and Cont conditions (n = 10, mean ± SD).

	Cont						CWI					
	Pre	Post	1h	2h	3h	6h	Pre	Post	1h	2h	3h	6h
Lactate, mmol·l^{-1}	0.9± 0.2	3.8± 1.1*	1.5± 0.8	1.4± 1.3	1.4± 1.1	1.1± 1.2	1.0± 0.2	4.2± 1.8*	1.3± 0.4	1.2± 0.7	1.2± 0.4	0.9± 0.4

A main effect for time (F = 38.665, $P < 0.05$) was found, along with no interaction between condition and time for plasma lactate (F = 0.563, P = 0.58). *$P < 0.05$; significant difference from pre-exercise.

Plasma adrenaline was similar in Cont (277 ± 65 pmol/l) and CWI (235 ± 90 pmol/l) at pre-exercise (P = 0.5). Plasma adrenaline significantly increased following exercise in both conditions (Cont, 437 ± 101 pmol/l; CWI, 492 ± 190 pmol/l; $P < 0.05$; Figure 7.3.5. A), with no difference between conditions (P = 0.36). Plasma adrenaline returned to pre level 1 h following exercise in both conditions (Cont, 300 ± 102 pmol/l; CWI, 282 ± 96 pmol/l; $P >$ 0.05) and gradually reduced through rest of period. Plasma noradrenaline were similar prior to exercise between Cont (230 ± 124 pmol/l) and CWI (212 ± 110 pmol/l), respectively (P = 0.8; Figure 7.3.5. B). Significant higher plasma noradrenaline concentration were observed immediately after exercise compared with base-line in both conditions (Cont, 1391 ± 453 pmol/l; CWI, 1439 ± 530 pmol/l; $P < 0.05$), with no significant interaction between conditions (P = 0.6). Exercise-induced increase in plasma noradrenaline reduced 1 h following exercise in both conditions. Gradual decline in plasma noradrenaline was observed during the 6 h post-exercise in Cont. However it was increased in CWI at 3 h and 6 h post-exercise, with significant interaction between conditions ($P < 0.05$).

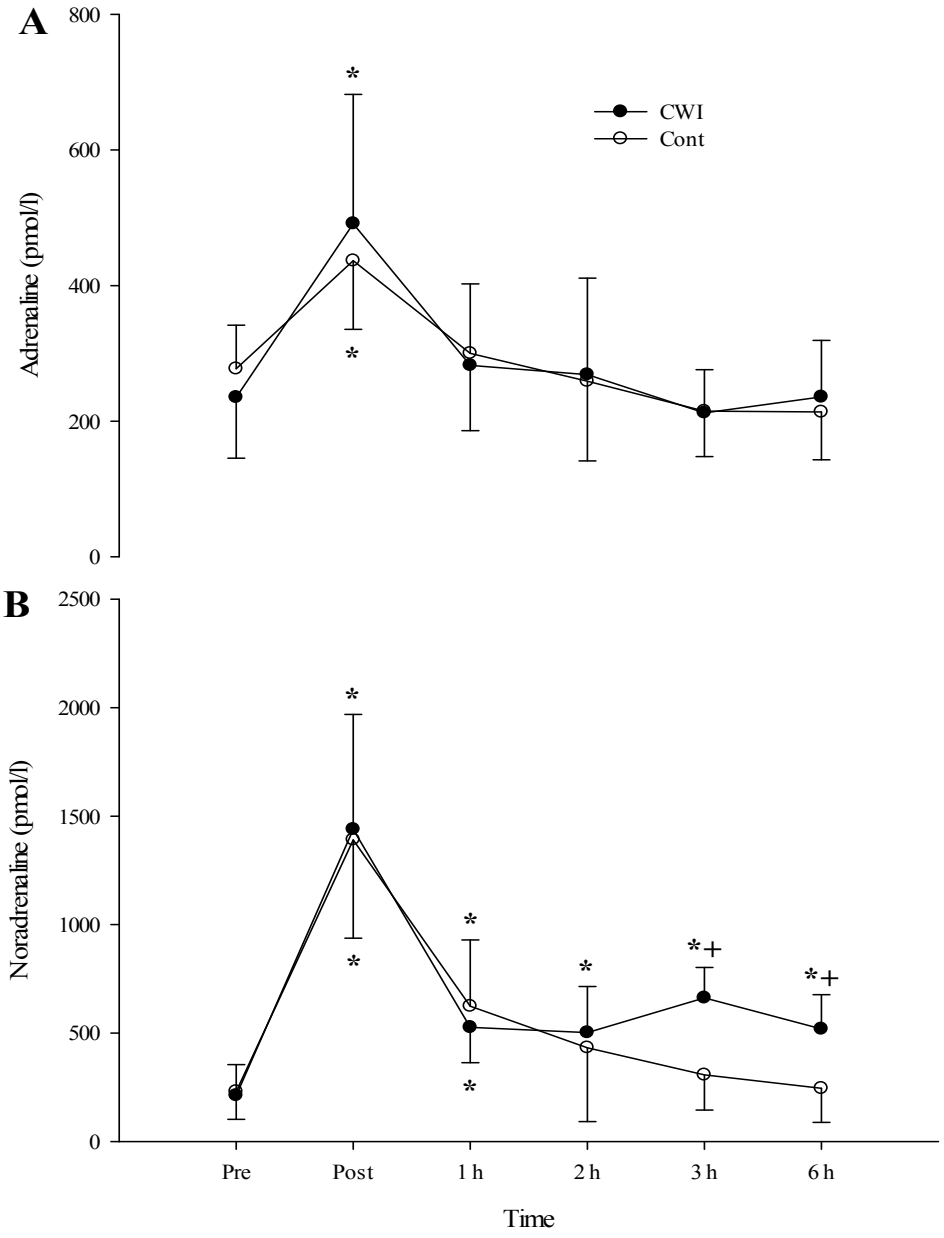

Figure 7.3.5. Plasma adrenaline (A) and noradrenaline (B) before, immediately after exercise, 1 h, 2 h, 3 h and 6 h post-exercise in the CWI and Cont conditions (n = 10, mean ± SD). A main effect for time was found for adrenaline (F = 34.914, P < 0.001). A main effect for time (F = 41.285, P < 0.001) was found, along with a significant interaction between condition and time for noradrenaline (F = 4.555, P = 0.01). *P < 0.05; significant difference from baseline. ^{+}P < 0.05; significant difference between conditions.

7.3.6. Phosphorylation of AMPK^{Thr172}

There were no changes in phosphorylation of AMPK^{Thr172} at any time during Cont and CWI trials ($P > 0.05$; Figure 7.3.6.).

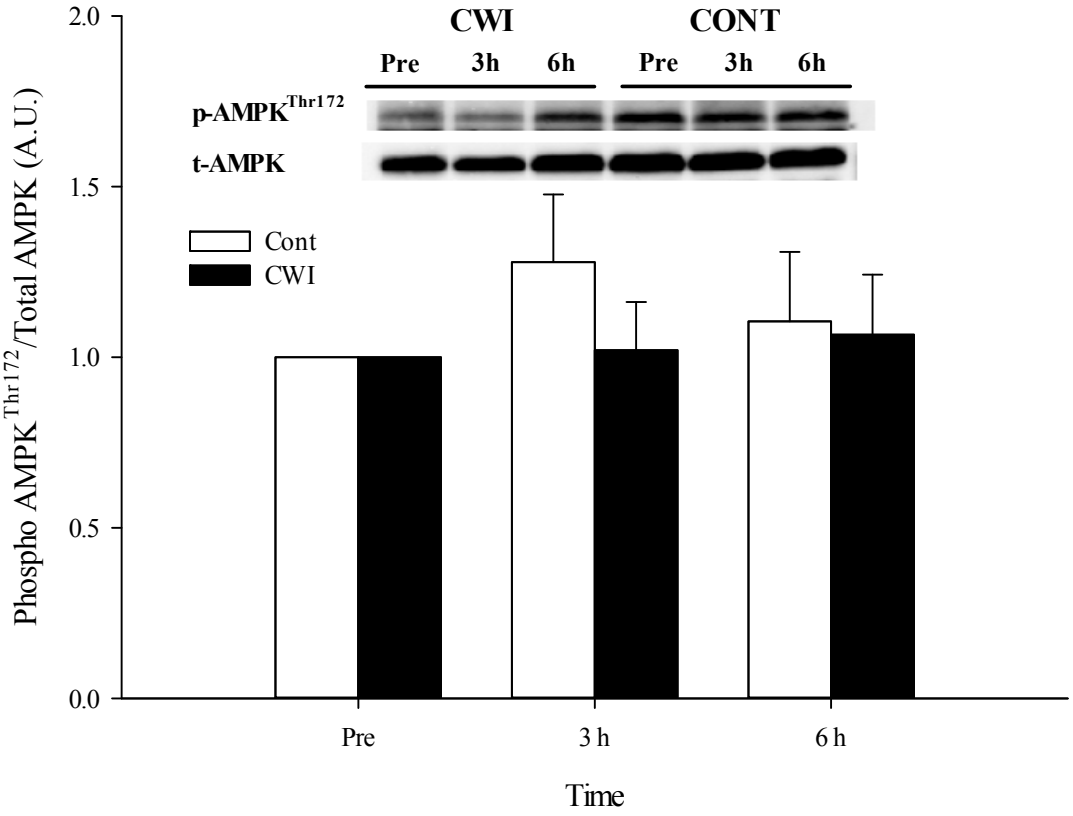

Figure 7.3.6. Phosphorylation of AMPK^{Thr172} before, 3 h and 6 h post-exercise in the CWI and Cont conditions ($n = 9$, mean ± SD).

7.3.7. Phosphorylation of p38MAPK$^{Thr180/Tyr182}$

Phosphorylation of p38MAPK$^{Thr180/Tyr182}$ increased 2-fold at 3 h post-exercise with no difference (P = 0.2; Figure 7.3.7.) between conditions. Phosphorylation of p38MAPK$^{Thr180/Tyr182}$ returned to basal level in Cont at 6 h following exercise, however, a further increase (3.4-fold) was observed in the CWI condition (P = 0.13).

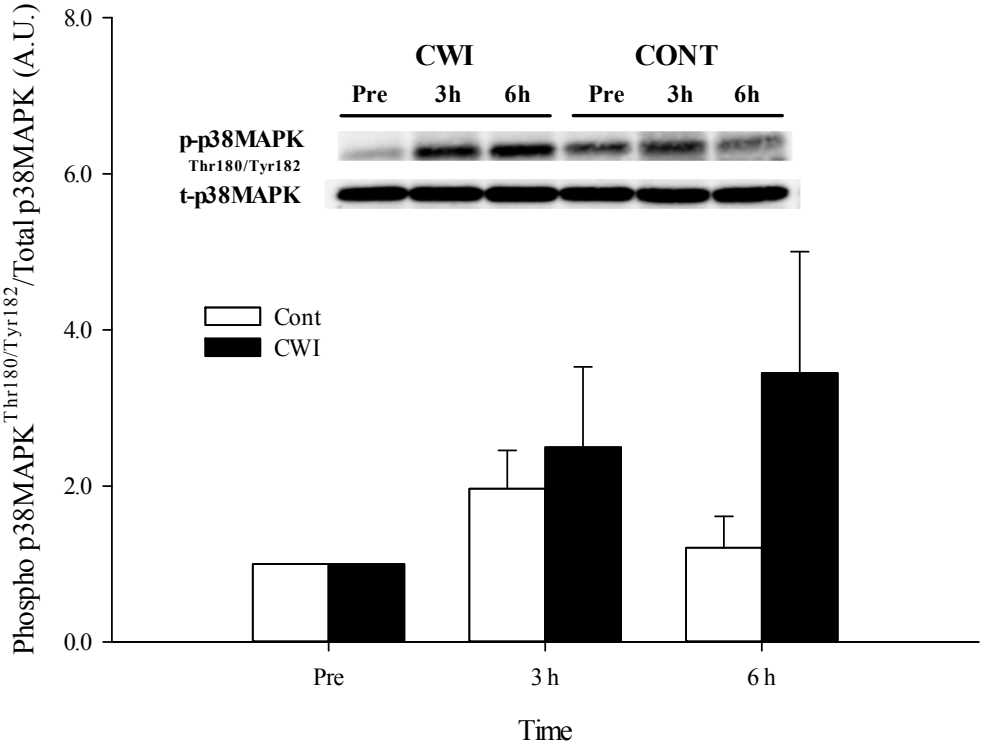

Figure 7.3.7. Phosphorylation of p38MAPK$^{Thr180/Tyr182}$ before, 3 h and 6 h post-exercise in the CWI and Cont conditions (n = 9, mean ± SD).

7.3.8. PGC-1α mRNA and protein content

PGC-1α mRNA increased similarly at 3 h post-exercise in the Cont and CWI conditions respectively ($P < 0.05$; Figure 7.3.8. A). In contrast, a further increase in PGC-1α mRNA was observed 6 h post-exercise with CWI, compared with a decrease in Cont ($P = 0.03$). There were no changes in total protein content of PGC-1α when expressed relative to GAPDH at any time during the CWI and Cont conditions ($P > 0.05$; Figure 7.3.8. B).

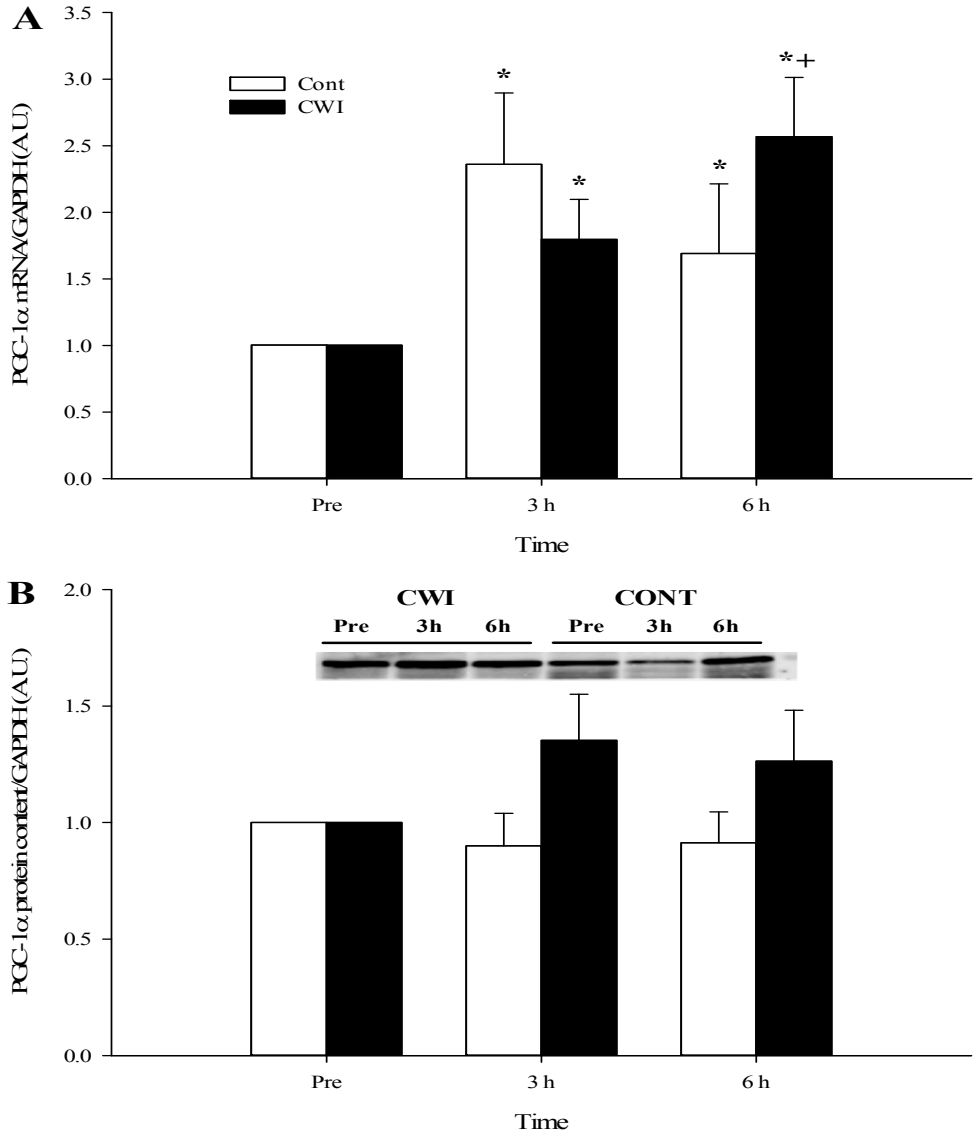

Figure 7.3.8. PGC-1α mRNA (A) and PGC-1α protein (B) before, 3 h and 6 h post-exercise in the CWI and Cont conditions ($n = 9$, mean ± SD). A main effect for time ($F = 4.584$, $P = 0.03$) was found, along with a significant interaction between condition and time for PGC-1α mRNA ($F = 6.663$, $P = 0.008$). *$P < 0.05$; significant difference from baseline. $^{+}P < 0.05$; significant difference between conditions.

7.3.9. VEGF mRNA and protein content

$VEGF_{165}$ and $VEGF_{total}$ mRNA increased more than ~1.6-fold relative to base-line at 3 h and 6 h following exercise ($P < 0.05$; Figure 7.3.9. A, B) with no difference between conditions ($P > 0.05$). There was no difference in VEGF protein content between conditions ($P > 0.05$; Figure 7.3.9. C).

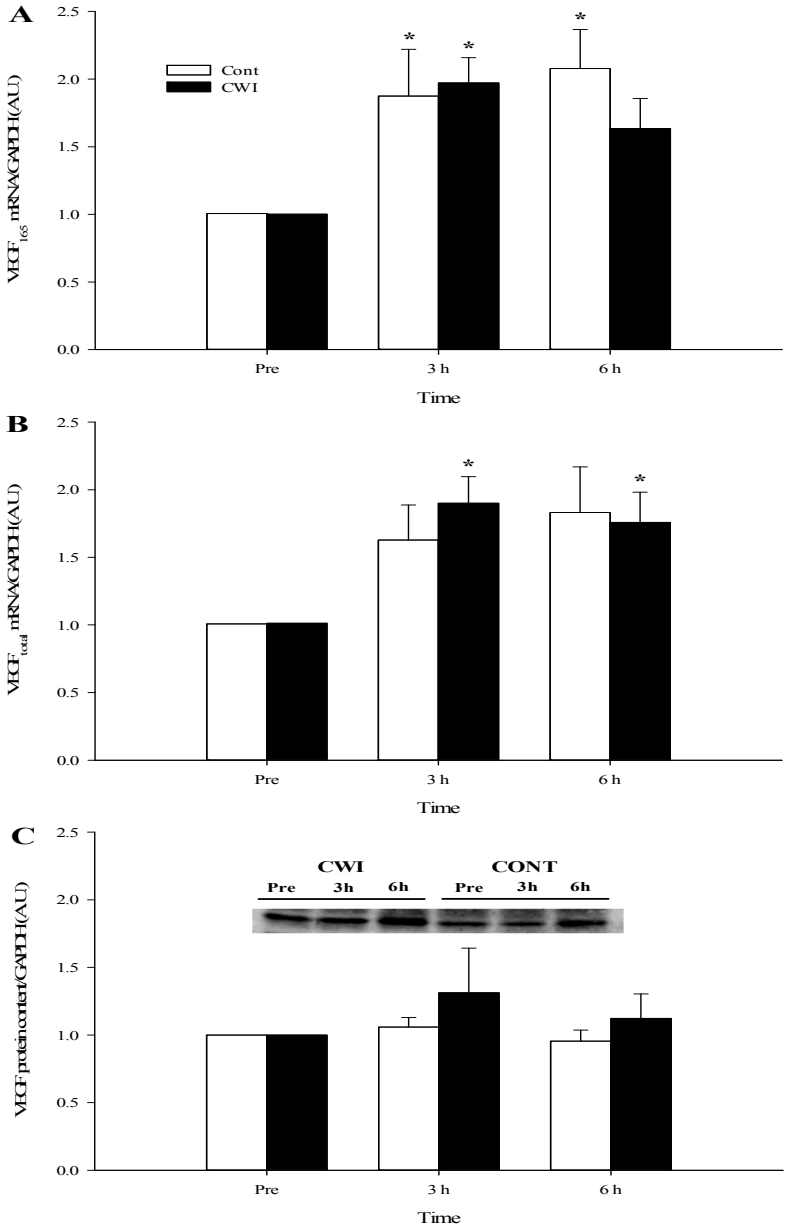

Figure 7.3.9. $VEGF_{165}$ (A), $VEGF_{total}$ mRNA (B) and VEGF protein (C) before, 3 h and 6 h post-exercise in the CWI and Cont conditions ($n = 9$, mean ± SD). A main effect for time was found for $VEGF_{165}$ ($F = 12.247$, $P = 0.001$) and $VEGF_{total}$ mRNA ($F = 13.969$, $P = 0.001$). *$P < 0.05$; significant difference from baseline.

7.4. DISCUSSION

The aim of the present study was to firstly determine whether repeatedly administering CWI following exercise further increases the expression PGC-1α and VEGF mRNA and secondly whether these alterations induce changes in their protein expression 6 h post-exercise in human skeletal muscle. Despite increasing the repeated CWI in the present study, fold changes of PGC-1α mRNA was less than one bout of CWI (Chapter 6) after 3 h exercise (1.8-fold vs. 5.9-fold). A greater increase in PGC-1α mRNA was presently observed following CWI at 6 h post-exercise compared with the Cont, however, these alterations did not induce any changes in protein expression 6 h post-exercise in human skeletal muscle.

AMPK and p38MAPK are not only associated with the regulation of mitochondrial biogenesis (Fan et al., 2004, Canto and Auwerx, 2009) but play an important role in the increased expression of VEGF (Ouchi et al., 2005). In present study, no changes in AMPK were observed at any time point following exercise. Although there was a 2-fold increase in p38MAPK at 3 h post-exercise in both conditions, this did not reach statistical significance. These findings are in agreement with recent observation from our laboratory in which similar subject populations and high-intensity intermittent exercise protocols have been studied (Bartlett et al., 2012). Despite no change in AMPK and p38MAPK, high-intensity intermittent exercise (1 h) increased PGC-1α mRNA content 3 h post-exercise. The relative increase in PGC-1α mRNA (2.4-fold) 3 h after exercise in the present study was lower than previously observed (3.4-fold; Chapter 6) following high-intensity intermittent exercise. The reasons for this discrepancy are unclear but could be associated with differences in subject training status and exercise intensity. Pilegaard et al. (2003) showed that the magnitude of increase in PGC-1α mRNA was greater in trained men (10-fold) compared with untrained (7-fold) 2 h post-exercise. PGC-1α mRNA was higher in high-intensity exercise (10-fold) compared to low-intensity exercise (4-fold) 3 h post-exercise (Egan et al., 2010). Indeed, subjects in the previous Chapter (58.1 ± 7.2mL.kg^{-1}.min^{-1}) demonstrated a higher $\dot{V}O_{2max}$ than subjects (55.5 ± 7.3mL.kg^{-1}.min^{-1}) in the present. Exercise intensity might be higher in the previous Chapter than in the present regarding changes in blood lactate data between pre-exercise and immediately post-exercise (5.2 mmol·l^{-1} vs. 2.9 mmol·l^{-1}). In the present study, high-intensity intermittent exercise increased $VEGF_{165}$ and $VEGF_{total}$ mRNA at 3 h and 6 h post-exercise. These findings are consistent with previous studies which showed that acute exercise increased VEGF mRNA in human skeletal muscle (Gavin et al., 2004, Ryan et al.,

2006). However, these results contrast with our previous observations (Chapter 6) were no significant change in $VEGF_{total}$ was observed after 3 h exercise. This may again reflect differences in subject training status between studies since the abundance of VEGF mRNA after acute exercise is more sensitive in untrained subjects (Richardson et al., 2000).

In the present study, we increased the cooling exposure following exercise compared to the experimental work in the previous Chapter. Interestingly, PGC-1α, $VEGF_{total}$ and $VEGF_{165}$ mRNA were not significantly different between conditions 3 h after exercise, however, PGC-1α mRNA expression was greater (2.6-fold vs. 1.7-fold) 6 h after exercise in CWI compared to Cont. The present findings observed at 3 h post-exercise contrast with the results from Chapter 6, where the exercise-induced increase in PGC-1α mRNA was further amplified with one bout of CWI. Interestingly in the present study, a greater reduction ($1^{o}C$) in muscle temperature at depths of 2 and 3 cm was observed at 3 h following exercise compared to the previous study. Furthermore, the magnitude of the increase in noreadrenaline content was also higher in present study at 3 h (~212% vs. ~54%) and 6 h (~145% vs. ~69%) post immersion. It is possible there may be a limit to which cold induces increases in PGC-1α mRNA in human skeletal muscle. In the present study, no change in PGC-1α protein content was observed 6 h post-exercise. Indeed, Perry et al. (2010) only reported increases in PGC-1α protein 24 h after exercise. Further research is therefore needed to investigate whether CWI may alter PGC-1α protein expression over extended periods of time.

The reduction tendency in muscle temperature in response to repeated CWI following the exercise is consistent with previous Chapter 6. However, muscle temperature increased at 3 h post-exercise, even if the last CWI was completed at 2 h post-exercise. Theoretically, it should decrease at the time point. The reasons for the results in the present study may be related to energy metabolism for thermogenesis. Indeed, metabolic responses (i.e.HR and $\dot{V}O_2$) and shivering were much greater according to accumulated cold stimulus. In support of the current findings, previous researches demonstrate that increasing rates of heat production induced by cold exposure is caused by shivering thermogenesis related to increase in HR and oxygen consumption (Iampietro et al., 1960, Sramek et al., 2000, Haman, 2006). Reduced skin temperature during and after CWI is consistent with the previous studies of CWI (Proulx et al., 2003, Gregson et al., 2011). These similar findings would suggest that reduced skin temperature via CWI stimulate thermoreceptors regulating vasoconstriction known to be located in the skin (Hensel and Boman, 1960). Indeed, Gregson et al. (2011) reported that a

significant reduction in cutaneous blood flow occurred after 10-min of immersion in 8^{o}C water. Together these findings provide evidence that thermoregulatory response of CWI is triggered by reduced body temperature and heat balance is maintained by reducing rates of heat loss via peripheral vasoconstriction and increasing rates of heat production via shivering (Haman, 2006).

In summary, the results of this study demonstrate that increasing the repeated post-exercise CWI does not further increase the expression of PGC-1α and VEGF mRNA in human skeletal muscle following exercise. One bout of CWI following high-intensity intermittent exercise may therefore maximise the expression of PGC-1α and VEGF mRNA.

CHAPTER 8

SYNTHESIS OF FINDINGS

The initial section of this Chapter presents an 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 CWI following high-intensity exercise. The thesis closes by outlining recommendations for future research based on questions that have arisen from this research project.

8.1. REALISATION OF AIMS AND OBJECTIVES

Aim 1: *To develop a laboratory based low-damaging high-intensity intermittent exercise protocol.*

This aim was achieved via the completion of Study 1 (Chapter 4). This study demonstrated that the high-intensity intermittent running protocol induced changes in physiological and subjective indices consistent with the effects of low muscle damaging as opposed to those changes normally associated with exercise-induced severe muscle damage. This protocol was therefore be used in future studies to determine the influence of CWI on adaptation in moderately trained individuals.

Aim 2: *To examine the effects of CWI ($8^{o}C$) on the acute skeletal muscle cell signalling responses at rest.*

This aim was achieved via the completion of Study 2 (Chapter 5). Resting CWI increased noradrenaline at 3 h (~54%) and 6 h (~69%) after immersion. Resting CWI increased the expression of PGC-1α (~1.3-fold and 1.4-fold) and $VEGF_{165}$ (~1.9-fold and ~2.2-fold) at 3 h and 6 h post-immersion. However, PGC-1α and $VEGF_{165}$ protein remained unaffected over time following resting CWI.

Aim 3: *To examine the effects of low-damaging high-intensity intermittent exercise on the acute skeletal muscle cell signalling responses.*

This aim was achieved via the completion of Study 3 and 4 (Chapter 6 and 7). High-intensity intermittent exercise increased PGC-1α and $VEGF_{165}$ mRNA 3 h and 6 h post-exercise. It was therefore suggested that high-intensity intermittent exercise upregulates acute signalling responses associated with mitochondrial biogenesis and angiogenesis.

Aim 4: *To examine the effects of CWI (8°C) on skeletal muscle cell signalling responses to acute high-intensity intermittent exercise.*

This aim was achieved via the completion of Study 3 (Chapter 6). High-intensity intermittent exercise increased PGC-1α mRNA. Furthermore, post-exercise CWI induced a further increase in PGC-1α mRNA, $VEGF_{165}$ and $VEGF_{total}$ mRNA 3 h post-exercise compared to Cont. It was therefore concluded that post-exercise CWI elevates acute signalling responses associated with mitochondrial biogenesis and angiogenesis.

Aim 5: *To examine the repeated CWI (8°C) on skeletal muscle cell signalling responses to acute high-intensity intermittent exercise.*

This aim was achieved via the completion of Study 4 (Chapter 7). Despite increasing the repeated CWI, there were no significant differences in PGC-1α, $VEGF_{total}$ and $VEGF_{165}$ mRNA between conditions 3 h post-exercise. PGC-1α mRNA was significantly higher following CWI compared to Cont at 6 h post-exercise. There was no change in PGC-1α protein content at any time point. It was therefore concluded that applying more than one bout of CWI immediately following high-intensity intermittent exercise does not lead to further changes in skeletal muscle cell signalling responses associated with mitochondrial biogenesis and angiogenesis.

8.2. GENERAL DISCUSSION

Recently, cold water immersion (CWI) has been widely used by athletes as recovery strategy after competition or training to recover from fatigue and muscle injury quickly and efficiently (Wilcock et al., 2006). Most researches about CWI as a means of recovery of a series of physiological stressors are related to physical capacity. However, little attention focused on its impact on adaptation at the cellular and molecular level. Therefore, the aim of this thesis was to investigate the impact of CWI on acute markers of adaptation in human skeletal muscle following intermittent exercise.

The three main investigations of the current thesis were undertaken in order to examine the influence of different modes of CWI on markers of adaptation responses to resting and acute exercise in human skeletal muscle. The initial investigation examined the effects of resting CWI on markers of adaptation (Chapter 5). Results indicated that resting CWI induced a significantly higher level of PGC-1α and $VEGF_{165}$ mRNA expression at 3 h post, which remained up-regulated for at least 3 h. These findings could have implications for the effects of CWI on muscle adaption since many athletes use CWI in rested states (e.g. during rehabilitation) and the days after competition when training stress may be minimal. Indeed, these results are consistent with previous researches demonstrating that acute (Puigserver et al., 1998) and chronic (Oliveira et al., 2004) whole body cold exposure promoted a significant increase of PGC-1α mRNA content in animal. Similarly in rats, acute (1 h) and chronic (1 h day^{-1}, 5 days $week^{-1}$ for 20 weeks) CWI (18^{o}C) increased VEGF mRNA under resting conditions (Kim et al., 2005). On the other hand, identification of increase in PGC-1α and VEGF mRNA in resting CWI in current study implicates resting CWI as a potential intervention to improve various indices of health. For example, defective or insufficient mitochondrial and endothelial cell function play a potentially pathogenic role in mediating risk of type 2 diabetes mellitus (Patti and Corvera, 2010, Kolluru et al., 2012). Therefore, these findings suggest that resting CWI may enhance the upstream signalling pathways associated to initiate mitochondrial biogenesis and angiogenesis and raise the question regarding the relative contribution of CWI following exercise at further increase in the exercise-induced expression of PGC-1α and VEGF in human skeletal muscle.

Athletes use CWI immediately following exercise to enhance the rate of recovery. Despite increase in popularity of post-exercise CWI in athletic training regimes, limited study has investigated its potential to improve the adaptive response to training. Therefore, this study

examined the effects of CWI on skeletal muscle cell signalling responses to acute high-intensity intermittent exercise. Post-exercise CWI (10-min at 8^{o}C) augments the exercise-induced expression of PGC-1α mRNA at 3 h post-exercise. These observations are largely consistent with recent work which reported that exercise and recovery in cold ambient temperatures (7^{o}C; Slivka et al., 2012) and recovery alone (Slivka et al., 2013) increases human skeletal muscle PGC-1α mRNA compared to exercise and recovery under moderate temperature (20^{o}C). In contrast with observations following one bout of post-exercise CWI, PGC-1α mRNA increased similarly at 3 h post-exercise in the Cont and repeated CWI conditions. However, there was a further increase in PGC-1α mRNA at 6 h post-exercise only in the repeated CWI condition compared to a decrease in Cont. While a mechanistic explanation for the difference in timing point of the further increase in PGC-1α mRNA between one bout of post-exercise CWI (3 h post-exercise) and repeated CWI conditions (6 h post-exercise) is presently lacking, it is possible that not only PGC-1α may be differently regulated by magnitude of cold stimulus such as duration and/or the number of CWI following exercise, but also further cold stimulus following exercise may induce slower adaptive responses in human skeletal muscle. Indeed, Peake and colleagues (2015) indicated that some biochemical messengers, which activate various signalling pathways that regulate gene expression, are either blocked or activated more slowly after cryotherapy. Consequently, the relative increase in PGC-1α mRNA in repeated CWI condition was lower than one bout of CWI following high-intensity intermittent exercise. These findings suggest that one bout of CWI may maximise the muscular adaptation to high-intensity intermittent exercise.

Despite post-exercise CWI resulted increase in PGC-1α and VEGF mRNA at 3 h post-exercise, there were no changes in AMPK at any time after exercise. Similarly, p38MAPK did not statically increase in exercise alone and exercise with CWI. These findings are in agreement with recent observation from our laboratory in which similar subject populations and high-intensity intermittent exercise protocols have been studied (Bartlett et al., 2012). These findings suggest that exercise intensity may not be enough to simulate an increase in AMPK and p38MAPK and these do not appear to be a mediator of the cold-induced increase in PGC-1α and VEGF mRNA in human skeletal muscle.

As described in section 2.4.3., exercise-induced inflammation is important for part of the process of muscle adaptation through the effects of increased inflammatory protein mediators on PGC-1α expression. However, post-exercise CWI may reduce muscle adaptation since post-exercise CWI induces reduction in inflammation response (Thorlacius et al., 1998,

Merrick et al., 1999, Lee et al., 2005) and increase in PGC-1α may attenuate exercise-induced inflammatory pathways for muscle adaption. In support of this, basal mRNA expression inflammatory factors including TNFα, IL-6, suppressor of cytokine signalling 1 and 3 in skeletal muscle was higher in PGC-1α knockout mice than in wild type (Handschin et al., 2007a, Handschin et al., 2007b). Moreover, significant increases in TNFα mRNA and serum TNFα content were observed in PGC-1α knockout mice compared to wild type (Handschin et al., 2007a). These findings are supported by observation in human model, PGC-1α mRNA was negatively correlated with IL-6 and TNFα mRNA expression in skeletal muscle (Handschin et al., 2007b). As described above, Post-exercise CWI (10-min at 8^{o}C) augments the exercise-induced expression of PGC-1α mRNA, despite it may reduce inflammatory pathways. Although this seems paradoxical at first, post-exercise CWI might exert muscle adaptation via other pathways in skeletal muscle.

The potential mechanism associated with muscle adaptation via an increase in PGC-1α and VEGF when CWI is applied post-exercise, can be explained via non-shivering thermogenesis, shivering thermogenesis and ROS stress related pathways. Cold temperatures induced increase in the activity of the sympathetic nervous system may be main pathway for the increase in PGC-1α and VEGF. Cold activates sympathetic nervous activity that leads to the release of catecholamine which triggers activation of the β-adrenergic receptors, resulting in the elevation of intracellular cAMP and a dramatic induction of PGC-1α in the muscle (Puigserver et al., 1998, Wu et al., 1999). Indeed, significant higher plasma noradrenaline concentration were observed in resting CWI and repeated CWI following high-intensity intermittent exercise in present studies compared to control condition. Although AMPK and p38MAPK are important for regulating PGC-1α and VEGF in skeletal muscle (Fan et al., 2004, Ouchi et al., 2005, Canto and Auwerx, 2009, Little et al., 2010), there were no significant differences in AMPK and p38MAPK between CWI and control in present study. The lack of difference in these factors despite obvious differences in PGC-1α and VEGF may be explained by other upstream signals. Another potential important pathway for the PGC-1α and VEGF may be CamK. Repeated muscle contraction involved in shivering can alter Ca^{2+} release and re-uptake in skeletal muscle (Arruda et al., 2008, Aydin et al., 2008), inducing increase in Ca^{2+} and resulting CamK induced PGC-1α activation and expression (Bruton et al., 2010).

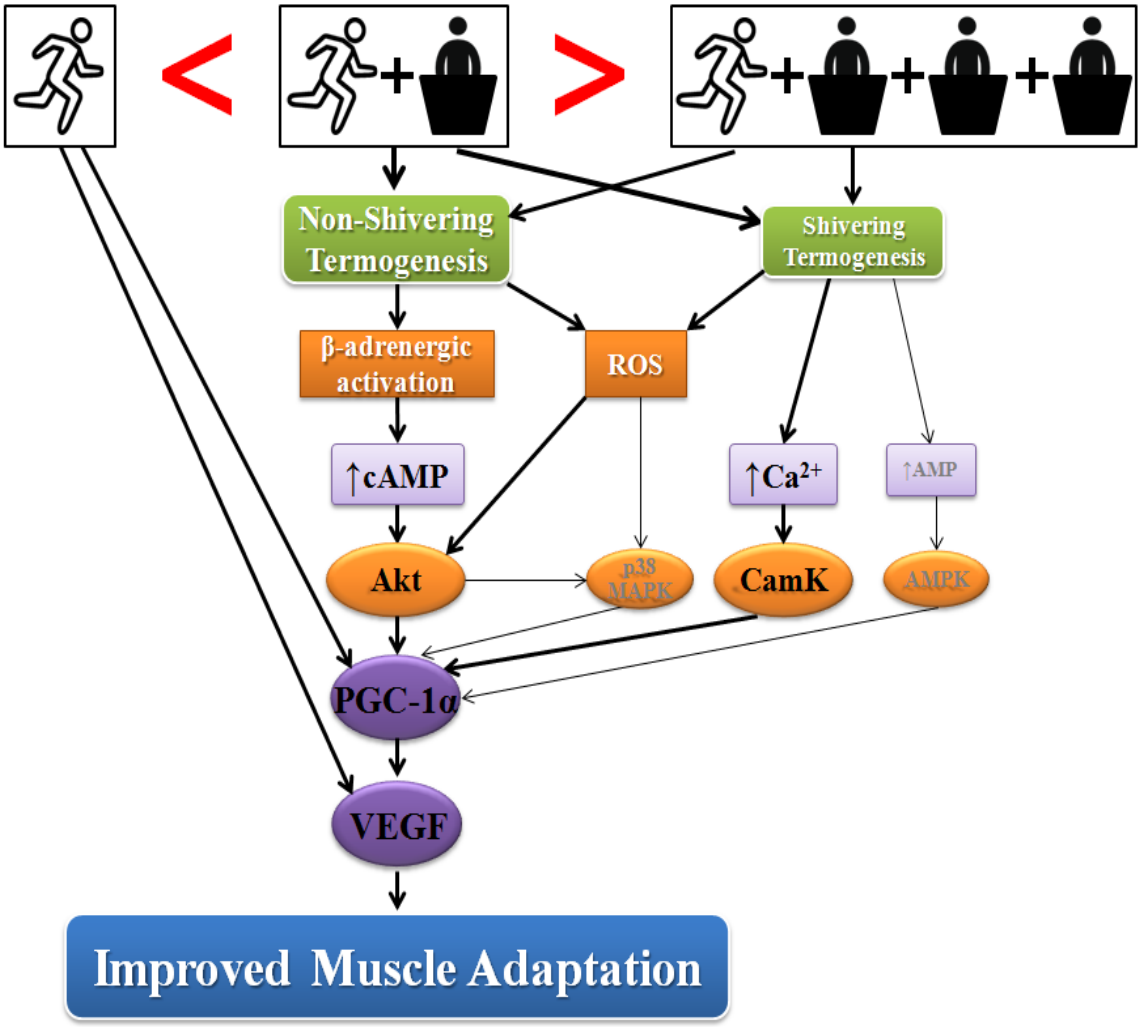

Figure 8.2.1. Post-exercise CWI induced signals involved in improved muscle adaptation. *AMP* adenosine monophosphate, *AMPK* 5'-AMP-activated protein kinase, *Akt* protein kinase B, *CamK* Ca^{2+}/calmodulin-dependent protein kinase, *Ca^{2+}* calcium, *cAMP* cycle AMP, *PGC-1α* peroxisome proliferator-activated receptor gamma co-activator-1 alpha, *p38MAPK* p38 mitogen-activated protein kinase, *ROS* reactive oxygen species, *VEGF* vascular endothelial growth factor.

The results from these investigations may have implications for the use of CWI strategies following the performance of high-intensity intermittent exercise. Current findings indicate that post-exercise CWI that promote a rise in catecholamine release and reduction in body temperature may have positive effects on mitochondrial biogenesis and angiogenesis. The current findings therefore suggest that athletes performing high-intensity intermittent exercise may benefit from post-exercise CWI that promote to maximise training adaptation.

8.3. LIMITATIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

There were several limitations within the current research including the use of MVCs without the application of twitch interpolation. MVCs used to quantify exercise-induced muscle damage in the study have potential sources of error since it is difficult to ascertain whether changes in muscle force are actually representative of structural damage to the muscle or simply a reduction in voluntary drive (Morton et al., 2005). Therefore, the twitch interpolation technique has been used to objectively measure muscle damage pre- and post-exercise (Morton et al., 2005). It is possible that MVCs with twitch interpolation in the study 1 (Chapter 4) may have different results in those being reported in this thesis.

The studies completed within this thesis provided an overview of the influence of post-exercise CWI on key signalling pathways which regulate adaptive responses to high-intensity intermittent exercise. In achieving this, some issues have arisen and certain findings have promoted the formulation of recommendations for further research. Future research is needed to shed some light on the following topics:

1. Effect of post-exercise CWI on PGC-1α and VEGF protein in human skeletal muscle.

The current experimental findings indicate that CWI following high-intensity intermittent exercise increases PGC-1α and VEGF mRNA. In contrast to mRNA, the post-exercise CWI failed to elicit any increase in protein content within our study timeline. Increase in PGC-1α and VEGF protein is typically only observed within days of exercise (Baar et al., 2002, Gustafsson et al., 2001, Perry et al., 2010). For example, PGC-1α protein was increased 24 h (+23%) after high-intensity cycle exercise and continued to increase, attaining a plateau (+42%) by the 5~7th (11~16th day) training session (Perry et al., 2010). Further research is needed to investigate the change in protein during a longer timeline.

2. Effects of CWI following muscle damage exercise on skeletal muscle cell signalling responses.

In the present studies, CWI following low-damaging high-intensity intermittent exercise further enhances the upstream signalling pathways associated with mitochondrial biogenesis in human skeletal muscle. However, CWI has utilized in the field (e.g., football, resistance training ect.) as recovery after competition or training which cause muscle damage. Exercise

protocols likely to induce muscle damage would elicit different effects on signalling pathways compared to non- or low-damaging exercise. For example, exercise-induced muscle damage increase ROS production that initiates a stress response and instigates the release of heat shock proteins (HSPs) (Baird et al., 2012), ultimately resulting in mitochondrial biogenesis (Ristow and Zarse, 2010). Cold exposure also increases the production of ROS via shivering and non-shivering thermogenesis (Wenz, 2013). It is possible that CWI following muscle damage exercise may cause greater mitochondrial biogenesis than CWI following non- or low-damaging exercise.

3. Effects of combination of recovery strategies following muscle damage exercise on skeletal muscle cell signalling responses.

Decreasing local tissue temperature via CWI is thought to reduce blood flow and the metabolic rate in injured tissues and has therefore been widely used by athletes to try and accelerate recovery following competition and training by alleviating the signs and symptoms associated with exercise-induced muscle damage (Mawhinney et al., 2013). In the present studies, post-exercise CWI also increases PGC-1α mRNA. In addition to environmental intervention, it is also possible to enhance exercise-induced increase in PGC-1α mRNA through physical interventions. Norrbom et al. (2004) observed that PGC-1α mRNA increased more after exercise with restricted blood flow than in the nonrestricted condition. The increase of the PGC-1α with restricted blood flow is associated with CamK, AMPK and p38MAPK pathways (Norrbom et al., 2004, Peake et al., 2015). Therefore, it is likely that CWI with restricted blood flow following exercise may cause further increase in PGC-1α expression than CWI alone.

4. Effects of CWI on exercise performance and markers of skeletal muscle adaptation following a period of exercise training.

In the current thesis we observed post-exercise CWI augments the exercise-induced expression of VEGF and PGC-1α in human skeletal muscle. CWI following exercise may influence training induced changes in performance and cellular and molecular responses. VEGF and PGC-1α markedly increased after 1 week of exposure to cold air ($4^{o}C$) in animal (Xue et al., 2009). Similarly chronic (1 h day^{-1}, 5 days $week^{-1}$ for 20 weeks) CWI ($18^{o}C$) increased VEGF mRNA and protein expression under resting conditions (Kim et al., 2005). Further work is required to determine the impact of CWI on exercise performance and markers of muscle adaptation following a period of exercise training.

CHAPTER 9

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Appendix 1

Cold water immersion augments the exercise-induced expression of PGC-1α in human skeletal muscle

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Introduction

Cold water immersion (CWI) enhances post-exercise recovery (Vaile et al., 2007), however, the mechanisms underpinning these responses have yet to be elucidated. The expression of peroxisome proliferator-activated receptor γ co-activator-1 α (PGC-1α), a key regulator of mitochondrial biogenesis, is increased in animals following chronic whole body cooling (Puigserver et al., 1998, Oliveira et al., 2004). However, it is currently unknown whether post-exercise CWI influences the expression of PGC-1α in human skeletal muscle. The aim of the present study was to therefore test the hypothesis that post-exercise CWI increases PGC-1α mRNA expression in human skeletal muscle following acute exercise.

Methods

Eight healthy males (25 ± 4 years) performed high-intensity interval running (8x3-min bouts at 90% $\dot{V}O_{2max}$ interspersed with 3-min recovery) on two separate occasions in a counterbalanced randomised crossover design. On each occasion, subjects rested passively (CON) or undertook 10-min of CWI (8^{o}C) immediately after exercise. Muscle biopsies (vastus lateralis), rectal, muscle and skin temperature were taken at regular intervals during the 3 h recovery period. All data were analysed using a two-factor (condition × time) within participants general linear model (GLM).

Results

Rectal temperature was similar between conditions during the 3 h recovery period (P=0.17), however, reductions in thigh skin and muscle (CON, -3.0 ± 0.9^{o}C; CWI, -4.6 ± 1.0^{o}C) temperature were significantly greater in the CWI condition compared to CON (P<0.05). PGC-1α mRNA expression did not change immediately post-exercise under both conditions (CON, 0.9 ± 0.7; CWI, 1.0 ± 0.5 fold; P>0.05). However, its expression was significantly increased 3 h post-exercise under both conditions (P=0.01) with greater expression observed in CWI (5.6 ± 3.4-fold) compared to CON (3.3 ± 2.6-fold; P<0.01).

Discussion

Data indicate that post-exercise CWI augments the exercise-induced expression of PGC-1α mRNA. These data suggest that post-exercise CWI may enhance the upstream signalling pathways associated with mitochondrial biogenesis and as such, our data have practical applications for athletes wishing to maximise training adaptations.

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Appendix 2

Title of project

The effects of cold water immersion on the cellular and molecular of human skeletal muscle to acute low-damaging high intensity intermittent exercise

Name of Researcher and School/Faculty

Mr. Chang Hwa Joo

The Research Institute for Sport and Exercise Sciences

1. I confirm that I have read and understand the information provided for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving a reason and that this will not affect my legal rights. ☐
3. I understand that any personal information collected during the study will be anonymised and remain confidential ☐
4. I agree to take part in the above study ☐
5. I consent to the removal and storage of tissue samples which will be collected during the study ☐
6. I agree to the storage of my tissue samples for future research purposes ☐

Name of Participant Date Signature

Name of Researcher Date Signature

Name of Person taking consent Date Signature
(if different from researcher)

Appendix 3

SSES REC readiness to exercise screening questionnaire

Participant ID NO :

		YES	NO
Q1	Has your doctor ever said you have a heart condition and/or should only participate in medically supervised physical activity?		
Q2	Do you ever feel pain in your chest during physical activity?		
Q3	Have you ever experienced chest pains when not doing physical activity?		
Q4	Do you suffer with palpitations?		
Q5	Do you experience dizziness or fainting?		
Q6	Have you ever been told that you have high blood pressure or are you taking medication for blood pressure or any other heart condition?		
Q7	Do you experience shortness of breath during only mild exertion?		
Q8	Do you ever get pains in your calves, buttocks or at the back of your legs during exercise which are not due to soreness or stiffness?		
Q9	Do you suffer from either asthma or Diabetes Mellitus?		
Q10	Do you have any live, kidney or thyroid disorders?		
Q11	Do you have epilepsy or have you ever had a seizure of any sort? Explain….		
Q12	Are you currently taking any prescribed or unprescribed medication? If so, please list all ?.....		
Q13	Do you have any existing bone or joint problems that could be made worse by physical activity?		
Q14	Are you pregnant or have you given birth in the last 6 months?		
Q15	Have you recently undergone surgery or are you carrying an injury?		
Q16	Do you currently have any incompletely healed injuries? Explain……		
Q17	Are you currently ill in any way? Please explain……..		
Q18	Are you aware of any other reason why you should not participate in physical exercise without medical supervision? If so, what?.....		

If you have answered yes to any of questions Q1 to Q18 we will require you to obtain written consent from your GP before agreeing to undertake any form of fitness test, physical activity, training or exercise with you.

YES	NO

Q19	Is your blood pressure known to be higher than average (over 120/80)?		
Q20	Is your level of cholesterol known to be high?		
Q21	Do you smoke regularly? If so, how many?……..		
Q22	Is there any history of coronary heart disease or coronary artery disease in either your parents or siblings before the age of 55?		

If you answered YES to 2 or more of the questions listed Q19 to Q22 above, we will, for reasons of safety, restrict any programme of exercise to a moderate intensity unless you obtain written consent from your GP to exercise at a higher intensity. Moderate intensity being a level at which you are able to comfortably sustain for up to at least 60 minutes (usually not exceeding 65% of your maximal heart rate).

Other considerations		YES	NO
Q23	Are you currently on a medically prescribed diet? Explain……..		
Q24	Do you have any allergies? Explain……		
Q25	Do you have vision in both eyes?		
Q26	Do you currently follow any specific diet restrictions (e.g. gluten free, vegetarian)? Explain….		
Q27	Do you take any dietary supplements? If yes, please state what and the frequency…….		

If you proceed with a programme of physical activity and, during that period, your health changes so that you would subsequently answer YES to any of the above questions Q1 to Q27, inform us immediately as you may need to suspend your physical activity. If you feel unwell because of a temporary illness such as cold or flu, it is advisable not to exercise.

I have read, fully understood and completed this questionnaire. The answers I have given are accurate to the best of my knowledge.

Signed…… Date……

Appendix 4

Physical Activity Readiness Questionnaire - PAR-Q (Revised - July 2007)

PAR-Q & YOU

(A Questionnaire for People Aged 15 to 69)

Regular physical activity is fun and healthy, and increasingly more people are starting to become more active every day. Being more active is very safe for most people. However, some people should check with their doctor before they start becoming much more physically active.

If you are planning to become much more physically active than you are now, start by answering the seven questions in the box below. If you are between the ages of 15 and 69, the PAR-Q will tell you if you should check with your doctor before you start. If you are over 69 years of age, and you are not used to being very active, check with your doctor.

Common sense is your best guide when you answer these questions. Please read the questions carefully and answer each one honestly : Check YES or NO.

YES	NO	
☐	☐	Has your doctor ever said that you have a heart condition and that you should only do physical activity recommended by a doctor?
☐	☐	Do you feel pain in your chest when you do physical activity?
☐	☐	In the past month, have you had chest pain when you were not doing physical activity?
☐	☐	Do you lose your balance because of dizziness or do you ever lose consciousness?
☐	☐	Do you have a bone or joint problem (for example, back, knee or hip) that could be made worse by a change in your physical activity?
☐	☐	Is your doctor currently prescribing drugs (for example, water pills) for your blood pressure or heart condition?
☐	☐	Do you know of any other reason why you should not do physical activity?

If you answered

YES to one or more questions

Talk with your doctor by phone or in person BEFORE you start becoming much more physically active or BEFORE you have a fitness appraisal. Tell your doctor about the PAR-Q and which questions you answered YES.

- You may be able to do any activity you want - as long as you start slowly and build up gradually. Or, you may need to restrict your activities to those which are safe for you. Talk with your doctor about the kinds of activities you wish to participate in and follow his/her advice.
- Find out which community programs are safe and helpful for you.

NO to all questions

If you answered NO honestly to all PAR-Q questions, you can be reasonably sure that you can :

- start becoming much more physically active - begin slowly and build up gradually. This is the safest and easiest way to go.
- take part in a fitness appraisal - this is an excellent way to determine your basic fitness so that you can plan the best way for you to live actively. It is also highly recommended that you have your blood pressure evaluated. If your reading is over 144/94, talk with your doctor before you start becoming much more physically active.

➔

DELAY BECOMING MUCH MORE ACTIVE:

- if you are not feeling well because of a temporary illness such as a cold or a fever - wait until you feel better; or
- if you are or may be pregnant - talk to your doctor before you start becoming more active.

Please note: If your health changes so that you then answer "YES" to any of the above questions, tell your fitness or health professional. Ask whether you should change your physical activity plan.

Informed Use of the PAR-Q: The Canadian Society for Exercise Physiology, Health Canada, and their agents assume no liability for persons who undertake physical activity, and if in doubt after completing this questionnaire, consult your doctor prior to physical activity.

No changes permitted. You are encouraged to photocopy the PAR-Q but only if you use the entire form.

NOTE: If the PAR-Q is being given to a person before he or she participates in a physical activity program or a fitness appraisal, this section may be used for legal or administrative purposes.

"I have read, understood and completed this questionnaire. Any questions I had were answered to my full satisfaction."

Signature:_____ Identity Document No.:_____

Name:_____ Date:_____

Signature of Parent or Guardian:_____ Witness:_____
(for participants under the age of majority)

Note: 1. The information provided on this form will only be used for the application for use of Leisure and Cultural Services Department's Fitness Rooms and enrolment of recreation and sports activities. For correction of or access to personal data collected by means of this form, please contact staff of the enrollment counter/district.

2. If you answer "yes" to one or more questions in the "PAR-Q & YOU", your physical condition may not be suitable for taking part in the activity concerned. For safety's sake, you should consult a doctor in advance and produce a medical certificate upon enrolment or hire of fitness equipment to prove that you are physically fit for taking part in the activity. If you fail to produce a medical certificate, you must submit the completed Declaration upon enrolment or hire of fitness equipment.

This physical activity clearance is valid for a maximum of 12 months from the date it is completed and becomes invalid if your condition changes so that you would answer YES to any of the seven questions.

 Supported by: Health Canada

Appendix 5

MUSCLE BIOPSY SUBJECT SCREENING FORM

Research Institute for Sport and Exercise Science
Liverpool John Moores University.
15-21 Webster Street Liverpool
0151-231-4330.

To help us ensure your safety and well-being please answer the following questions.

1. Have you ever had a negative or allergic reaction to local freezing (e.g. during dental procedures)?

   No □ Yes □

2. Do you have any tendency toward easy bleeding or bruising (e.g with minor cuts or shaving)?

   No □ Yes □

3. Are you currently taking any medications that may increase the chance of bleeding or bruising (e.g. Aspirin, Anti-inflammatories)?

   No □ Yes □

4. Have you ever fainted or do you have a tendency to faint when undergoing or watching medical procedures?

   No □ Yes □

5. Will you contact the physician who did the biopsy directly if you have any concerns about the biopsy site including: excessive redness, swelling, infection, pain or stiffness of the leg?

   No □ Yes □

6. Are you willing to visit the physician who did the biopsy 7 – 10 days following the biopsy for an assessment of the biopsy site?

   No □ Yes □

Subject Name (print) :_____

Subject Signature :_____

Date :_____

Signature of Person Conducting Assessment: _____

Appendix 6

Name

Date

48 Hour Diet and Exercise Activity Log

Guidance notes

The aim of this booklet is to assess what you have eaten, your individual eating patterns and physical activity. For 48 hours, you will need to record **EVERYTHING** you eat and drink and **ALL** forms of physical activity and exercise undertaken.

Diet Logs

One page is allocated for each day's nutritional intake. Please provide as detailed as information as possible when describing food and drink intake. Where possible, provide precise weights of foods / volumes of fluids (if not appropriate, describe the amount according to plate, portion, glass and cup size etc). If known, please also record the 'brand' of food and fluid consumed (e.g. Tesco, Uncle Ben's etc). The more detailed information you provide, the more detailed analysis can be undertaken. **It is extremely important to be completely HONEST when recording your food and drink intake!** Examples are provided in the grey boxes in the table provided overleaf.

Please refrain from exercise, alcohol, tobacco and caffeine in the 72-hour period prior to your first test and throughout the subsequent 7 day period.

On the morning of the exercise trial please refrain from eating until completion of the days testing (overnight fast)

In order to ensure you are completely hydrated prior to testing can you please consume at least 5 ml of water per kg 2 h prior to arriving at the laboratory. NB. Only consume water on the morning of the exercise trial

Activity Logs

In addition to recording your nutritional intake, you also need to record all exercise and physical activity undertaken for the 48-hours prior to each exercise trial, where one page is also allocated for each day. Examples are provided in the grey boxes in the table provided overleaf. Please provide as detailed information as possible and once again, **please be HONEST!**

Contact

If you unsure how to complete this booklet and require further information at any time, please contact:

DAY 1:

TIME	FOOD / BRAND	COOKING METHOD	QUANTITY
Example **7.00 am**	**Weetabix** **Semi-skimmed milk** **Brown toast with butter** **Eggs** **Tea with 2 sugars**	 **Boiled**	**2** **200 ml** **2 slices** **3** **1 cup**

TIME	ACTIVITY	INTENSITY	DURATION
7 am	**Walk/cycle to work**	**Moderate**	**10 mins**
6 pm	**Walk/cycle home from work**	**Moderate**	**10 mins**

In general, how were you feeling today e.g. tired, full of energy, hungry, alert, moody, good?

DAY 2:

TIME	FOOD / BRAND	COOKING METHOD	QUANTITY
Example **7.00 am**	**Weetabix** **Semi-skimmed milk** **Brown toast with butter** **Eggs** **Tea with 2 sugars**	 **Boiled**	**2** **200 ml** **2 slices** **3** **1 cup**

TIME	ACTIVITY	INTENSITY	DURATION
7 am	**Walk/cycle to work**	**Moderate**	**10 mins**
6 pm	**Walk/cycle home from work**	**Moderate**	**10 mins**

In general, how were you feeling today e.g. tired, full of energy, hungry, alert, moody, good?