Modulation of exercise-induced gene expression in human skeletal muscle by exercise intensity, training status and short-term endurance training

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By

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Declaration

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Table of contents

List of figures

List of tables

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Abbreviations

ACC	acetyl CoA carboxylase
AICAR	5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside
Akt	acute transforming retrovirus thymoma (a.k.a. protein kinase B)
δ-ALAS	δ-aminolevulinate synthase
AMPK	adenosine monophosphate-activated protein kinase
AS160	Akt substrate at 160 kDa
ATF2	activating transcription factor 2
BM	body mass
BMI	body mass index
BMR	basal metabolic rate
CaM	calmodulin
CaMKII	calcium/calmodulin-dependent protein kinase II
CBP	CREB binding protein
CHO	carbohydrate
COXIV	cytochrome c oxidase subunit IV
CPT1	carnitine palmitoyltransferase 1
CRE	cyclic AMP response element
CREB	CRE binding protein
CtBP	carboxyl-terminal binding protein
DRIP	vitamin D receptor-interacting protein
EE	energy expenditure
ERRα	estrogen-related receptor α
ERK	extracellular signal-regulated kinase
FABPpm	membrane-associated fatty acid binding protein
$FADH_2$	reduced form of flavin adenine dinucleotide (FAD)
FAT/CD36	fatty acid translocase
FOXO1A	forkhead transcription factor, O-box subfamily, 1A (a.k.a. FKHR)
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCN5	general control of amino-acid synthesis, yeast, homolog
GEF	GLUT4 enhancer factor
GLUT4	glucose transporter type 4
β-GPA	β-guanidinopropionic acid
β-HAD	β-hydroxyacyl CoA dehydrogenase
HAT	histone acetyltransferase
HDAC	histone deacetylase
HK	hexokinase
JNK	c-Jun N-terminal kinase
LDH	lactate dehydrogenase
LPL	lipoprotein lipase

MAPK	mitogen-activated protein kinase
MCAD	medium-chain acyl-coenzyme A dehydrogenase
MCIP1	myocyte-enriched calcineurin interating protein
MCT1	monocarboxylate transporter 1
MDH	malate dehydrogenase
MEF2	myocyte enhancer factor 2
Mfn2	mitofusin 2
MHC	myosin heavy chain
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced form of NAD^+
NRF-1	nuclear respiratory factor 1
PCr	phosphorylcreatine
PCR	polymerase chain reaction
PDC	pyruvate dehydrogenase compex
PDK4	pyruvate dehydrogenase kinase 4
PFK	phosphofructokinase
PK	pyruvate kinase
PPARγ	peroxisome proliferator-activated receptor γ
PGC-1α	PPARγ coactivator 1α
PRC	PGC-1α-related coactivator
PVDF	polyvinylidine fluoride
RER	respiratory exchange ratio
RIP140	nuclear receptor interacting protein 1
RSV	resveratrol
SDH	succinate dehydrogenase
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Sirt1	sirtuin (silent mating type information regulation 2 homolog) 1
SRC	steroid receptor coactivator
SRF	serum response factor
Tfam	mitochondrial transcription factor A
TRAP	thyroid hormone receptor-associated protein
UCP3	uncoupling protein 3
VO_{2peak}	maximal oxygen uptake

Abstract

Modulation of exercise-induced gene expression in human skeletal muscle by exercise intensity, training status and short-term endurance training

Brendan Egan

A single bout of exercise generates a robust, but transient, increase in mRNA abundance for a multitude of genes, which is thought to contribute to the recovery from and adaptation to exercise. Adaptation to exercise training is mediated by the accumulation of pulses of elevated mRNA after individual exercise bout within a training program, leading to longer term increases in protein abundance that culminate in physiological adaptations. Several signalling pathways involving cytoplasmic protein kinases, transcription factors and their coregulators are recognised as regulators by which activation transduces physiological stimuli into transcriptional adaptations.

This thesis examined the modulation of gene expression in human skeletal muscle under varying physiological conditions, including divergent exercise intensities, untrained and trained muscle, and short-term endurance training.

Compared to low intensity isocaloric exercise (400 kcal, 40%VO_{2peak}), a single bout of high intensity exercise (80% VO_{2peak}) resulted in greater activation of the signalling kinases AMPK and CaMKII, coincident with a larger increase in mRNA abundance of PGC-1α and FOXO1A during recovery. A single bout exercise induced an increase in PGC-1α. FOXO1A, and PDK4 mRNA abundance in both untrained and trained muscle. This may form part of a transcriptional response that contributes to exercise-induced alterations in skeletal muscle metabolism such as glucose sparing and increased fat oxidation during recovery from exercise. Fourteen consecutive days of endurance training resulted in the accumulation of mRNA and corresponding protein for some (ERRα, COXIV), but not all (FOXO1A, PDK4), reportedly acute exercise-responsive genes. This suggests that certain genes are involved in the restoration of homeostasis after acute exercise, whereas others are involved in adaptation to regular exercise.

Our results illustrate the well-described phenomenology of skeletal muscle plasticity and suggest that transcript level adjustments underlie modulation of skeletal muscle metabolism and phenotype by regular exercise.

Chapter I Introduction

All living organisms possess the inherent capacity to alter the structural and functional properties of their organ systems in accordance with the environmental conditions imposed on a particular system; i.e. systems are malleable in the presence of physiological stimuli. Phenotypic plasticity thus refers to the property of a given genotype to produce different phenotypes in response to distinct environmental conditions (Pigliucci, 2001), ultimately yielding phenomena observed in living beings. To the exercise physiologist, phenotypic plasticity is most obviously observed in the remarkable exercise performance capacity of elite athletes after years of exercise training (Coyle, 2005a), or the ability of regular exercise to prevent or ameliorate pathophysiological disease states to which physical inactivity contributes (Hawley, 2004).

1.1 Metabolic dysfunction in human skeletal muscle

Skeletal muscle is a tissue richly endowed with mitochondria and strongly reliant on oxidative phosphorylation for energy production. Quantitatively, it is the largest organ in the body, contributing 30–40% of the resting metabolic rate in adults (De Lange *et al.*, 2007). One of the striking physiological characteristics of skeletal muscle is metabolic flexibility, expressed by a high capacity to modulate rates of energy production, blood flow, and substrate utilization (Saltin & Gollnick, 1983). Impaired metabolic flexibility is manifested as reduced insulin-stimulated skeletal muscle glucose uptake, increased intramuscular triglyceride accumulation, elevated respiratory exchange ratio at rest and an inability to switch between fuel sources appropriately when challenged by feeding or fasting (Kelley *et al.*, 1999). These phenomena are closely associated with chronic metabolic diseases (Storlien *et al.*, 2004).

Impaired mitochondrial function and reduced oxidative capacity in skeletal muscle contribute to the pathophysiology of insulin resistance, type 2 diabetes mellitus and obesity (Storlien *et al.*, 2004;Lowell & Shulman, 2005). A coordinated downregulation of genes involved in mitochondrial oxidative phosphorylation and a reduction in oxidative metabolism has been demonstrated in skeletal muscle of obese (Kelley *et al.*, 2002), insulin resistant (Patti *et al.*, 2003), and type 2 diabetic (Mootha *et al.*, 2003) individuals and insulin resistant offspring of type 2 diabetic patients (Petersen *et al.*, 2004). However, the causative or compensatory nature of this pattern of expression is not established and is debated (Pospisilik *et al.*, 2007). In rats selectively bred for divergent endurance exercise capacities, rats with high intrinsic aerobic fitness levels and commensurate elevation in resting skeletal muscle mitochondrial oxidative capacity have reduced cardiovascular risk factors (Wisloff *et al.*, 2005) and exhibit resistance to development of insulin resistance when challenged with a high fat diet (Noland *et al.*, 2007), when compared to rats with lower intrinsic aerobic capacity and reduced skeletal muscle mitochondrial oxidative capacity.

1.2 Exercise as therapeutic intervention for metabolic dysfunction

Therefore, therapeutic strategies that increase oxidative capacity and the expression of mitochondrial genes in skeletal muscle are essential for the amelioration of metabolic

dysfunction and insulin resistance associated with obesity and type 2 diabetes mellitus. Fortunately, skeletal muscle shows a remarkable malleability to remodel its phenotype and to adapt functionally in response to contractile stimuli (Hood *et al*., 2006;Fluck, 2006). A single bout of exercise is known to acutely increase lipid metabolism and glucose disposal in skeletal muscle (Wasserman & Cherrington, 1996) and improve whole body insulin sensitivity (Richter *et al*., 1989). Repeated myofibrillar contraction associated with physical activity and exercise training is a potent stimulus for physiological adaptation. For instance, exercise training can improve indices of metabolic flexibility in both obese and type 2 diabetic individuals (Toledo *et al*., 2006;Menshikova *et al*., 2007). Regular physical activity is therefore a critical tool in the treatment and prevention of these chronic diseases (Booth *et al*., 2002;Hawley, 2004), although the molecular mechanisms underlying these benefits are still being elucidated. However, a direct association between chronic physical activity, increases in oxidative capacity and improvements in mitochondrial function has long been established (Holloszy, 1967). As little as six sessions of exercise training can almost double the endurance capacity of untrained individuals when completing a cycle to exhaustion at a fixed workload (Burgomaster *et al*., 2005). Importantly from a metabolic perspective, this is accompanied by an increase in muscle oxidative capacity and mitochondrial protein expression (Gibala *et al*., 2006).

1.3 Regulation of skeletal muscle gene expression

The expression of genes encoding proteins regulating skeletal muscle mitochondrial metabolism is under control of an increasingly well-defined subset of transcription factors and their coregulators. Peroxisome proliferator-activated receptor (PPAR) γ co-activator 1α (PGC-1α) is a transcriptional coactivator that is critical in controlling many aspects of cellular metabolism in a variety of tissues and thought to be a “master regulator” of transcriptional processes (Finck & Kelly, 2006;Handschin & Spiegelman, 2006). In muscle cells, altering the transcriptional activity of PGC-1α affects physiological responses that equip the cell to meet the energy demands of a changing environment including augmentation of mitochondrial biogenesis, cellular respiration rates and energy substrate uptake and utilisation (Wu *et al*., 1999;Michael *et al*., 2001;Lin *et al*., 2002;St-Pierre *et al*., 2003;Wende *et al*., 2005;Rohas *et al*., 2007;Wende *et al*., 2007). The molecular mechanism underlying these functional adaptations may be the ability of PGC-1α to coordinate alterations in the expression of genes involved in carbohydrate and lipid metabolism, oxidative phosphorylation and mitochondrial biogenesis in response to metabolic stimuli.

In recent years, it has become clear that transcriptional coregulator proteins, apart from being crucial to the regulation of gene expression in response to cellular signals, can actually be primary targets for signal transduction pathways (Gerhart-Hines *et al*., 2007;Jager *et al*., 2007). This characterisation provides a framework for understanding the continuity between signalling events affecting cellular energetics and the expression of both nuclear- and mitochondrial-encoded genes that dictate skeletal muscle phenotype (Scarpulla, 2008). Therefore, transcription factors, nuclear receptors and their transcriptional coregulators are key players in

integrating signals from physiological stimuli into adaptive tissue responses to coordinate metabolic processes (Spiegelman & Heinrich, 2004;Desvergne *et al.*, 2006;Feige & Auwerx, 2007). Transcription factors and coregulator proteins can be activated in response to specific cellular signals, cocomplex with a variety of other factors and demonstrate selective activation of only certain gene promoters containing binding sites for the given transcription factor. These highly regulated functions suggest that the coregulator proteins play a crucial role in selecting the subset of genes that make up a given biological program (Spiegelman & Heinrich, 2004;Desvergne *et al.*, 2006). Regulation of transcriptional activity occurs via change in the amounts (protein content) or activities (e.g. posttranslational modifications) of transcription factors (Spiegelman & Heinrich, 2004).

1.4 Contraction-mediated regulation of skeletal muscle gene expression

Of particular interest is the fact that the activation of these cellular signals and the expression and/or function of these transcriptional regulators can be markedly and rapidly induced by acute exercise. A single bout of exercise presents multiple stimuli to and within the contracting muscle mass, which underlines the difficulty in delineating the signalling pathway from the exercise signal and ultimately the molecular mechanisms linking exercise to the transcription of metabolic genes. Acute contractile activity increases calcium flux, ATP turnover, mechanical stress, glycogen utilisation, catecholamine concentration and oxidative stress (Fluck, 2004;Freyssenet, 2007;Coffey & Hawley, 2007). Any one of these changes may, in principle, activate a signalling pathway that may ultimately alter the expression of downstream gene targets (Williams & Neufer, 1996). Exercise-induced phenotypic alterations are generally accepted to involve the sensation and transduction of contraction-induced stimuli through intracellular signal transduction pathways including protein kinases, phosphatases and deacetylases, their integration into physiological processes by downstream targets including transcription factors and transcriptional coregulators (Fluck, 2004;Hood *et al.*, 2006;Freyssenet, 2007). The consequent alterations in mRNA abundance and protein accretion result in functional and structural adaptations localised to the exercised muscle.

These functional adaptations to exercise training have been well characterised and include proteins involved in mitochondrial ATP production (Holloszy, 1967), mobilisation, transport and oxidation of fatty acids (Bonen *et al.*, 1999), glucose transport and glycogen synthesis (Houmard *et al.*, 1993), antioxidant defences (Ookawara *et al.*, 2003), and oxygen delivery to and extraction from skeletal muscle (Hickson, 1981). These adaptations have long been known but many of the detailed molecular mechanisms remain to be identified. Contraction-induced changes in skeletal muscle gene expression and activity of key metabolic and mitochondrial proteins are largely responsible for the pleiotropic effects of exercise on skeletal muscle metabolism. A widely accepted hypothesis is that adaptation is mediated largely at the level of transcription (Mahoney & Tarnopolsky, 2005). A single exercise session carries a “molecular signature” which is typical for the type of stimulus (i.e. the nature of the exercise session) as well as the actual condition of the muscle tissue (i.e. untrained, trained) (Hoppeler *et al.*, 2007).

This signature is evident as rapid, transient changes in mRNA abundance and protein expression during recovery from exercise. The transcriptome response to a single bout of exercise includes an upregulation of mRNAs involved in carbohydrate and lipid metabolism, oxidative phosphorylation and mitochondrial biogenesis (Pilegaard *et al*., 2000;Hildebrandt & Neufer, 2000;Pilegaard *et al*., 2003;Hildebrandt *et al*., 2003;Russell *et al*., 2005;Cartoni *et al*., 2005;Cluberton *et al*., 2005;Pilegaard *et al*., 2005;Mahoney *et al*., 2005). Repeated exercise sessions thus lead to concerted accretion of expressed mRNAs which upon translation results in a corresponding protein accretion. This process has been identified as the major molecular strategy of muscle for governing structural and functional adaptations with exercise training (Booth & Baldwin, 1996;Williams & Neufer, 1996;Hood, 2001;Fluck & Hoppeler, 2003). Therefore, regulation of gene expression at the transcriptional level is accepted as a key process in understanding the overall adaptive response to exercise.

Furthering our understanding of both the transcriptome response to acute and chronic exercise and the molecular mechanisms underpinning these responses is important for establishing therapeutic strategies for disorders involving skeletal muscle. The aim of this thesis is to examine the modulation of gene expression in human skeletal muscle under varying physiological conditions, including divergent exercise intensities, untrained and trained muscle, and short-term endurance training, to further our understanding of the molecular biology of acute exercise and exercise training.

1.5 Thesis overview: aims, objectives and hypotheses

This thesis will examine the modulation of gene expression in human skeletal muscle under varying physiological conditions. Firstly, the effect of different exercise intensities on contraction-induced signalling cascades and gene expression of metabolic genes and transcriptional regulators in untrained men will be investigated. Secondly, the transcriptional response to a single bout of exercise in well-trained men will be examined in comparison to results from untrained men. Thirdly, the mRNA and protein abundance of a subset of metabolic genes and transcriptional regulators in response to short-term endurance training in previously untrained men will be examined.

1.5.1 Experiment I

Contraction-induced signalling and gene expression of metabolic genes and transcriptional regulators in human skeletal muscle: influence of exercise intensity

Overview

Eight healthy, sedentary males performed two isocaloric (400 kcal) cycle exercise trials, once each at either 40% or 80% VO_{2peak}. Skeletal muscle biopsies from the *m. vastus lateralis* were taken at rest and at +0 h, +3 h and +19 h after exercise.

Specific aims

(i) To investigate the effect of divergent exercise intensities on the expression of genes encoding transcriptional regulators and proteins involved in mitochondrial and substrate metabolism in untrained skeletal muscle in response to a single bout of exercise.

(ii) To examine the potential for differential activation of contraction-induced signal transduction pathways in response to divergent exercise intensities

Hypothesis

(i) There will be greater activation of contraction-induced signalling kinases (AMPK, CaMKII) and transcript expression of selected gene targets (PGC-1α, FOXO1A, PDK4) after a single bout of high intensity compared to low intensity isocaloric exercise

1.5.2 Experiment II

The effect of glycogen-depleting exercise on contraction-induced signalling and gene expression of metabolic genes and transcriptional regulators in well-trained human skeletal muscle

Overview

Eight healthy well-trained males cycled for 90 min at 73% VO_{2peak}. Skeletal muscle biopsies from the *m. vastus lateralis* were taken at rest and at +0 h and +1 h after exercise.

Specific aims

(i) To examine the effect of a single bout of glycogen-depleting exercise on the mRNA abundance of transcription factors and transcriptional coregulators in well-trained human skeletal muscle

Hypothesis

(i) The exercise-induced alterations in mRNA expression of selected gene targets will be qualitatively similar to those reported in untrained muscle

1.5.3 Experiment III

The effect of fourteen consecutive days of exercise training on the expression of metabolic and mitochondrial genes and their transcriptional regulators in skeletal muscle of previously untrained men

Overview

Eight healthy, sedentary males performed 60 min of exercise at 80% VO_{2peak}, once per day for fourteen consecutive days. Skeletal muscle biopsies from the *m. vastus lateralis* were taken

prior to the start of training, and on the morning after the 1st, 3rd, 7th, 10th and 14th training session.

Specific aims

(i) To examine whether proposed accumulation of mRNA underlying muscular adaptation after repeated bouts of exercise training is evident after fourteen days of training.

(ii) To examine the continuity between alterations in mRNA and translational changes in protein content during training.

(iii) To examine the time course of adaptation at both the transcriptional and translational level during fourteen consecutive days of training.

Hypotheses

(i) There will be an increase in steady-state mRNA content of selected genes will be consistent with those that are reported to be transiently elevated after a single bout of exercise

(ii) The steady-state increases in the expression of gene transcripts will be paralleled by increases at the translational level manifested as increased protein content of the encoded protein

(iii) Sampling skeletal muscle at multiple time points during fourteen days of training will reveal a sequential accumulation of mRNA and protein abundance of selected exercise-responsive gene targets

Chapter II Review of literature

2.1 Transcriptional regulation of metabolism in skeletal muscle

The flow of information in biological systems is generally such that for a gene of interest, DNA is copied into mRNA by transcription and proteins are synthesised by translation using mRNA as the template. Unlike DNA and protein, mRNA is synthesised rapidly and on-demand, and changes in mRNA concentration generally reflect a change in the rate of transcription (under the control of the opposing effects of activation and repression) or the stability of mRNA (Koulmann & Bigard, 2006). The human genome contains approximately 20-25,000 genes and encodes approximately 85,000 different mRNAs, due to alternative mRNA splicing and variable polyadenylation (International Human Genome Sequencing Consortium, 2004). Transcriptional regulation of metabolism is critical to maintenance of homeostasis in living organisms in response to physiological cues (Desvergne *et al.*, 2006;Feige & Auwerx, 2007). Furthermore, this process has been identified as the major molecular strategy of muscle governing the induction of structural and functional adaptations to exercise training (Booth & Baldwin, 1996;Williams & Neufer, 1996;Hood, 2001;Fluck & Hoppeler, 2003). Therefore, regulation of gene expression at the transcriptional level is accepted as a key process in understanding the overall adaptive response to exercise.

Transcriptional regulation may be thought of as the key step in integrating signals from dietary, metabolic and endocrine pathways to control target gene expression (Desvergne *et al.*, 2006). The activation of gene transcription results in the transient synthesis of mRNA, which may undergo modification to generate the mature transcript. The mature transcript exits the nucleus via the nuclear pores and, when coupled to the translational machinery of the ribosome, is translated into the encoded peptide. However, gene activation is a multistep process involving a very large number of proteins functioning in discrete complexes (Spiegelman & Heinrich, 2004). Therefore, understanding transcriptional control in a biological system requires information on (i) events upstream of transcriptional activity, which define the signals involved and their route to the nucleus; (ii) the molecular mechanisms by which transcription factors operate; and (iii) events downstream of transcriptional activity, which depend on the groups of genes that are targeted and how further signals are generated to reach the dynamic equilibrium of homeostasis (Desvergne *et al.*, 2006). Exercise-induced phenotypic alterations involve the sensation and transduction of contraction-induced stimuli through intracellular signal transduction pathways including protein kinases, phosphatases and deacetylases, their integration into physiological processes by downstream targets including transcription factors and transcriptional coregulators (Fluck, 2004;Hood *et al.*, 2006;Freyssenet, 2007). The consequent alterations in mRNA abundance and protein accretion result in functional and structural adaptations localised to the exercised muscle. This review of literature will focus on the regulation and effectors of each of these nodes of control in skeletal muscle metabolism at rest and in response to acute exercise and exercise training.

Transcriptional control requires specific signals to be transduced to the cell nucleus where defined sets of genes are targeted. The factors controlling the activation of these target genes

under the influence of these signals encompass a modest subset of well-defined transcription factors, nuclear receptors, and transcriptional coregulators (Scarpulla, 2008). These factors coordinate metabolic processes by adapting tissue responses to various challenges through the integration of endogenous and exogenous signals (Feige & Auwerx, 2007). To this end, the terminal point of many complex signal transduction cascades is the activation of transcriptional regulator proteins that bind to promoter and enhancer regions of target genes, which in turn activates transcription of that gene. The regulatory sequences of most genes contain binding sites for multiple transcription factors, allowing each gene to respond to multiple signalling pathways and facilitating the fine-tuning of transcript levels (McKenna & O'Malley, 2002;Orphanides & Reinberg, 2002).

2.1.1 Activation and repression of transcription

In order for a transcription factor to be relevant to a particular gene encoding for a particular protein, there must be a binding site for that factor on the promoter region of that gene (Hood, 2001). Transcription factors bind directly to DNA in a sequence-specific manner and essentially mark a gene for activation or repression through the recruitment of coactivator or corepressor proteins (Spiegelman & Heinrich, 2004). These coregulators are characterised by their ability to increase or decrease transcription without direct binding to DNA. Transcription factors bound to their specific site in the promoter region of the target gene recruit transcriptional coregulators in response to a cellular signal. Once recruited, these coregulators alter the transcriptional machinery mainly through the modification of local histones and recruitment of transcriptional enzymes. Coactivators are generally associated with histone acetyltransferase (HAT) activity resulting in histone acetylation and alteration in chromatin structure, which allows greater access to DNA for the activated transcription factor. Corepressors generally have the opposite effect on chromatin structure associated with histone deacetylase (HDAC) activity reducing the effect of transcription factors on the target gene. The unique local chromatin structure and histone acetylation state at enhancer/silencer and promoter regions provide critical regulatory input that allows the cell to greatly increase the range of transcriptional responses (Naar *et al*., 2001). The global impact of coregulators on metabolism depends on their capacity to modulate metabolic balance by promoting or inhibiting anabolic and catabolic functions (Feige & Auwerx, 2007).

The regulation of gene activity at the transcriptional level has generally been thought to occur via changes in the abundance or activities (function) of transcription factors. This means that genes encoding transcription factors may themselves be transcriptionally induced or repressed or the existing transcription factor protein may be activated or deactivated. Similarly, coactivator and corepressor proteins may participate in gene regulation, not merely by being necessary gears in the transcriptional machinery but by being primary targets of physiological signals (Spiegelman & Heinrich, 2004). Protein phosphorylation and acetylation are prevalent posttranslational modifications by which the functions of transcription factors and coregulators are regulated in response to cellular stimuli (Orphanides & Reinberg, 2002), and are discussed in the context of skeletal muscle later.

2.1.2 Changes in mRNA abundance

Increments in mRNA abundance are often referred to as increases in gene expression. This is technically incorrect, because increased gene expression and its associated phenotypic/functional manifestations do not take place until there is an increase in the concentration of the protein encoded by the gene (Baar *et al*., 2002). Gene expression in its broadest sense therefore refers to a process encompassing the activation of transcriptional regulators to the synthesis of a functional protein (Orphanides & Reinberg, 2002). Gene expression can be controlled at various points beyond transcription, so the extent to which a protein will increase in response to an adaptive stimulus cannot always be predicted from the increase in mRNA. In skeletal muscle, expression of mRNA does not always accurately reflect the abundance of proteins and can give no information regarding their posttranslational modifications (Hojlund *et al*., 2008). In addition, it is established in yeast (Gygi *et al*., 1999) that the correlation between mRNA and protein levels was insufficient to predict protein expression levels from quantitative mRNA data. Indeed, for some genes, while the mRNA levels were of the same value the protein levels varied by more than 20-fold. Conversely, invariant steady-state levels of certain proteins were observed with respective mRNA transcript levels that varied by as much as 30-fold. Therefore, when considering alterations in transcriptional regulation or mRNA content in terms of muscle metabolism, although mRNA abundance is a strong determinant of protein synthesis, this relationship is neither simple nor linear.

In summary, homeostatic perturbations, such as those induced by exercise in skeletal muscle, are integrated via intracellular signalling cascades into alterations in gene transcription. The diffusible gene copies produced then provide the message for the instruction of muscle tissue remodelling via translation and assembly of the encoded proteins. Based upon this relationship, it is hypothesized that the systematic exploration of differences in transcript levels relative to phenotypic adjustments arising from the impact of exercise will reveal the strategy underlying muscle plasticity (Fluck, 2006).

2.2 PGC-1α and its associated transcriptional regulators in the regulation skeletal muscle metabolism

In skeletal muscle and other metabolic tissues, an increasingly well defined network of transcription factors and coregulator proteins provide a critical level of control in a wide range of metabolic processes. This is illustrated by their ability to target and alter the expression of key metabolic enzymes and mitochondrial proteins and coordinate the process of mitochondrial biogenesis. The most well-characterised of these proteins that can be thought of as metabolic regulators in skeletal muscle include, but is not limited to, PGC-1α, PPARs, NRF-1, NRF-2, ERRα, FOXO1A, MEF2 and RIP140 and their subsequent coordination of alterations the expression of genes involved in carbohydrate metabolism (HKII, PDK4, GLUT4), lipid metabolism (CPT1, MCAD, LPL), oxidative phosphorylation (cytochrome c, COXIV) and mitochondrial biogenesis (Tfam).

2.2.1 Discovery and expression of PGC-1α

PGC-1α is a transcriptional coactivator that is critical in controlling many aspects of cellular metabolism in a variety of tissues (Finck & Kelly, 2006;Handschin & Spiegelman, 2006). In muscle cells, altering the transcriptional activity of PGC-1α affects physiological responses that equip the cell to meet the energy demands of a changing environment including augmentation of mitochondrial biogenesis, cellular respiration rates and energy substrate uptake and utilisation (Wu *et al.*, 1999;Michael *et al.*, 2001;Lin *et al.*, 2002;St-Pierre *et al.*, 2003;Wende *et al.*, 2005;Rohas *et al.*, 2007;Wende *et al.*, 2007). PGC-1α was first identified in brown fat as a cofactor for PPARγ that is required for adaptive thermogenesis in response to cold exposure (Puigserver *et al.*, 1998). PGC-1α protein expression is highly expressed in mitochondria-enriched tissues including skeletal muscle and its rapid and pronounced response to cold exposure indicated that it responded to physiological cues. This prompted investigations on the regulation of its activity by cellular signals in response to physiological stimuli. In human skeletal muscle, PGC-1α protein content is highest in type IIa, lower in type I and lowest in type IIx fibres (type IIa 2.2-fold > type I and 6-fold > type IIx; type I 2.5-fold > type IIx)(Russell *et al.*, 2003). In terms of mRNA, whereas large differences in PGC-1α mRNA levels are seen between muscle types in rodents (up to five times higher in type I fibres) (Lin *et al.*, 2002), there is no difference in PGC-1α mRNA levels between different human skeletal muscles (Plomgaard *et al.*, 2006;Mortensen *et al.*, 2007). PGC-1α mRNA (Pilegaard *et al.*, 2003), protein expression (Baar *et al.*, 2002) and activation (Wright *et al.*, 2007b) are stimulated by a single bout of exercise in skeletal muscle. Similar effects can be induced by fasting in liver (Rhee *et al.*, 2003;Rodgers *et al.*, 2005) but this chapter will be limited to skeletal muscle metabolism where possible.

2.2.2 Interaction with transcription factors

No intrinsic chromatin-modifying capabilities have been identified in PGC-1α but it can associate with many proteins that either alter chromatin structure or recruit other essential transcriptional components. After docking with a transcription factor on the promoter of a target gene, PGC-1α recruits several powerful HATs including CBP/p300 and SRC-1, leading histone acetylation, chromatin remodelling and access for additional factors for gene activation. Additionally, a mediator known as the TRAP/DRIP complex is recruited to PGC-1α and enhances the transcription process by recruiting RNA polymerase II. These two features are though to be the main mechanisms by which PGC-1α alters the transcriptional control of target genes (Lin *et al.*, 2005).

MEF2 is a transcription factor with response elements on a variety of genes involved in muscle remodelling (Potthoff & Olson, 2007). MEF2 relies on the recruitment of other transcriptional regulators to mediate its effects. Coactivation of MEF2 by PGC-1α provides a positive feed-forward signal that rapidly induces PGC-1α expression in an autoregulatory loop (Handschin *et al.*, 2003). This provides a mechanism to sustain PGC-1α levels in response to physiological stimuli (Finck & Kelly, 2006). MEF2 binds to PGC-1α on at least two binding sites and

modulates its expression through MEF2 interaction with HDAC5 (Czubryt *et al.*, 2003). The importance of MEF2-PGC-1α interaction is underscored by the observation that MEF2 and CRE sites in the PGC-1α promoter previously described (Handschin *et al.*, 2003;Czubryt *et al.*, 2003) are indispensable in the activation of the PGC-1α promoter in response to contractile activity (Akimoto *et al.*, 2004b). This suggests that contractile activity-induced PGC-1α gene transcription must be mediated through signals that are transduced through MEF2 and/or CRE binding proteins or certainly regulatory factors that interact with MEF2 and CRE sequence elements on the PGC-1α promoter.

Ectopic expression of PGC-1α in muscle cells induces an increase in mitochondrial DNA and mitochondrial biogenesis and promotes the expression of a large number of nuclear- and mitochondrial-encoded mitochondrial genes. Since this phenomenon was first observed (Wu *et al.*, 1999), the mechanisms underlying this response have delineated a specific pathway involving PGC-1α, NRF-1, NRF-2, ERRα and Tfam (Wu *et al.*, 1999;Schreiber *et al.*, 2004;Huss *et al.*, 2004;Mootha *et al.*, 2004). NRF-1 and NRF-2 are nuclear-encoded transcription factors linked to the transcriptional control of many mitochondrial genes by virtue of the presence of NRF binding sites within their promoters (Kelly & Scarpulla, 2004). ERRα is an orphan nuclear receptor expressed in high levels in oxidative tissues including cardiac and skeletal muscle. Mechanistically, PGC-1α induces expression of mitochondrial genes by increasing the levels of NRF-1, NRF-2 and ERRα (Wu *et al.*, 1999;Schreiber *et al.*, 2004;Mootha *et al.*, 2004). When coactivated by PGC-1α, NRF-2 and ERRα promote their own, as well as each others' expression, in a PGC-1α-dependent manner and subsequently elevate the levels of NRF-1 (Schreiber *et al.*, 2004). Subsequently, PGC-1α coactivates NRF-1 and NRF-2 binding to regulatory regions of the Tfam promoter (Wu *et al.*, 1999). Tfam is a nuclear-encoded transcription factor essential for the replication, maintenance, and transcription of mitochondrial DNA (Finck & Kelly, 2006), and possesses the ability to bend and unwind DNA during stimulation of transcription after its binding to target DNA (Scarpulla, 2008). Tfam is required and responsible for normal expression of mitochondrial DNA and function (Larsson *et al.*, 1998;Ekstrand *et al.*, 2004). Contractile activity-induced mitochondrial biogenesis is thought to occur through the stimulation of this pathway in skeletal muscle (Baar, 2004;Hood *et al.*, 2006).

Multiple roles for these factors and several other PGC-1α partners have been identified in the regulation of skeletal muscle metabolism (Fig. 2.1) and are considered below. Our current understanding of the physiological role of PGC-1α in skeletal muscle metabolism has largely been established based on the results of overexpression/gain-of-function and knockout/loss-of-function experiments.

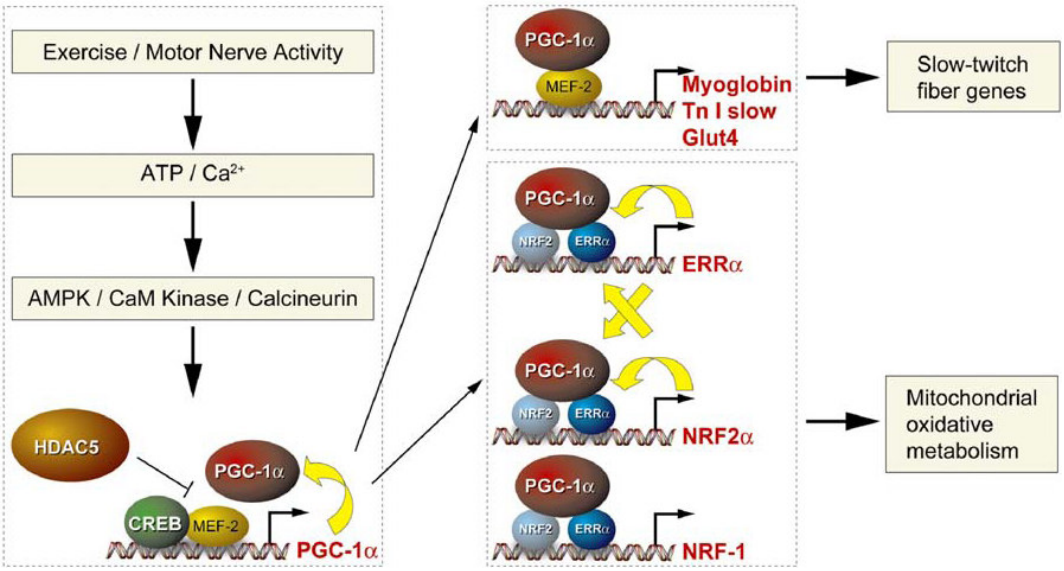

Figure 2.1 Regulation of PGC-1α expression in skeletal muscle and mechanisms by which PGC-1α stimulates mitochondrial gene expression. Coactivation of MEF2 by PGC-1α provides a positive feed-forward signal to rapidly induce PGC-1α expression following muscle contraction. PGC-1α induces the expression of ERRα, which activates the expression of NRF-1, NRF-2, and ERRα itself. These molecular events lead to the stimulation of nuclear-encoded mitochondrial genes, (reproduced from Lin et al., 2005).

2.2.3 Regulation of glucose disposal

The insulin-responsive GLUT4 transporter is highly expressed in skeletal muscle and its activation and translocation the cell membrane accounts for the majority of insulin- and contraction-mediated skeletal muscle glucose uptake (Dohm, 2002). Muscle-specific GLUT4 knockout markedly attenuates insulin-mediated glucose disposal (Kim *et al.*, 2001), whereas skeletal muscle GLUT4 overexpression improves whole body insulin sensitivity (Ren *et al.*, 1995). In addition, a single bout of exercise in known to increase the transcription and expression of GLUT4 mRNA and protein content (Neufer & Dohm, 1993;Kraniou *et al.*, 2006). Expression of the GLUT4 gene requires two distinct binding regions on the GLUT4 promoter for the MEF2 (Thai *et al.*, 1998) and GEF transcription factors (Oshel *et al.*, 2000). The molecular regulation of the GLUT4 gene and its response to exercise is thought to be associated with PGC-1α (McGee & Hargreaves, 2006). Adenoviral-induced expression of PGC-1α induces expression of GLUT4 and increases glucose transport in C_2C_{12} cells (Michael *et al.*, 2001) whereas conditional overexpression of PGC-1α in murine muscle increases muscle glycogen storage via several mechanisms including stimulation of glucose import, suppression of glycolytic flux, and by downregulation of the expression of glycogen phosphorylase (Wende *et al.*, 2007). This effect is potentially mediated by a MEF2-HDAC-PGC-1α regulatory mechanism. Association with class II HDACs in the basal state represses MEF2 transcriptional activity. Removal of HDAC repression on MEF2 via HDAC phosphorylation allows MEF2 to act on its binding site in the promoter region of the GLUT4 gene and recruit PGC-1α to enhance the transcription process. A single bout of exercise reduces HDAC5 association with MEF2 (McGee & Hargreaves, 2004), possibly through phosphorylation of HDAC5 by exercise-induced kinases

such as AMPK and CaMKII (McGee & Hargreaves, 2006). Similarly, HDAC4-associated repression of MEF2 transcriptional activity is decreased coincident with HDAC4 nuclear efflux in electrically stimulated muscle fibres (Liu *et al.*, 2005). In addition, acute exercise increases physical MEF2/PGC-1α association (McGee & Hargreaves, 2004), which taken together would result in exercise-induced enhancement in MEF2 transcriptional function, thereby potentially leading to increased PGC-1α and GLUT4 expression by the mechanisms described.

2.2.4 Regulation of substrate utilisation

In addition to regulating glucose transport at the level of transcription, forced expression of PGC-1α in C_2C_{12} cells regulates the balance between carbohydrate and lipid metabolism by inducing PDK4 mRNA and protein expression leading to a reduction in glucose oxidation (Wende *et al.*, 2005). The induction of GLUT4 expression is critical to the uptake of glucose under stimulated conditions. However, the ability of the muscle to switch effectively between fuel sources for oxidation and storage when challenged with fasting and feeding is an essential feature of metabolic flexibility (Storlien *et al.*, 2004). This is also true in the post-exercise period wherein rapid glycogen repletion following a bout of intense exercise is an important adaptive response, preparing the muscle for subsequent bouts of activity. Thus the post-exercise period is characterised by a reduction in glucose oxidation, increase in glycogen synthesis and increase mitochondrial fatty acid oxidation (Kimber *et al.*, 2003). This acute metabolic response is thought to be PGC-1α-mediated through the coordinated induction of genes encoding proteins involved in these processes. The pyruvate dehydrogenase complex (PDC) is the key step regulating the complete oxidation of glucose proceeding from glycolysis to the Kreb cycle. Inactivation of this complex via phosphorylation by PDK4 shuts down the conversion of pyruvate to acetyl CoA, resulting in allosteric inhibition of glycolysis and suppression of glucose oxidation leading to the diversion of imported glycogen precursors to storage (Sugden & Holness, 2006). PGC-1α-associated induction of PDK4 expression is mediated by the activity of ERRα and FOXO1A (Furuyama *et al.*, 2003;Wende *et al.*, 2005;Araki & Motojima, 2006). FOXO1A is a forkhead transcription factor, regulated by posttranslational modifications including acetylation and phosphorylation and known to regulate genes involved in energy metabolism (Accili & Arden, 2004;Gross *et al.*, 2008). Induction of ERRα and subsequent binding to the PDK4 gene promoter in a complex with PGC-1α is induced by PGC-1α and results in increased PDK4 expression, a pathway in which ERRα is indispensable (Wende *et al.*, 2005).

In transgenic mice overexpressing muscle-specific FOXO1A, PGC-1α mRNA levels are increased at rest suggesting that FOXO1A promotes PGC-1α gene expression in muscle (Kamei *et al.*, 2004). In skeletal muscle, FOXO1A is known to positively regulate fat metabolism and glucose sparing through the induction of LPL and PDK4 gene transcription (Kamei *et al.*, 2003;Furuyama *et al.*, 2003) and fat transport by enhancing FAT/CD36 expression in the plasma membrane (Bastie *et al.*, 2005). FOXO1A binds directly to the PDK4 promoter (Kwon *et al.*, 2004) and overexpression of inducible FOXO1A in C_2C_{12} cells increases PDK4 expression, leading to decreased pyruvate dehydrogenase activity and glycolytic flux (Bastie *et al.*, 2005).

2.2.5 Regulation of lipid metabolism

In addition to the role of ERRα in the induction of PDK4 expression and reduction of glucose oxidation, ERRα is capable of regulating the lipid metabolism by inducing the expression of MCAD, an enzyme catalysing the first step of the β-oxidation pathway (Sladek *et al.*, 1997). The results of ERRα overexpression and cotransfection studies demonstrate that MCAD is an endogenous target for ERRα and but that only in the presence of PGC-1α does ERRα transactivate the MCAD promoter (Huss *et al.*, 2002;Huss *et al.*, 2004).

PGC-1α was identified as a regulator of lipid metabolism by virtue of its ability to increase mitochondrial fatty acid oxidative enzyme gene expression and cellular oxidation rates in concert with PPARα in preadipocytes (Vega *et al.*, 2000). Similar findings have been reported in L6 muscle cells where PGC-1α overexpression upregulates PPAR-target genes involved in fatty acid catabolism and β-oxidation (Koves *et al.*, 2005). These results have been recapitulated *in vivo* with the additional observation that fatty acid transporters and the rate of mitochondrial fatty acid oxidation were increased after PGC-1α transfection (Benton *et al.*, 2008). The resultant modest overexpression of PGC-1α protein in rodent muscle upregulates expression of proteins involved in lipid metabolism at the level of membrane (FABPpm, FAT/CD36) and mitochondrial transport (CPT1).

These results suggest that induction of PGC-1α controls muscle fuel selection by increasing glucose uptake but reducing glucose oxidation, thereby increasing glucose storage, while at the same time reciprocally upregulating mitochondrial fatty acid oxidation. Therefore, PGC-1α has a critical role in fuel selection in resting skeletal muscle and in response to physiological stimuli.

2.2.6 Overview of mitochondrial biogenesis

2.2.6.1 Fundamentals of mitochondrial biogenesis

The process of mitochondrial biogenesis refers to increase in muscle mitochondrial volume and alterations in organelle composition, a well-established consequence of endurance training (Holloszy, 1967;Hoppeler *et al.*, 1973). This is a complex and highly regulated process requiring the coordination and coexpression of both the nuclear and mitochondrial genomes for the assembly and expansion of the reticulum (Hood, 2001;Goffart & Wiesner, 2003). These processes include the transcription of nuclear genes, translation of the newly formed mRNAs and import of proteins into mitochondria, replication of mtDNA, transcription and translation of mitochondrial genes, biosynthesis of mitochondrial membrane phospholipids, and assembly of the enzyme complexes (Freyssenet *et al.*, 1996). The expression of genes promoting mitochondrial biogenesis is predominantly controlled by the global principles of gene expression (Goffart & Wiesner, 2003) described in section 2.1. In the context of skeletal, mitochondrial biogenesis is regulated by signals intrinsic to the contracting muscle (Hood, 2001) described later in section 2.6. These signals are known to activate protein kinases and phosphatases that modify the activity of transcription factors acting in the nucleus, as well as mRNA stability factors

acting within the cytosol, resulting in an increase in the mRNA expression of nuclear-encoded mitochondrial proteins (Fig. 2.2). The translated proteins are then appropriately chaperoned to the mitochondria and imported into the different organelle compartments, such as the matrix space, the inner or outer membranes. A small subgroup of nuclear-encoded mitochondrial proteins comprises transcription factors that act directly on mitochondrial DNA (mtDNA) to increase the mRNA expression of mitochondrial gene products, as well as mtDNA copy number (Scarpulla, 2008). One such factor, Tfam, regulates the expression of the thirteen mtDNA gene products, including proteins such as COX. Nuclear-encoded mitochondrial proteins and mtDNA-encoded proteins are assembled to form multi-subunit enzyme complexes required for oxygen consumption and ATP synthesis. This coordination between nuclear and mitochondrial genomes is necessary for organelle biogenesis.

Exercise-induced modulation of these processes either directly or indirectly by targeting other regulatory factors (e.g. ERRα, MEF2), and therefore has the ability to modulate the mitochondrial phenotype. Although the modulation of mitochondrial phenotype is important to the function of skeletal muscle during exercise, dysfunctional mitochondria have been implicated in the age-related loss of muscle mass i.e. sarcopenia (Bua *et al.*, 2002) and insulin resistance (Lowell & Shulman, 2005). Thus, mitochondrial biogenesis induced by chronic exercise is now recognized to have implications for a broader range of health issues than just the enhancement of endurance performance.

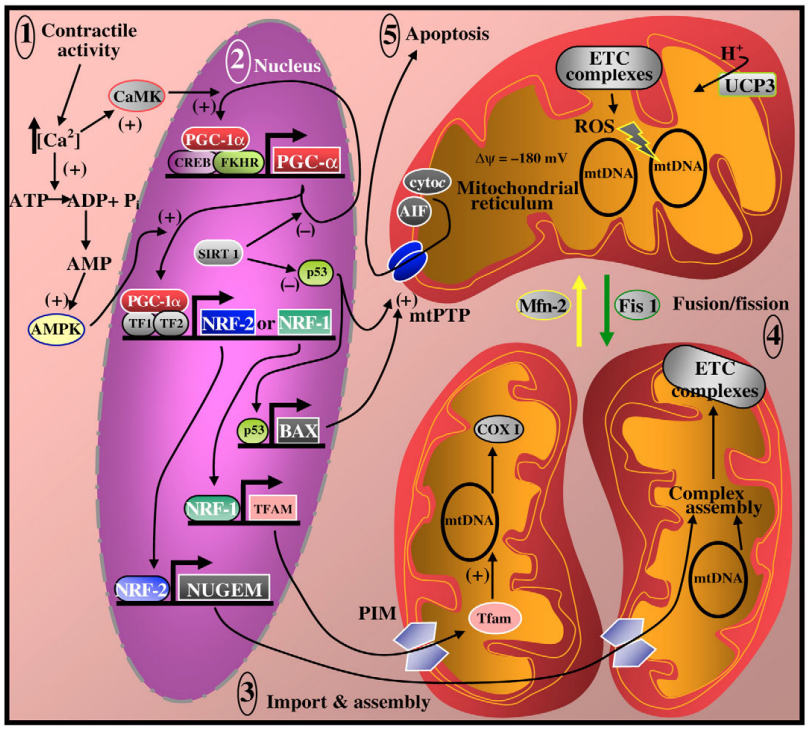

Figure 2.2 Mitochondrial biogenesis is a component of contractile activity-induced muscle plasticity (reproduced from Hood et al., 2006)

2.2.6.2 PGC-1α in the regulation of mitochondrial biogenesis

PGC-1α overexpression both *in vivo* and *in vitro* is associated with an increase in the induction of mitochondrial gene expression program including increased respiratory chain subunit expression, mitochondrial density and mitochondrial enzyme activity (Wende *et al.*, 2007;Benton *et al.*, 2008). Skeletal muscle-specific PGC-1α overexpression driven by the muscle creatine kinase promoter results in the transition from type II fibres to an increased proportion of type I muscle fibres coincident with increased oxidative phenotype, expression of mitochondrial markers and resistance to electrically stimulated fatigue (Lin *et al.*, 2002). The regulation of this phenomenon through PGC-1α, NRFs and ERRα was described in section 2.2.2. In addition to the stimulatory effect of NRFs on Tfam expression, these transcription factors also bind to target sequences on nuclear- and mitochondrial-encoded mitochondrial genes, including cytochrome c and COXIV (Scarpulla, 2008). Similarly, ERRα has recently been associated with PGC-1α-induced mitochondrial biogenesis (Schreiber *et al.*, 2004;Mootha *et al.*, 2004) in association with NRF-2. ERRα was shown to be essential for PGC-1α-mediated mitochondrial biogenesis and function upstream of the previously mentioned NRF-1 action in the program of oxidative phosphorylation gene expression (Mootha *et al.*, 2004). To this end, ERRα binding sites are present in the promoters of genes such as cytochrome c and β-ATP synthase (Scarpulla, 2008).

2.2.6.3 Functional consequences of mitochondrial biogenesis

The major functional adaptation that occurs within skeletal muscle fibre types as a result of endurance training is an improvement in fatigue resistance, which is strongly correlated with an increased mitochondrial enzyme activity (Holloszy, 1967;Baldwin *et al.*, 1972;Walsh *et al.*, 2001). This is evidenced by an increased capacity to oxidize respiratory substrates in whole homogenates (Baldwin *et al.*, 1972), isolated mitochondria (Holloszy, 1967;Bizeau *et al.*, 1998;Tonkonogi *et al.*, 2000) and permeabilised muscle fibres (Walsh *et al.*, 2001). Studies of mitochondria isolated from endurance-trained muscle have revealed a reduced sensitivity to ADP-stimulated respiration (Walsh *et al.*, 2001), but an enhanced creatine-stimulated response (Tonkonogi & Sahlin, 2002). The combination of a greater response to creatine, along with the higher overall mitochondrial content of trained muscle, leads to a greater sensitivity of whole muscle mitochondrial respiration to ADP (Dudley *et al.*, 1987). The greater mitochondrial content means that a lower ADP is required to achieve the same level of oxygen consumption per gram of muscle (Dudley *et al.*, 1987). This reduces the consumption of phosphorylcreatine, attenuates the rate of glycolysis and lactic acid production, reduces the formation of AMP and ammonia, improves the fraction of energy obtained aerobically, and decreases the activation of AMPK. It also means that each mitochondrial electron transport chain operates at a lower fraction of its maximal rate to produce the same oxygen consumption as before the adaptation took place (Hood, 2001).

These are the molecular mechanisms underpinning the functional consequences of endurance training i.e. a reduction on the reliance of carbohydrate as a fuel and an increase in the

contribution from lipid sources when exercise is performed at the same relative exercise intensity after a period of training (Holloszy & Coyle, 1984). These adaptations are considered specifically in section 2.5.1. From a disease perspective, a current hypothesis is that inefficiency to couple "complete" β-oxidation of fatty acids to mitochondrial respiration in skeletal muscle is a possible cause of insulin resistance and type 2 diabetes (Koves *et al*., 2008). Exercise training-induced and PGC-1α-associated mitochondrial remodelling is proposed to favourably alter the efficiency of mitochondrial fatty acid oxidation resulting in a reduction in the accumulation of intramuscular acylcarnitines, metabolic by-products of incomplete fatty acid oxidation, thus ameliorating lipid-induced glucose intolerance (Koves *et al*., 2005).

2.2.7 Metabolic consequences of PGC-1α knockout

PGC-1α overexpression/gain-of-function studies demonstrate the capability of PGC-1α induction to alter skeletal muscle metabolism, but the necessity of PGC-1α in regulating metabolic processes is better established in knockout/loss-of-function studies. Whole body PGC-1α knockout mice display multiple pathologies indicative of altered energy metabolism including an inability to evoke a thermogenic response upon exposure to cold, and reduced mitochondrial content and oxidative capacity in type I muscle fibres leading to a reduction in exercise tolerance (Lin *et al*., 2004a;Leone *et al*., 2005). Muscle-specific PGC-1α knockout mice (Handschin *et al*., 2007b) have been generated to examine the isolated effects on skeletal muscle in the absence of the influence of whole body phenotypical changes such as hyperactivity exhibited by whole body knockouts (Lin *et al*., 2004a). These muscle-specific knockout mice have a higher percentage of the glycolytic type IIx and IIb fibres at the apparent expense of a loss of oxidative type I and IIa fibres, reduced voluntary physical activity and impaired exercise endurance and strength performance (Handschin *et al*., 2007a). The metabolic phenotype of these mice is one of impaired glucose tolerance but not peripheral insulin resistance despite impaired mitochondrial function (Handschin *et al*., 2007b). The transcriptional mechanisms underlying these alterations in muscle metabolism may be the coordinated downregulation of mRNAs for transcription factors regulating mitochondrial biogenesis (ERRα, NRF-2), mRNAs encoding proteins of oxidative phosphorylation (cytochrome c, COXVb, β-ATP synthase) and mRNA of the glucose transporter GLUT4. Mitochondrial enzymatic activity was also reduced in these knockouts coupling the decrease in mitochondrial gene expression with defects in mitochondrial function (Handschin *et al*., 2007b). The effect of muscle-specific PGC-1α knockout on the regulation of metabolic and mitochondrial gene expression at rest and in response to exercise is considered in section 2.6.8.

2.2.8 Summary

Collectively this section demonstrates that PGC-1α has a critical role in the control of skeletal muscle metabolism at rest and in the induction of appropriate cellular responses to physiological stimuli. In addition, this level of control resides in its ability to coordinate the expression of mitochondrial and metabolic genes. Although the data do not fully demonstrate causation, PGC-

1α is necessary for the normal expression of nuclear- and mitochondrial-encoded mitochondrial gene expression. A critical aspect to this control is its versatility in its ability to respond to cellular signals by interacting with a variety of transcription factors and other regulators to induce the expression of well-defined gene subsets depending on the physiological response required. The functional interactions of PGC-1α with its transcriptional partners in effecting the responses described above are in turn regulated by the functional activity of existing PGC-1α protein as described below.

2.3 Regulation of PGC-1α-associated transcriptional activity

As would be predicted by its inducibility, the activity of PGC-1α is linked to variety of upstream signal transduction pathways that modulate PGC-1α function and protein stability through posttranslational modifications. These modifications are one means of modulating metabolic function through the activation or inactivation of enzymes, or by affecting protein stability. In the context of transcriptional regulation, posttranslational modifications are a means of altering transcriptional regulators either by affecting transcription factor/coregulator nuclear translocation or exclusion, or by affecting activation status of the factor within the nucleus (Desvergne *et al*., 2006). This is of major importance to muscle metabolism as it links intracellular signalling to transcriptional regulation (Feige & Auwerx, 2007). Phosphorylation-dependent regulation of PGC-1α stability and activity, and the regulation of acetylation-mediated metabolic regulation through the Sirt1 deacetylase have been established (Fig. 2.3)(Rodgers *et al*., 2008).

2.3.1 Regulation by phosphorylation

The half-life of PGC-1α protein is relatively short (2.3 h) (Handschin & Spiegelman, 2006). Therefore, upon cessation of stimulatory pathways on PGC-1α expression, PGC-1α protein is rapidly degraded and induction of many PGC-1α downstream target genes returns to basal levels. Phosphorylation of PGC-1α by p38 MAPK (section 2.6.4), leads to a tripling of protein half-life, and thus higher PGC-1α protein levels are observed (Puigserver *et al*., 2001). Conversely, in liver cells, PGC-1α protein stability is reduced by phosphorylation by Akt (Li *et al*., 2007). As well as affecting PGC-1α protein stability, phosphorylation by p38 MAPK enhances the transcriptional activity of PGC-1α (Puigserver *et al*., 2001). Phosphorylation at these p38 MAPK sites removes the repressive effect of inhibitory factor known as p160 myb binding protein on PGC-1α activity (Fan *et al*., 2004). In skeletal muscle, AMPK (section 2.6.2) is known to directly phosphorylate PGC-1α at two sites that are indispensable to the effect of AMPK on PGC-1α mRNA induction and protein function, and the expression of metabolic and mitochondrial genes (Jager *et al*., 2007). Phosphorylation by AMPK enhances the functional activity of PGC-1α on its own promoter. Similarly, although untested, AMPK-mediated phosphorylation could modulate the ability of PGC-1α to dock on certain transcription factors or affect the binding or function of other cofactors in the PGC-1α coactivator complex. This might provide a simple explanation by which this kinase could activate certain PGC-1α functions in

muscle (Zong *et al*., 2002). The activation of calcium signalling cascades in skeletal muscle has also been implicated in the induction of PGC-1α activity, although the precise mechanism is not well defined (Wu *et al*., 2002;Wright *et al*., 2007a). That acute exercise results in the activation of each of these signal transduction kinases (section 2.6) strongly implicates rapid alterations in PGC-1α protein as a key regulatory mechanism in the exercise-induced modulation of skeletal muscle metabolism (Atherton *et al*., 2005;Akimoto *et al*., 2005;Wright *et al*., 2007b).

2.3.2 Regulation by acetylation

The acetylation status of PGC-1α protein is the most prominent modification in terms of control of its activity and physiological output (Feige & Auwerx, 2007;Rodgers *et al*., 2008). Functional activity of PGC-1α is inhibited by acetylation and the PGC-1α acetylation status is directly balanced by the action of the GCN5 acetyltransferase and the NAD^+-dependent Sirt1 deacetylase in liver (Rodgers *et al*., 2005;Lerin *et al*., 2006) and skeletal muscle cells . GCN5 directly acetylates PGC-1α, negatively regulating its transcriptional activity in part through nuclear sublocalisation, at least in liver cells (Lerin *et al*., 2006;Gerhart-Hines *et al*., 2007). Sirt1 is capable of direct physical interaction with PGC-1α, deacetylating the PGC-1α protein and increasing its transcriptional activity (Nemoto *et al*., 2005). Sirt1 is a member of the sirtuin family of NAD^+-dependent class III HDACs, which are capable of coupling the cleavage of NAD^+ to nuclear gene expression (Gerhart-Hines *et al*., 2007;Dali-Youcef *et al*., 2007). In this model of metabolic regulation, increases in NAD^+ levels are sensed by Sirt1, which is in turn activated. Sirt1 then targets and deacetylates PGC-1α at promoter regions of target genes to induce gene expression of mitochondrial and fatty acid oxidation to maintain the bioenergetic state of the cell (Rodgers *et al*., 2008). This model provides a molecular mechanism by which fluctuations in energy status of the cell induce cellular adaptation. During muscle contraction, the NAD^+/NADH ratio is subject to dynamic fluctuations imposed by the rate of NAD^+ reduction to NADH and rate of NADH oxidisation to NAD^+ (Robergs *et al*., 2004). However, our understanding of Sirt1 regulation in skeletal muscle is limited.

2.3.3 Sirt1-mediated metabolic regulation

The regulation of Sirt1 at a translational level is currently unknown. However, Sirt1 enzymatic activity is directly affected by fluctuations in NAD^+ as well as the ratio of NAD^+/NADH. NAD^+ is an obligate cosubstrate (Imai *et al*., 2000), whereas NADH (Lin *et al*., 2004b) and nicotinamide (Bitterman *et al*., 2002) are inhibitors of Sirt1. Thus, the redox state of the cell controlled by the activation of different metabolic pathways that change these variables will affect Sirt1 activity (Rodgers *et al*., 2008). The potential role of Sirt1 was first shown in the liver, where Sirt1 in a complex including HNF-4 deacetylates and activates PGC-1α, promoting gluconeogenesis and expression of fatty acid oxidation genes following fasting (Rodgers *et al*., 2005). Fasting increases in the levels of pyruvate and NAD^+ in the liver which resulted in elevated Sirt1 protein and enzymatic activity.

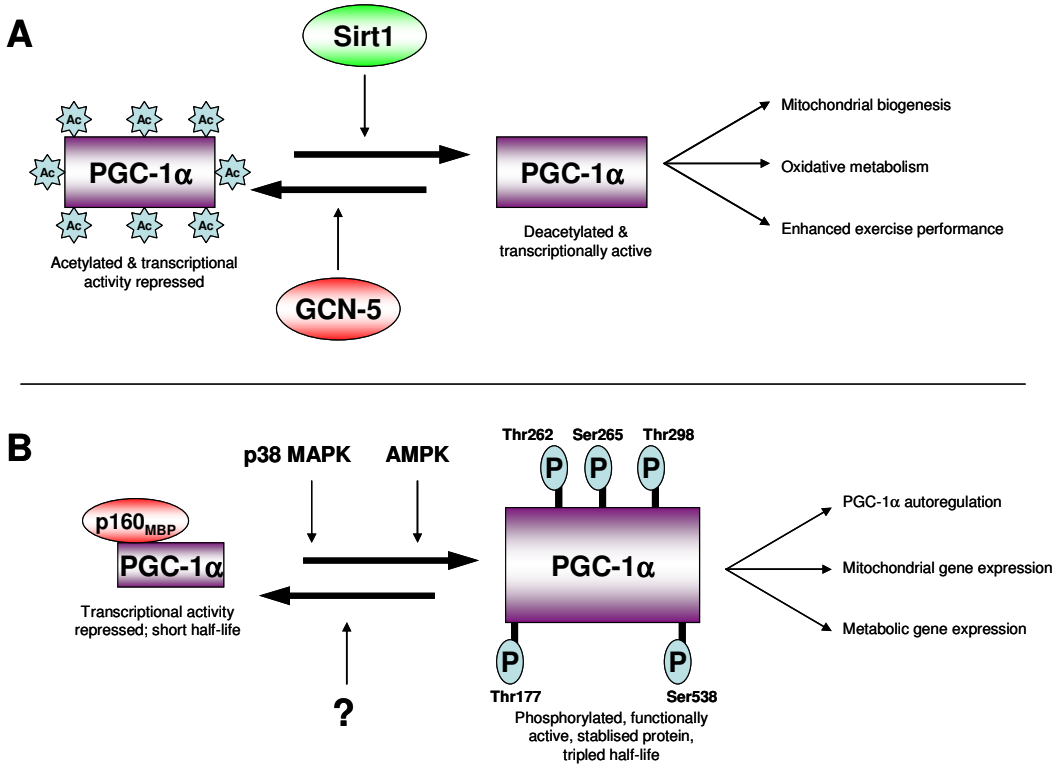

Figure 2.3 Regulation of the physiological output of PGC-1α by (A) acetylation, and (B) phosphorylation. See text for detailed discussion.

A compound known as resveratrol significantly increase Sirt1 activity through an allosteric interaction, resulting in the increase of Sirt1 affinity for both NAD^+ and the acetylated substrate (Howitz *et al.*, 2003). In skeletal muscle, Sirt1-mediated effects on PGC-1α regulation and target gene expression *in vivo* have recently been established (Lagouge *et al.*, 2006). Myofibres from resveratrol-treated mice were enriched in mitochondria, exhibited enhanced oxidative capacity, and displayed a greater resistance to fatigue because of the concerted activation of a genetic program geared for aerobic metabolism (Lagouge *et al.*, 2006). The effects of resveratrol on PGC-1α target gene expression were dependent on the presence of the wild-type PGC-1α protein. In addition, these effects were lost in cases where the acetylation sites in PGC-1α that are targeted by Sirt1 were mutated or when Sirt1 expression was disrupted. Gene-expression analysis supported an increase in fatty-acid oxidation since MCAD expression was increased and glucose utilization reduced as PDK4 levels were increased. Gerhart-Hines et al. (2007) have since shown that Sirt1 was bound to promoters of PGC-1α targets such as cytochrome c, PDK4 and PGC-1α and ectopic expression of PGC-1α induced a 7-10-fold recruitment of Sirt1 to these promoters. In addition, this ectopic PGC-1α expression increased ERRα expression and increased mitochondrial gene expression, including respiratory chain (cytochrome c, COXV) and fatty acid utilization (MCAD, CPT1 and PDK4) genes, an effect that was largely prevented by knocking down Sirt1 (Gerhart-Hines *et al.*, 2007).

However, it cannot be concluded that PGC-1α is the sole target of Sirt1, as Sirt1 interacts with and deacetylates other substrates (Dali-Youcef *et al*., 2007), including potential regulators of metabolism and mitochondrial function such as FOXO1A (Brunet *et al*., 2004;Motta *et al*., 2004). The role of acetylation of FOXO1A is less clear; acetylation impairs FOXO1A factor activity on reporter constructs but has also been reported to enhance FOXO1A activity (van der Heide & Smidt, 2005). It is possible that acetylation either enhances or represses FOXO factors in a target-gene-specific context or tissue-specific context. In general, acetylation of FOXO1A reduces its DNA binding activity and makes its more sensitive to phosphorylation, resulting in nuclear exclusion and cytosolic proteasomal degradation (Matsuzaki *et al*., 2005). However, p300-mediated FOXO1A acetylation results in transcriptional activation (Perrot & Rechler, 2005). Deacetylation of FOXO1A renders the protein resistant to phosphorylation and results in nuclear accumulation of the protein (Frescas *et al*., 2005). Reports demonstrating Sirt1-mediated effects on FOXO1A transcriptional activity are conflicting, with activity repressed in prostate cancer cells (Yang *et al*., 2005b) but activated in liver cells (Nakae *et al*., 2006). The role of FOXO1A acetylation in skeletal muscle metabolism or an exercise context is not established. In rodents, FOXO proteins act as switch from carbohydrate to predominantly lipid utilisation as a fuel substrate in skeletal muscle during periods of starvation (Furuyama *et al*., 2003). However, in humans fasted for 48 h, FOXO1A mRNA, total protein expression and Ser^{256} phosphorylation were unaltered despite marked changes in whole body metabolism and the expression of the FOXO1A target, PDK4 (Tsintzas *et al*., 2006).

2.4 The role of corepression in metabolic regulation

Whereas the importance of activating gene transcription in the regulation of skeletal muscle metabolism is well established, the contribution of transcriptional inhibition is less well defined (Christian *et al*., 2006). Recently it has been shown that a transcriptional corepressor known as RIP140 exerts critical physiologic roles by actively repressing gene transcription (Powelka *et al*., 2006;Seth *et al*., 2007;White *et al*., 2008). RIP140 is a ligand-dependent corepressor for most, if not all, nuclear receptors by binding to nuclear receptors through their LXXLL motifs. Because several nuclear receptor coactivators also possess and utilize these motifs, RIP140 may compete with the coactivators of the same binding sites on the ligand-bound receptors (Christian *et al*., 2006). RIP140 acts as scaffold protein in a similar manner to PGC-1α but has the opposite effects, recruiting chromatin remodelling enzymes leading to chromatin condensation and transcriptional repression (White *et al*., 2008). The repressive function of RIP140 is mediated by the presence of four repression domains which recruit HDACs leading to increased repression (Wei *et al*., 2000). The recruitment of HDACs is mediated by the binding of the carboxyl-terminal binding protein (CtBP). CtBP is a functional dehydrogenase that binds both NAD^+ and NADH (Zhang *et al*., 2002) and is associated with repression of transcription by RIP140 by facilitating HDAC recruitment (White *et al*., 2008). Therefore changes in cellular redox potential may affect RIP140 repressive activity mediated by CtBP acting as a sensor of redox state (White *et al*., 2008). This may have functional consequences for control of gene

expression in skeletal muscle after exercise as acute exercise is known to alter redox state (see section 2.6.5).

Like PGC-1α, the control of RIP140 repressive activity is largely under the control of posttranslational modifications including phosphorylation and acetylation (White *et al.*, 2008). Phosphorylation of RIP140 results in greater HDAC recruitment and enhanced repression (Gupta *et al.*, 2005), whereas acetylation of RIP140 prevents CtBP recruitment and relieves transcriptional repression (Vo *et al.*, 2001). Therefore it is likely that cellular signal transduction cascades regulate its activity but acute exercise-induced effects are entirely unexplored at this time. The expression pattern in skeletal muscle is opposite to PGC-1α expression; expression in lowest in muscles with high oxidative capacity and highest in muscles with higher glycolytic activity (Seth *et al.*, 2007).

RIP140 plays a crucial role in lipid metabolism demonstrated by the fact that RIP140-null mice are lean and resistant to obesity (Leonardsson *et al.*, 2004), an effect mediated by the upregulation of a number of metabolic genes (Powelka *et al.*, 2006). The increased expression of GLUT4 and the mitochondrial proteins COXVb and SDH in cultured RIP140-null adipocytes is ERRα-dependent (Powelka *et al.*, 2006). In skeletal muscle, RIP140 depletion markedly upregulates genes encoding metabolic enzymes and proteins that catalyze mitochondrial respiration, a phenomenon that may also be ERRα-dependent. The phenotype of RIP140-null mice is remarkably similar to PGC-1α overexpression experiments, with RIP140-depletion of muscle previously high in RIP140 expression exhibiting a more oxidative fibre type coincident with increased mitochondrial number and an upregulation of genes involved in oxidative metabolism, most notably fatty acid oxidation (Seth *et al.*, 2007). Conversely, a reduction in mitochondrial gene expression is seen in transgenic mice overexpressing RIP140. Therefore the effects of RIP140 on transcriptional repression may be dependent on the relative expression of RIP140 evidenced by the differences and the intermediate phenotypes observed in heterozygous animals (White *et al.*, 2008). Finally, overexpression of RIP140 in transgenic mice does not impair muscle fibre remodelling in response to six weeks of voluntary exercise training compared to wild-type mice (Seth *et al.*, 2007), suggesting that RIP140 does not alter the exercise training response but does determine the relative oxidative capacity.

The mechanism by which these effects are controlled is not fully understood. In the context of skeletal muscle fat oxidation, RIP140 has been shown to bind directly to MCAD and MCAD expression is increased in the RIP140-null muscle, coincident with an increase in ERRα (Seth *et al.*, 2007). As described earlier, ERRα induction of MCAD is PGC-1α-dependent. In general, there is a remarkable similarity between RIP140 and PGC-1α target genes, with RIP140 primarily repressing and PGC-1α activating, suggesting a functional interplay between the coregulators could be an integral part of the regulation of metabolic processes (White *et al.*, 2008).

2.5 Skeletal muscle plasticity in response to contractile activity

The phenomenon of skeletal muscle plasticity is illustrated by the remodelling of the muscle's structure and functional make-up, as seen in muscular force, endurance and contractile velocity of mammalian skeletal muscle, as a result of alterations in functional demand (Booth & Baldwin, 1996;Fluck, 2006). The adaptation to endurance exercise is characterised by functional adaptations in proteins involved in mitochondrial ATP production (Holloszy, 1967), mobilisation, transport and oxidation of fatty acids (Bonen *et al.*, 1999), glucose transport and glycogen synthesis (Houmard *et al.*, 1993), and oxygen delivery to and extraction from skeletal muscle (Hickson, 1981). These adaptations collectively contribute towards maximising substrate delivery, respiratory capacity and contractile parameters during submaximal exercise. The net effect is an enhanced resistance to fatigue, or what is thought of as a more robust defence of homeostasis in the face of metabolic perturbation (Mahoney & Tarnopolsky, 2005).

Although each individual bout of exercise is necessary as a stimulus for adaptation, it alone is not sufficient to alter muscle phenotype. A change in muscle phenotype is the result of repeated bouts of exercise. The adaptation is mediated at transcriptional level, where pulses of elevated mRNA abundance after acute bouts of exercise may accumulate to new steady-state level, which after translation leads to a change in protein abundance and associated physiological adaptation (Fig. 2.4)(Booth & Baldwin, 1996;Williams & Neufer, 1996;Hood *et al.*, 2006;Fluck, 2006). In support of this hypothesis, endurance-trained athletes with approximately two-fold higher VO_{2peak} and muscle mitochondrial volume compared to sedentary individuals have elevated mRNA concentrations for genes encoding six enzymes involved in oxidative phosphorylation (Puntschart *et al.*, 1995). These elevations were in direct proportion to the higher mitochondrial content of the muscle. Enhanced levels of gene transcripts therefore support the synthesis of protein components and provoke structural remodelling and functional adjustments in the long term (Fluck, 2006).

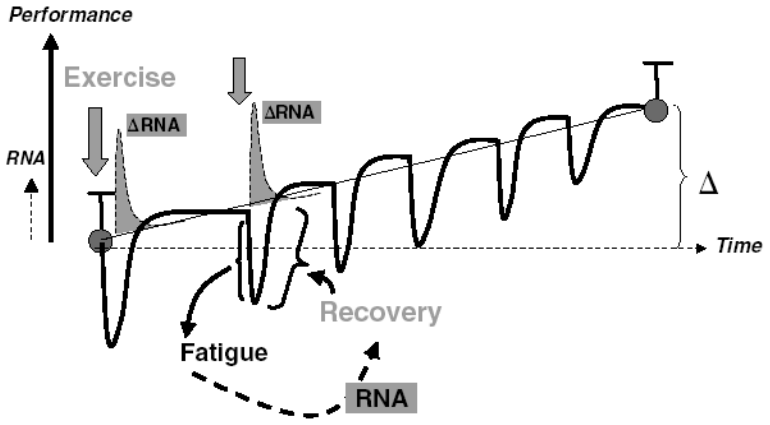

Figure 2.4. Microadaptations of transcript expression relate to the training effect. Model of the increase in RNA and endurance performance with repetition of exercise. Each bout of exercise leads to an overshoot of transcript levels in the recovery phase from fatiguing exercise, which leads via translation to a microadaptation of the encoded protein and related structure. This relays to the gradual accumulation (Δ) of mitochondrial volume density and the improved oxidative capacity with repetition of endurance exercise. A match of transcriptional, structural and functional parameters is observed in recruited muscle groups between untrained and endurance-trained steady-states, (reproduced from Fluck, 2006).

2.5.1 Adaptations to exercise training

2.5.1.1 Mitochondrial and biochemical adaptations

Regular endurance training improves performance during tasks that rely mainly on aerobic energy metabolism, in large part by increasing the body's ability to transport and use oxygen and altering substrate metabolism by working skeletal muscle. The adaptive increase in skeletal muscle oxidative capacity after exercise training was first established over forty years ago (Holloszy, 1967). Twelve weeks of training (5 d/wk) resulted in an approximately two-fold increase in mitochondrial enzymes (SDH, NADH dehydrogenase, COX activities per gram of muscle) and in the capacity of skeletal muscle to oxidize pyruvate. This provided evidence of mitochondrial biogenesis in skeletal muscle in response to exercise training, a fact that was confirmed in human muscle when it was demonstrated that an increase in mitochondrial size and number was evident after several years of endurance training (Hoppeler *et al.*, 1973). Protein concentration of cytochrome c in skeletal muscle was also increased two-fold and the protein fraction of mitochondria in total muscle was increased by approximately 60% (Holloszy, 1967). This was reflective of an increase in mitochondrial number. Endurance capacity was improved by approximately six-fold in these animals. In humans, an increase in total mitochondrial volume density by 40% in *m. vastus lateralis* and increase in maximal oxygen uptake capacity increase by 14% has been reported when sedentary subjects were trained for six weeks (5 d/wk for 30 min) and are typical of results observed (Hoppeler *et al.*, 1985). These changes were accompanied by an increase of the volume density of mitochondria in all three fibre types, the difference being somewhat larger in type IIa than in type I and type IIx fibres (Howald *et al.*, 1985).

2.5.1.2 Effects on glycolytic metabolism

Endurance training generally has little effect on or reduces the enzymatic activity of glycolytic enzymes with the exception of hexokinase, which increases after a period of exercise training (Baldwin *et al.*, 1973;Green *et al.*, 1983;Phillips *et al.*, 1996b). Intense endurance training leads to a reduction in the enzymatic activity of glycogen phosphorylase, GAPDH, pyruvate kinase and LDH in rodent skeletal muscle (Green *et al.*, 1983). However, in human skeletal muscle, no change is observed in the enzymatic activity of LDH, PFK, PK, GAPDH, HK or glycogen phosphorylase after ten to twelve days of training (Green *et al.*, 1991). Comparison of skeletal muscle from elite distance runners and untrained men shows no difference in glycogen phosphorylase activity but a reduction in LDH activity in the trained muscle (Fink *et al.*, 1977) consistent with data from rodent muscle. Similarly, no difference existed in PFK activity when comparing trained and untrained muscle (Gollnick *et al.*, 1972), although these authors subsequently reported that PFK activity was increased in response to five months of endurance training (Gollnick *et al.*, 1973). Although many enzymes of the glycolytic pathway may not be upregulated by endurance exercise training, the concentration of the muscle glucose transport protein GLUT4 is consistently increased with acute exercise (Kraniou *et al.*, 2004), short- (Gulve & Spina, 1995), and long-term (Houmard *et al.*, 1993) training. Therefore, it is likely that the

training effect on glucose metabolism enhances the ability to store muscle glycogen but has little effect on the catabolism of glucose at the level of the glycolytic pathway.

2.5.1.3 Effects on lipid metabolism

In addition to increased enzymatic activity of mitochondrial β-oxidation enzymes observed after endurance training (e.g. β-HAD) (Spina *et al*., 1996), exercise training alters several regulatory proteins of the fatty acid oxidation pathway. A 70% higher muscle LPL activity in the *vastus* lateralis muscle was demonstrated after eight weeks of regular, dynamic knee-extensor exercise training compared with the untrained muscle in the same individuals (Kiens & Lithell, 1989). Similarly, individuals who engage in regular physical exercise training for several years have significantly higher muscle LPL activity than sedentary-matched males and females (Kiens *et al*., 2004). In the same study, FAT/CD36 protein content was not different between groups, but short-term training for nine days (Tunstall *et al*., 2002) as well as a single exercise bout (Roepstorff *et al*., 2004) increased FAT/CD36 protein content slightly (20–25%) in muscle. This suggests that increased FAT/CD36 expression is an early adaptation to increased muscle activity. Higher FABPpm protein expression in homogenates from the *vastus lateralis* muscle was also demonstrated with exercise training (Kiens *et al*., 1997). Regulation of fatty acyl-CoA entry into the mitochondria by CPT1 has been identified as a rate-limiting step in the oxidation of fatty acids (McGarry & Brown, 1997). The activity of CPT1 is higher in muscle from trained compared to untrained humans (Berthon *et al*., 1998;Jong-Yeon *et al*., 2002), and this was strongly correlated with citrate synthase activity and VO_{2peak} (Berthon *et al*., 1998). In addition, a period of training in previously sedentary individuals results in an increase in CPT1 activity (Bruce *et al*., 2006) and FAT/CD36 associated CPT1 protein complexes (Schenk & Horowitz, 2006). Thus, the net effect exercise training on lipid metabolism is to increase the mitochondrial capacity to oxidise fatty acids but also increase the capacity for lipid mobilisation and transport across the plasma and mitochondrial membrane.

2.5.1.4 Effects on fuel storage

Longitudinal training studies have shown higher concentration of intramuscular triglyceride and glycogen in the *vastus lateralis* muscle in healthy subjects after endurance training (Phillips *et al*., 1996b;Gibala *et al*., 2006;Schrauwen-Hinderling *et al*., 2006;Tarnopolsky *et al*., 2007). Cross-sectional data confirm these observations: muscle glycogen and intramuscular triglyceride concentrations are higher in endurance-trained athletes compared to sedentary individuals (Gollnick *et al*., 1972;van Loon *et al*., 2004).

2.5.2 Metabolic and physiological consequences of endurance training

When previously sedentary individuals adapt to endurance training, maximal oxygen uptake increases and endurance at submaximal intensities increases (Holloszy & Coyle, 1984). The increase is maximal oxygen uptake is largely due to an increased ability of the vascular system to deliver oxygenated blood and the increase in the muscle's ability to extract and utilise the supplied oxygen (Holloszy & Coyle, 1984). The increased endurance at submaximal exercise

intensity is attributed to enhanced fatigue resistance to exercise by virtue of reduced muscle glycogen depletion and smaller disturbances to homeostasis with a consequent reduction in metabolic by-products (Coggan & Williams, 1995). Well-trained athletes deplete their muscle glycogen stores less rapidly during submaximal standardized exercise compared with untrained individuals (Hermansen *et al.*, 1967). Endurance training reduces the production, uptake and oxidation of plasma glucose during both moderate and intense exercise (Coggan *et al.*, 1990;Coggan *et al.*, 1995). The decreased carbohydrate utilization during submaximal exercise in the trained state is compensated for by a proportional increase in fat oxidation rates at the same absolute and relative intensity (Coggan & Williams, 1995).

2.5.3 Adaptation in skeletal muscle after short-term training (14 days or less)

The time course of adaptation to endurance exercise is not well described. Although an increase in mitochondrial content of approximately 50-100% is generally observed after six weeks of training (Hood, 2001), measurable changes in skeletal muscle metabolism such as enzymatic activity and altered substrate metabolism during exercise are observed after as little as three (Green *et al.*, 1989), five (Green *et al.*, 1992), seven (Spina *et al.*, 1996), ten (Mendenhall *et al.*, 1994), fourteen (Gibala *et al.*, 2006) and thirty-one (Phillips *et al.*, 1996b) days of training. In addition, adaptations in this time frame are observed at mitochondrial (Spina *et al.*, 1996), metabolic (Green *et al.*, 1991), intracellular signalling (McConell *et al.*, 2005) and transcriptional (Pilegaard *et al.*, 2003) levels.

Short-term training has been shown to induce similar metabolic effects to longer term endurance training, namely an increase in maximal oxygen uptake, and when exercise was performed at the same absolute intensity after training, smaller decreases in high-energy phosphates, smaller increases in inorganic phosphate and creatine, a reduction in exercising RER, muscle glycogen utilisation and blood lactate accumulation and concomitant increase in lipid utilisation was observed (Green *et al.*, 1991;Green *et al.*, 1992;Phillips *et al.*, 1995;Phillips *et al.*, 1996a). In these studies, seven to fourteen days of training for 2 h/d at 60-70% VO_{2peak} did not result in an increase in mitochondrial enzymes but the training regimen resulted in a delayed increase in skeletal muscle mitochondria that did not occur until sometime between the second and fourth weeks of training (Green *et al.*, 1995).

These findings led to the suggestion that metabolic adaptations, such as changes in the concentration of allosteric modulators of glycolysis, are largely responsible for the initial metabolic consequences of exercise training and occur in the absence of mitochondrial adaptation (Green *et al.*, 1992;Phillips *et al.*, 1995). The adaptation in mitochondrial phenotype is apparent however after thirty-one days of the same training program (Phillips *et al.*, 1996b). However, Spina et al. (1996) have since shown that seven to ten days of the same training regimen is sufficient to induce mitochondrial adaptation, as measured by citrate synthase and β-HAD enzymatic activity, coincident with an increase in maximal oxygen consumption. These findings were confirmed by Starritt et al. (1999) who demonstrated increases in citrate synthase

activity and mitochondrial ATP production rate in isolated mitochondria after both five and seven days of training (Starritt *et al.*, 1999).

An absence of mitochondrial adaptation would be in some ways contradictory to what intuitively would be expected based on our current knowledge of molecular adaptation. The adaptive increase in proteins with a short half-life, such as GLUT-4 or δ-ALAS, can occur within two days (Holloszy & Winder, 1979;Ren *et al.*, 1994) in response to exercise. In the case of enzymes with a longer half-life, such as the mitochondrial enzymes cytochrome c, COX, and citrate synthase that have a half-life of seven days, one-half of the adaptive increase in response to a constant stimulus occurs within the first week, 75% in the second week, and so on, in skeletal muscle of rats (Booth, 1977;Holloszy & Winder, 1979;Town & Essig, 1993). In fact, significant increases in mitochondrial respiratory enzymes were observed within two to three days in skeletal muscle of rats (Booth, 1977;Holloszy & Winder, 1979).

Other proteins involved in skeletal muscle metabolism have been shown to be upregulated at the protein content or activity level after short-term exercise training. In terms of glucose metabolism, resting muscle glycogen (Chesley *et al.*, 1996), hexokinase activity (Spina *et al.*, 1996) and GLUT4 protein content (Gulve & Spina, 1995) are increased after one week of training whereas PFK activity is decreased (Green *et al.*, 1992). The regulation of lipid metabolism in response to short-term training is not well defined despite increased fatty acid oxidation rates and decreased RER observed during exercise after training (McConell *et al.*, 2005;Talanian *et al.*, 2007). Increased activity of β-HAD after short-term training (Spina *et al.*, 1996) indicates a greater capacity for β-oxidation of fatty acids, whereas an increase in FAT/CD36 (Tunstall *et al.*, 2002) and FABPpm (Talanian *et al.*, 2007) protein content indicates adaptation at the level of lipid transport into the muscle cell.

Muscle glycogenolysis is regulated by glycogen phosphorylase, which itself is regulated by activation by epinephrine via the cAMP second messenger system and the release of calcium during contractions (Connett & Sahlin, 1996). The activity of phosphorylase in the active “a” form is also stimulated via the contraction-induced accumulation of allosteric regulators, free ADP and AMP. Accumulations of free ADP and AMP and consequent activation of glycogenolysis are reduced after short-term training (McConell *et al.*, 2005;Talanian *et al.*, 2007). This suggests there is less homeostatic perturbation associated with exercise after training, a fact that is emphasised by the reduction in AMPK activation after short-term training (McConell *et al.*, 2005). Little is known about the regulation of other signalling cascades after short-term training (section 2.6.7).

2.5.3.1 Alterations in mRNA abundance in response to short-term training

What remains unclear is the role of transient induction or incremental accumulation of mRNA and the function of transcriptional regulators during the adaptive response to short-term training. Coupling increments in mRNA to changes in enzymatic activity has not been demonstrated. For instance, HKII mRNA was unchanged after two weeks of exercise training that would be expected to increase its enzymatic activity (Schrauwen-Hinderling *et al.*, 2006) whereas Kraniou et al. (2004) demonstrated an increase in GLUT4 protein content in the absence of any change

in mRNA after seven days of training. In contrast, Tunstall et al. (2002) demonstrated coincident increases in both FAT/CD36 mRNA and protein content after nine days of training. These results may simply reflect the transient nature of mRNA and protein expression, where the sampling points for muscle biopsies may conflict with measurable and functional changes in mRNA and protein due to variations in respective half-lives or stability. Thus, although the metabolic effects of short-term training are reasonably well-established, the molecular mechanisms at the level of mRNA or regulation by transcription factor networks are not described despite this fact being widely accepted (Cameron-Smith, 2002;Hood *et al.*, 2006;Fluck, 2006). However, it does appear that a training-induced accretion of mRNA (1.5-2.2-fold) is evident after short-term training for metabolic enzymes including HKII, citrate synthase and β-HAD, and apparent, but non-significant, increases in steady-state mRNA for factors such as NRF-2 and Tfam (Pilegaard *et al.*, 2003). In addition, PGC-1α protein content is increased after two weeks of exercise training sessions that would be expected to repeatedly transiently upregulate its mRNA expression (Burgomaster *et al.*, 2008). However, to date no data exists on the time course of incremental increases in mRNA or their possible role in the adaptive process. Similarly, the effect of short-term exercise training on the expression of transcription factors and transcriptional coregulators purported to modulate skeletal muscle metabolism is not described.

2.6 Molecular mechanisms regulating skeletal muscle plasticity and exercise-induced phenotypes

2.6.1 Contraction-induced signal transduction pathways

Signal transduction refers to the transfer of signals and stresses from the outside or inside of a cell, usually by kinase or phosphatase cascades or other signalling processes, to cytosolic or nuclear targets (Wackerhage & Woods, 2002). In the context of exercising skeletal muscle, the effect on these targets can be either to affect an acute process such as substrate metabolism or a delayed process such as alterations in gene expression. For instance, a decline in cellular energy status through ATP depletion can activate the AMPK pathway which increases fuel metabolism in the short term but alters metabolic gene expression in the longer term (next section). In this regard, the benefits of acute or regular exercise can be manifested either through improvements in glucose and lipid homeostasis or through functional remodelling of the skeletal muscle tissue (Sakamoto & Goodyear, 2002). How these signals are deciphered and integrated into functional alterations in gene expression is not fully understood at present. In addition, the contribution or redundancy of different signal transduction cascades in response to various metabolic perturbations and which subsets of transcriptional regulators are responsive to each signal are poorly described *in vivo*. Acute muscle contraction alters calcium flux, ATP turnover, cellular stress, redox potential, oxygen tension and reactive oxygen species production, each of which have been implicated in the activation of signal transduction cascades regulating skeletal muscle plasticity (Fig. 2.5)(Fluck, 2004;Hood *et al.*, 2006;Freyssenet, 2007;Coffey & Hawley, 2007).

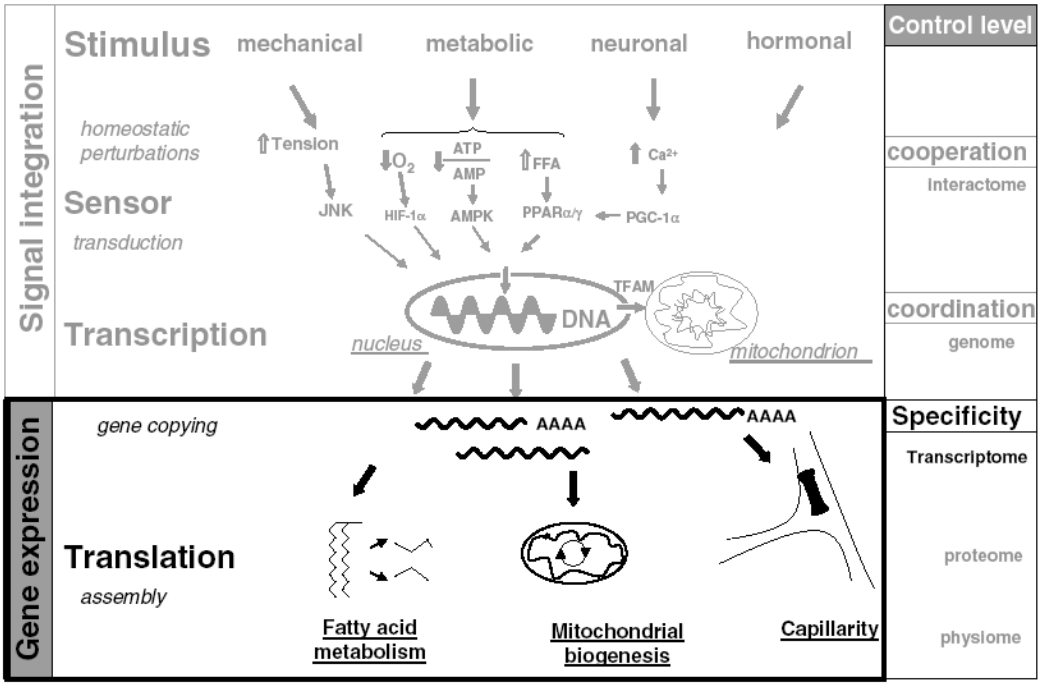

Figure 2.5 Scheme visualizing the integration of the complex stimulus of exercise in recruited skeletal muscle. Different homeostatic perturbations, such as those related to metabolic flux, loading, hormonal and neuronal alterations, are converted by specific sensory molecules into the activation of signalling cascades. These ultimately control muscle fate via the regulation of gene expression. Consequently, gene expression represents an important layer of control for the processing of physiological information towards a biological outcome, (reproduced from Fluck, 2006).

2.6.2 Cellular energy status and AMP-activated protein kinase

One of the most fundamental parameters that any healthy cell must maintain is a high ratio of ATP to ADP (of the order of 10:1). Almost all energy-requiring processes in the cell are driven, either directly or indirectly, by hydrolysis of one or other of the acid anhydride bonds in ATP, yielding ADP or AMP. Eukaryotic cells have a very active adenylate kinase that interconverts ATP, ADP and AMP, and maintains this reaction close to equilibrium. If it is at equilibrium, the AMP/ATP ratio will vary as the square of the ADP/ATP ratio (Hardie & Hawley, 2001). If a cellular stress causes the rate of ATPases to exceed that of the ATP synthases, the ADP:ATP ratio will rise, and the adenylate kinase reaction will increase AMP generation (Hardie & Hawley, 2001). If the ADP/ATP ratio rises by 5-fold, the AMP:ATP ratio will rise 25-fold and thus, the cellular concentrations of AMP change much more dramatically than do those of ATP or ADP (Hardie & Hawley, 2001). It therefore makes sense for any system that monitors cellular energy status to respond to AMP (or the AMP/ATP ratio) rather than ADP (or the ADP/ATP ratio). As it name suggests, AMPK is allosterically activated by 5'-AMP, an effect that is antagonised by high concentrations of ATP (Hardie & Hawley, 2001). Therefore, an important feature of AMPK as a signal transducer for skeletal muscle metabolic adaptations is its capacity to monitor and respond to the cellular energy status.

AMPK exists as a heterotrimer, consisting of α, β and γ subunits. The α subunit, of which there are two known isoforms (α_1 and α_2), contains the catalytic domain that transfers a high-energy

phosphate from ATP to serine and threonine residues on a number of different target proteins. The α subunit also contains a specific threonine residue (Thr^{172}) that functions as an activating phosphorylation site for one of several 'upstream' AMPK kinases. Multiple isoforms of β (β_1, β_2) and γ (γ_1, γ_2, γ_3) regulatory subunits have also been identified that are essential for full enzymatic activity, and also seem to function in localising the AMPK molecule within cells (Kahn *et al.*, 2005). The binding of AMP to AMPK at the γ subunit brings about a conformational change which allows phosphorylation (and activation) by the major upstream AMPK kinase, LKB1 (Hawley *et al.*, 2003). Recently, CaMKKs have been identified as upstream AMPK kinases capable of inducing phosphorylation at Thr^{172} and thus activating AMPK (Hurley *et al.*, 2005;Hawley *et al.*, 2005). In fact, AMPK activation occurs directly as a result of calcium signalling in myotubes (Freyssenet *et al.*, 2004). AMPK activation is regulated allosterically by changes in energy status of the cell (i.e. 'low' energy status), reflected by increases in the AMP/ATP and creatine/phosphocreatine ratios. A wide range of cellular stresses that deplete ATP (such as metabolic poisons) or increase the cellular AMP/ATP ratio (such as glucose deprivation or oxidative stress) are known to activate AMPK (Kahn *et al.*, 2005). Given the increase in ATP turnover and increase in production of AMP associated with muscle contraction (Spriet, 1995), it is not surprising that acute exercise has consistently been shown to increase phosphorylation of AMPK and its enzymatic activity (Rasmussen & Winder, 1997;Wojtaszewski *et al.*, 2000;Fujii *et al.*, 2000;Wojtaszewski *et al.*, 2002;Chen *et al.*, 2003), as has electrical stimulation (Atherton *et al.*, 2005).

2.6.2.1 Effect of exercise on AMPK activity

Available evidence suggests that activation of AMPK during exercise occurs in an intensity-dependent and isoform-specific manner (Aschenbach *et al.*, 2004), with activation reported at intensities greater than ~60% VO_{2peak} and during exercise lasting between five minutes and 3.5 hours. Initial studies investigating the effect of various exercise intensities on AMPK activity in skeletal muscle were conducted on rats (Rasmussen & Winder, 1997;Rasmussen *et al.*, 1998). Six-fold increases in AMPK activity were induced by only five minutes of exercise at greater than 20 $m \cdot min^{-1}$. The increase in kinase activity was relatively transient and returned to baseline within 90 min after the cessation of exercise.

Based on the results of subsequent human experiments, the AMPK response to exercise may be summarised as follows (Wojtaszewski *et al.*, 2000;Fujii *et al.*, 2000;Wojtaszewski *et al.*, 2002;Chen *et al.*, 2003;Sriwijitkamol *et al.*, 2007)

(i) activation of AMPK during exercise occurs in an intensity-dependent and isoform-specific manner

(ii) AMPK is not activated at intensities less than 50-60% VO_{2peak} unless exercise prolonged for more than 2 h but can be activated during exercise as short as five minutes when intensity is greater than 70% VO_{2peak} (almost exclusively α_2 AMPK in each instance)

(iii) α_2 AMPK is more readily activated in skeletal muscle to facilitate metabolism during 'prolonged' exercise; α_1 AMPK is less readily activated and may serve as an additional ATP regeneration boost during low energy status in the cell like during maximal and supramaximal exercise

(iv) the fact that α_1 AMPK is less sensitive to AMP than α_2 AMPK, and thus would require greater metabolic changes to become active, supports this view

(v) AMPK activation during exercise *in vivo* is likely better explained by the recruitment of muscle fibres according to the ramp theory of threshold activation and not fibre type per se (Aschenbach *et al.*, 2004).

(vi) the respective roles of blood-borne and intramuscular substrates in the activation of AMPK are not clear currently.

There appears to be a threshold of activation for AMPK at approximately 60% VO_{2peak} (Wojtaszewski *et al.*, 2000;Sriwijitkamol *et al.*, 2007). This intensity-dependence of AMPK activation is to be expected since the intracellular changes in the AMP/ATP ratio do not change appreciably at intensities similar to 50% VO_{2peak} (Howlett *et al.*, 1998). However, activation of α_2 AMPK (2.5-fold increase) at exhaustion (2:39-4:00 h) after cycling at 45% VO_{2peak} has been demonstrated (Wojtaszewski *et al.*, 2002). This increase in activity was tightly associated with the degree of Thr^{172} phosphorylation. As with previous studies there was no activation of the α_1 isoform, and α_2 AMPK activity had returned to basal levels at 1 h after exercise. At this intensity, it is likely that decreases in blood glucose and muscle glycogen exert primary influence on AMPK activity given the fact that the AMP/ATP ratio was unchanged throughout exercise and that AMPK activity increased as fuel stores decreased, reaching a peak and nadir, respectively, at the same time points. Muscle glycogen is thought to partly regulate the activity of AMPK during exercise. The AMPK β subunit possesses a glycogen binding domain and glycogen loading of muscle suppresses AMPK signalling in response to both exercise/contraction and pharmacological activation (Derave *et al.*, 2000;Wojtaszewski *et al.*, 2003). However, when preexercise glycogen levels are similar, the rate of muscle glycogen utilisation does not directly regulate the extent of AMPK activation during exercise in humans (Wadley *et al.*, 2006).

2.6.2.2 Putative role in skeletal muscle metabolism

The generalised effects of AMPK activation on skeletal muscle metabolism are thought to occur in such a manner as to conserve ATP by inhibiting biosynthetic pathways and anabolic pathways, while stimulating catabolic pathways that generate ATP in a control mechanism that acts to restore cellular energy (ATP) stores (Hardie *et al.*, 2006). In the context of skeletal muscle, this is observed acutely as a suppressive effect of AMPK on glycogen synthesis and protein synthesis and a permissive effect on glucose transport and fatty acid oxidation, whereas chronic effects of AMPK activation lead to alterations in metabolic gene expression and mitochondrial biogenesis.

Briefly, AMPK is thought to inhibit protein synthesis by inhibition of the mTOR pathway (Bolster *et al*., 2002), thereby preventing the initiation of mRNA translation to a synthesised protein (Bolster *et al*., 2004). AMPK phosphorylates glycogen synthase, reducing its enzymatic activity (Carling & Hardie, 1989;Jorgensen *et al*., 2004), and thereby attenuating glycogen synthesis, which is consistent with a role for AMPK in defending cellular energy homeostasis by limiting anabolic processes. Pharmacological activation of AMPK using AICAR increases glucose uptake and AMPK activity in perfused rat muscle (Merrill *et al*., 1997) and in general, it is thought that AMPK activity regulates glucose uptake in resting muscle and is at least partly responsible for contraction-mediated glucose uptake (Jorgensen *et al*., 2004). AMPK-stimulated increases in glucose transport are mediated via the translocation of existing GLUT4 to the plasma membrane (Koistinen et al., 2003), possibly in an AS160-dependent manner (Bruss *et al*., 2005). Lastly, activation of AMPK is associated with increase fatty acid uptake to the cell via FAT/CD36 translocation to the plasma membrane (Raney *et al*., 2005) and increases the rate of fatty acid oxidation by relieving inhibition of fatty acid entry into the mitochondria (Osler & Zierath, 2008). The concentration of malonyl CoA is a potent inhibitor of CPT1, which is the rate limiting enzyme in mitochondrial fatty acid uptake. AMPK phosphorylates the muscle isoform of acetyl CoA carboxylase, ACCβ, making it more sensitive to inhibition which in turn decreases the formation of malonyl CoA. In addition, AMPK can lower malonyl CoA content by phosphorylating and activating malonyl CoA decarboxylase, the enzyme responsible for decarboxylating malonyl CoA to acetyl CoA. These combined effects lower malonyl CoA content upon AMPK activation and relieve CPT1 inhibition, thereby increasing mitochondrial fatty acid oxidation (Hardie & Hawley, 2001).

Given the critical role for the AMPK in regulating intracellular energy metabolism in response to acute energy crises, it is perhaps not surprising that AMPK has also been identified as a major regulator of mitochondrial biogenesis in response to chronic energy depletion (Kahn *et al*., 2005). Feeding rats β-guanadinopropionic acid (β-GPA), a chronic pharmacological activator of AMPK that works by chronically depleting muscle phosphocreatine stores, for eight weeks resulted in chronic AMPK activation in skeletal muscle and increases in NRF-1 binding activity, δ-ALAS mRNA expression, cytochrome c protein expression, and mitochondrial content, thus clearly demonstrating that AMPK activation promotes mitochondrial biogenesis through PGC-1α and the NRFs (Bergeron *et al*., 2001). Other pharmacological studies have also established a link between chronic AMPK activation and the upregulation of key mitochondrial enzymes in skeletal muscle. AICAR administration to rats for four weeks increased protein expression of cytochrome c and δ-ALAS and increased activities of citrate synthase, MDH and SDH in skeletal muscle (Winder *et al*., 2000).

To determine if AMPK is required for mitochondrial biogenesis, transgenic mice expressing a dominant-negative mutant form of AMPK in skeletal muscle and wild-type mice were fed β-GPA for eight weeks. While AMPK was activated in the skeletal muscle of the wild-type mice in response to β-GPA feeding, the transgenic mice fed β-GPA showed no similar increase in AMPK activation. Moreover, PGC-1α mRNA expression, cytochrome c protein expression

levels, mitochondrial DNA content and mitochondrial density were all increased in the wild-type mice, and none of these parameters were increased in the β-GPA transgenic mice lacking a functional form of AMPK (Zong *et al*., 2002). Clearly, these data demonstrate that AMPK is necessary for mitochondrial biogenesis in response to chronic energy deprivation, and it appears likely that pharmacologically activated AMPK conveys its signal to induce mitochondrial biogenesis via the PGC-1α–NRF pathway. In terms of metabolic gene expression, pharmacological activation of AMPK enhances the expression of FOXO1A, CPT1, GLUT4 and HKII (Holmes *et al*., 1999;Jorgensen *et al*., 2005), a program of gene expression that would increase substrate transport to, and utilisation by, the muscle cell. This indicates a role for AMPK in defending cellular energy status by initiating an adaptive response that favours enhancement of ATP producing pathways.

Exercise studies demonstrate that AMPK activation is followed by increased PGC-1α expression, suggesting AMPK signalling may work through PGC-1α to promote mitochondrial biogenesis. Six hours of low intensity swimming resulted in increases in AMPK activation and PGC-1α mRNA expression (Terada *et al*., 2002). Electrical stimulation designed to mimic endurance exercise also activated AMPK and increased PGC-1α protein expression (Atherton *et al*., 2005). However, Coffey et al. (2006a) have shown that PGC-1α mRNA expression is induced in the muscle of both endurance- and resistance-trained athletes in response to a single bout of endurance- but not resistance-type exercise. This finding is notable because despite the fact that AMPK activation was not activated in endurance-trained muscle, PGC-1α mRNA was elevated in the same condition. AMPK activation was increased by resistance exercise in endurance-trained muscle but no increase in PGC-1α mRNA was observed. Furthermore, exercise has been shown to increase PGC-1α mRNA expression in the absence of a functional copy of α_2 AMPK (Jorgensen *et al*., 2005), which suggests that AMPK is dispensable in the adaptive response to exercise. Similarly, in response to exercise training, α_2 AMPK knockout and α_2 AMPK kinase dead mice show no defect in mitochondrial increases (Jorgensen *et al*., 2007;Rockl *et al*., 2008). Thus, despite its apparent capacity to increase mitochondrial biogenesis, AMPK is not required for the exercise-induced training responses.

Whereas these α_2 AMPK knockout mice have a similar exercise-induced increase in PGC-1α, FOXO1A and PDK4 mRNA to wild-type littermates, this response is suppressed in the knockout animals following AICAR stimulation. This highlights two critical points when interpreting studies delineating the role of AMPK in skeletal muscle metabolism: (i) pharmacological activation using AICAR or other means may not accurately represent the metabolic milieu of contracting muscle, and (ii) results obtained in transgenic animals may be affected by compensatory alterations in AMPK isoform activity or alternative signalling pathways (Jorgensen *et al*., 2006). Therefore, the precise role for AMPK in contraction-mediated regulation of metabolic gene expression remains to be fully elucidated. Exploring further how exercise affects AMPK and PGC-1α and whether this leads to an increase in mitochondrial biogenesis are important issues that need to be addressed.

2.6.2.3 Downstream targets of AMPK

If AMPK activation is causally linked to the reported effects on gene expression and mitochondrial adaptation, AMPK activity must be capable of directly or indirectly altering mechanisms of transcriptional regulation. This is consistent with the function of the AMPK yeast homologue Snf1, which is known to regulate a range of metabolic genes (Lo *et al.*, 2001). Snf1 regulates gene transcription by directly phosphorylating nuclear transcription factors (Lo *et al.*, 2001). Similarly, AMPK complexes containing the α_2 subunit are preferentially localized to the nucleus (Salt *et al.*, 1998). This suggests that AMPK translocates to the nucleus where it could then interact with transcriptional regulators or DNA directly to control gene expression. Exercise has been shown to increase AMPK nuclear abundance (McGee *et al.*, 2003) and it has been proposed that AMPK directly links cellular metabolism to gene regulation by phosphorylation of nuclear proteins (Leff, 2003). Recently, it has been shown that AMPK directly phosphorylates PGC-1α (Jager *et al.*, 2007). Many effects of activated AMPK on gene expression in skeletal muscle, including the inductions of the PGC-1α, GLUT4, and mitochondrial genes, required the presence of the PGC-1α protein. PDK4 induction by AMPK activation did not require the presence of PGC-1α protein, indicating the existence of PGC-1α-independent but AMPK-dependent pathways regulating gene expression in muscle cells (Jager *et al.*, 2007). AMPK is also thought to phosphorylate and activate CREB, which would theoretically enhance the transcription of genes with CRE binding sites in their promoter region, such as HKII (Thomson *et al.*, 2008)

AMPK activation alters transcriptional regulation by modulating the DNA binding activities of transcription factors including NRF-1 and MEF2 (Bergeron *et al.*, 2001;Zheng *et al.*, 2001), a mechanism that would explain alterations in mitochondrial and GLUT4 gene expression mediated by AMPK. Finally, AMPK can directly phosphorylate HDAC5 leading to its nuclear exclusion and relieving its repression of MEF2-mediated GLUT4 transcription (McGee *et al.*, 2008). This is a very similar mechanism described previously for CaMK/HDAC4/MEF2 in response to stimulated contractions (Liu *et al.*, 2005). Although much more work is required to fully characterise the pathway of contraction-induced AMPK activation and modulation of gene expression, the available data suggest that this pathway is important in the acute and adaptive response of skeletal muscle gene expression to exercise.

2.6.3 Calcium flux and calcium/calmodulin-dependent protein kinases

Calcium is essential for facilitating the interaction between myosin and actin during myofibrillar contraction. Neural activation of skeletal muscle results in the release of acetylcholine from the neuromuscular junction and depolarisation of the plasma membrane, which activates force production. A transient increase in cytosolic calcium concentration is triggered by each wave of sarcolemmal depolarization during muscle contraction and mounting evidence links these calcium transients and associated activation of calcium-dependent protein kinases and phosphatases with adaptive response to exercise (Chin, 2004;Ojuka, 2004). The frequency, intensity and duration of stimulation determine the amplitude and duration of the calcium

transients and as a result the level of force output by the muscle (Westerblad & Allen, 1991). Intracellular calcium concentrations can be elevated by up to five- and fifty-fold in slow and fast twitch muscle fibres, respectively, during muscle contraction (Westerblad & Allen, 1991). Experiments using agents to elevate intracellular calcium levels *in vitro* (Ojuka *et al*., 2003;Freyssenet *et al*., 2004;Kusuhara *et al*., 2007) or with transgenic mice with altered calcium homeostasis (Chen *et al*., 2001;Wu *et al*., 2002;Chin *et al*., 2003) provide unequivocal evidence linking calcium flux to alterations in metabolic and mitochondrial gene expression and muscle plasticity. The expression of many genes encoding mitochondrial proteins are increased in response to elevations in intracellular calcium (Ojuka *et al*., 2003). However, transcript expression of certain genes is not induced by elevated calcium, whereas others follow a distinct time-course of induction (Ojuka *et al*., 2003;Freyssenet *et al*., 2004;Wright *et al*., 2007a). Elevations in calcium may only partially mediate the contractile-activity induced changes in mitochondrial biogenesis and may act in concert with other signalling pathways (Ojuka *et al*., 2002;Hood *et al*., 2006).

Elevations in intracellular calcium are initially decoded by an intermediate binding protein, calmodulin (CaM). CaM is a multifunctional signal transducer that undergoes conformational changes before activating other CaM binding proteins, primarily downstream kinases and phosphatases (Chin, 2005). The mechanism by which the nature of the calcium oscillations is decoded by CaM includes changes in CaM conformation and CaM subcellular localisation upon binding of the calcium ion (Chin, 2005). Calcium/calmodulin-dependent protein kinases (CaMK) are multifunctional serine/threonine protein kinases that are activated in a calmodulin-dependent manner and that translate calcium oscillations into discrete levels of kinase activity. In human skeletal muscle, CaMKII has been shown to the dominant isoform expressed, and this isoform and the CaMKIV isoform have been implicated in the regulation of skeletal muscle plasticity (Fluck *et al*., 2000;Wu *et al*., 2002). Initial activation of CaMKII is facilitated by calcium-dependent calmodulin binding, resulting in autophosphorylation on Thr^{287} and activation of a calcium/calmodulin independent form of CaMKII (Chin, 2005).

Phosphorylation of CaMKII has been previously shown to increase in an intensity-dependent manner in response to exercise in human skeletal muscle (Rose & Hargreaves, 2003;Rose *et al*., 2006), with evidently no further increases with an increase in exercise duration. The greater increase in CaMKII activity may be related to the greater proportion of muscle fibres activated, the different types of fibre recruited, or the higher calcium concentrations expected at higher force outputs. Phosphorylation of CaMKII at Thr^{287} is highly correlated with autonomous enzymatic activity (Rose *et al*., 2006;Rose *et al*., 2007a), although these authors suggest that it is obligatory for autonomous CaMKII activity rather than necessarily reflecting *in vivo* kinase activity. Phosphorylation of CaMKII makes the kinase partially independent of calcium/CaM and thus, when a calcium transient is over, the kinase retains heightened activity above basal (Hudmon & Schulman, 2002). Another important feature conserved in skeletal muscle CaMKII is that the activation of CaMKII is sensitive to the frequency of calcium oscillations (De Koninck & Schulman, 1998). The functional consequence of autophosphorylation is believed to be maintenance of enzymatic activity between calcium transients, thereby allowing persistent

phosphorylation of downstream substrates during repeated stimulation (Hudmon & Schulman, 2002). The intensity-dependent increase in CaMKII activation is consistent with CaMKII being a stimulation-frequency decoder in skeletal muscle (Chin, 2005).

2.6.3.1 Putative role in skeletal muscle metabolism

Conclusive mechanistic data from human skeletal muscle linking the effect of CaMK activation to skeletal muscle adaptation is currently lacking but there are several lines of evidence based on *in vitro* and animal models. However, the limited data from exercising human skeletal muscle indicates that as well as being rapidly activated by contraction, it phosphorylates downstream targets including the transcription factor, SRF (serum response factor) (Rose *et al.*, 2006). Several putative CaMK targets and mechanisms have been implicated in the regulation of skeletal muscle gene transcription including CREB and MEF2, two transcription factors critical in the regulation of PGC-1α expression (Handschin *et al.*, 2003;Akimoto *et al.*, 2004b). In addition, endurance exercise training leads to an increase in CaMKII expression and autonomous activity at rest, an effect which may increase the sensitivity of CaMKII to calcium transients (Rose *et al.*, 2007b). Similar results were reported in wheel running rats after two weeks of training (Fluck *et al.*, 2000). These findings have been taken to suggest that an increase in resting autonomous CaMKII activity is sustained beyond the cessation of contractile activity and may be mediating a calcium-related change in muscle plasticity. This data would at least suggest that there is some potential role for CaMKII in acute exercise metabolism and adaptation to exercise training. When considered in concert with several other lines of evidence, the role is more convincing.

Overexpression of a constitutively active form of CaMKIV in skeletal muscle resulted in increased mitochondrial volume, increased expression of both nuclear- and mitochondrial-encoded mitochondrial enzymes involved in fatty acid metabolism and electron transport, and enhanced recovery from fatigue (Wu *et al.*, 2002). These changes were associated with increased expression of PGC-1α, and it is thought that the CaMKIV signal was transduced directly or indirectly through PGC-1α. However, CaMKIV protein expression is not detectable in human skeletal muscle (Rose & Hargreaves, 2003;Rose *et al.*, 2006), which precludes this pathway in human muscle. In addition, CaMKIV null mice exhibit similar increases in PGC-1α and COXIV protein content as do wild-type mice in response to voluntary wheel running (Akimoto *et al.*, 2004a). The generalised influence of the calcium ion on skeletal muscle plasticity is demonstrated in mice with alterations in calcium handling via modulation of parvalbumin, a calcium buffering protein. Parvalbumin-deficient mice exhibit increased mitochondrial volume and COX activity coincident with elevations in intracellular calcium concentrations (Chen *et al.*, 2001). Conversely, mice overexpressing parvalbumin (and consequently reduced calcium levels) have increased mitochondrial enzyme activity of SDH (Chin *et al.*, 2003).

In studies using a combination of calcium releasing agents and CaMK inhibitors, the role of CaMK in skeletal muscle metabolism has been elegantly described (Ojuka *et al.*, 2002;Ojuka *et al.*, 2003;Wright *et al.*, 2004;Freyssenet *et al.*, 2004;Kusuhara *et al.*, 2007;Wright *et al.*, 2007a). In terms of glucose metabolism, elevating calcium concentrations in myotubes leads to acute

increases in glucose uptake (Wright *et al*., 2004). This effect may be mediated by chronic increases in the mRNA and protein abundance of GLUT4 and its regulatory transcription factor MEF2 (Ojuka *et al*., 2003), and HKII expression (Kusuhara *et al*., 2007). These stimulatory effects of elevated calcium on glucose metabolism are diminished by incubation with either an inhibitor of calcium release (e.g. dantrolene) or CaMK activity (e.g. KN62). Similar effects are reported when markers of mitochondrial biogenesis are examined. Caffeine-treatment of myotubes increased the mRNA and protein expression of PGC-1α and Tfam and increased NRF-1 and NRF-2 DNA binding (Ojuka *et al*., 2003), whereas similar results were reported for PGC-1α, COXI and δ-ALAS (Wright *et al*., 2007a). Again, these effects are reversed when incubation with dantrolene or KN93 (Ojuka *et al*., 2003). Finally, a recent report has shown that the elevation observed in PGC-1α mRNA after 40 min of electrical stimulation is reduced when KN62 is added to the incubation medium, providing evidence that the contraction-induced increase in PGC-1α mRNA is in part mediated by a CaMK-dependent mechanism (Kusuhara *et al*., 2007).

The exact mechanism by which CaMKs regulate skeletal muscle gene expression is not defined. Direct modulation of transcription factor function by protein phosphorylation is one possible mechanism. Transcription factors such as CREB and SRF are known CaMK targets (Shaywitz & Greenberg, 1999;Rose *et al*., 2006). The presence of CRE motifs in the promoter region of a large number of functionally diverse genes involved in cellular metabolism is evidence for an essential role for CREB as an activator of stimulus-induced gene transcription (Mayr & Montminy, 2001). For instance, PDK4 and PGC-1α both contain consensus sites for CREB binding, although expression of PDK4 doesn't appear to be under the control of calcium-dependent regulation (Kusuhara *et al*., 2007). Activation of CREB via phosphorylation at Ser^{133} appears to be an important step in a cascade of events that results in the integration of several signaling pathways leading to the execution of PGC-1α-mediated actions in various tissues (Akimoto *et al*., 2005;Hood *et al*., 2006;Wu *et al*., 2006). Phosphorylation at this residue is necessary, but not always sufficient, for stimulus-induced activation of CREB and consequent gene transcription (Mayr & Montminy, 2001). Another potential mechanism of CaMKII function is by modulating HDAC activity. One report has demonstrated a causal link between the activation of CaMKII by electrical stimulation of cultured muscle cells, its phosphorylation and subsequent nuclear exclusion of HDAC4, thereby relieving repression of MEF2 transcriptional activity (Liu *et al*., 2005). This model would thus couple contraction-induced calcium signaling to an increase in the rate of transcription of MEF2 target genes such as PGC-1α and GLUT4.

In summary, there is strong circumstantial evidence that exercise-induced intracellular calcium flux mediates so-called 'excitation-transcription coupling (Chin, 2005)' in skeletal muscle. However, the majority of our understanding is based on *in vitro* and animal experiments and so remains to be established in *in vivo* exercising human experiments.

2.6.4 Cellular stress and the mitogen-activated protein kinases

Because of the wide variety of biochemical and biophysical processes activated by muscle contraction, it is unsurprising that the MAPK family of signalling kinases are activated by acute exercise. Growth factors, cytokines, and cellular stress lead to changes in activity of various members of the MAPK family (Long *et al.*, 2004), and these signal transduction cascades are well established in physiological processes such as cell proliferation, differentiation, hypertrophy, inflammation, gene expression and apoptosis (Kyriakis & Avruch, 2001). Three main subfamilies of MAPK have been implicated in altering skeletal muscle metabolism: (i) the extracellular-regulated kinase (ERK1/2), (ii) c-jun N-terminal kinase (JNK), and (iii) p38 MAPK (Koulmann & Bigard, 2006). MAPKs phosphorylate diverse substrates, including transcription factors and coactivators localized in the cytoplasm or nucleus, thereby forming a basis for the regulation of transcriptional events (Long *et al.*, 2004). Exercise leads to the activation of at least three MAPK signalling pathways, i.e. ERK1/2, p38 MAPK and JNK, in skeletal muscle (Zierath, 2002). Regulation of PGC-1α-dependent gene transcription in skeletal muscle is mediated through p38 MAPK (Yan *et al.*, 2007), and therefore discussion will be limited to this kinase.

2.6.4.1 Activation by contraction

In human subjects p38 MAPK is phosphorylated in skeletal muscle in response to cycle exercise (Widegren *et al.*, 1998;Chan *et al.*, 2004) and marathon running (Boppart *et al.*, 2000;Yu *et al.*, 2001). In contrast to marked but transient ERK1/2 activation, one-leg cycling exercise leads to a smaller increase but more persistent p38 MAPK activation. p38 MAPK phosphorylation is also increased in the resting leg, indicating the potential influence of a systemic factor or perhaps the stress of muscle sampling (Widegren *et al.*, 1998). In addition, in well-trained skeletal muscle, p38 MAPK is activated when muscle performs unaccustomed exercise i.e. endurance-trained athletes perform resistance exercise or resistance-trained athletes perform endurance exercise (Coffey *et al.*, 2006b). Therefore, the level of activation is somewhat dependent on the nature of exercise. In isolated rat skeletal muscle concentric contractions increase ERK1/2 phosphorylation with no effect on p38 MAPK, whereas eccentric contractions increase phosphorylation along both kinase cascades (Wretman *et al.*, 2001). In human subjects, one session of eccentric contractions during leg press resistance exercise is associated with an increase in ERK1/2 phosphorylation, with no effect on p38 MAPK phosphorylation (Williamson *et al.*, 2003).

2.6.4.2 Role in skeletal muscle metabolism

Although MAPK pathways are candidate systems that may convert mechanical/biochemical stimuli into modulations of muscle gene expression, the transcription factors activated by these intracellular cascades and their integration into other signalling pathways involved in the changes of muscle gene expression remain to be elucidated (Koulmann & Bigard, 2006). The phosphorylation of transcription factors by MAPK signalling pathways can regulate their activities in several ways including intracellular localisation, transcription factor protein levels,

binding to DNA, and their interactions with regulatory proteins (Yang *et al*., 2003). In terms of skeletal muscle metabolism, the most pertinent interaction is the effect of p38 MAPK on PGC-1α function as described earlier. Phosphorylation by p38 MAPK increases PGC-1α protein stability and removes the repressive effect of inhibitory factors such as p160 myb binding protein (Puigserver *et al*., 2001;Fan *et al*., 2004). p38 MAPK can directly stimulate upstream transcription factors of the PGC-1α gene, such as ATF2 and MEF2 (Akimoto *et al*., 2005). ATF2 is nuclear CRE-element binding transcription factor that is activated by p38 MAPK and binds to the CRE binding site on the PGC-1α promoter, enhancing PGC-1α transcription (Cao *et al*., 2004). This pathway has recently been shown to be critical to the exercise-induced increase in PGC-1α mRNA. Contractile activity was associated with p38 MAPK and ATF2 phosphorylation, coincident with an increase in PGC-1α mRNA (Akimoto *et al*., 2005;Wright *et al*., 2007b). Constitutive activation of p38 MAPK induced increases in PGC-1α and COXIV protein content, whereas the acute increase in PGC-1α mRNA induced by p38 activation was ATF2-dependent (Akimoto *et al*., 2005).

MAPKs are known to phosphorylate CREB at Ser^{133} (Deak *et al*., 1998), which could potentially increase the transcription of CREB target genes. Yet, exercise is not associated with increased CREB phosphorylation in the working skeletal muscle; CREB phosphorylation in skeletal muscle after exercise is either unchanged (Widegren *et al*., 2000) or repressed (Widegren *et al*., 1998), despite increases in MAPK phosphorylation described above. Thus, the role of CREB phosphorylation under control of MAPKs or other kinases in skeletal muscle after exercise is not clear.

2.6.5 Cellular redox state: NAD^+/NADH ratio

The degradation of glucose and fatty acids produces acetyl CoA, which is rapidly shuttled through the TCA cycle to generate ATP and the reduced equivalents NADH and $FADH_2$, which in turn yield more ATP through oxidative phosphorylation and the electron transport chain. During muscle contractions, the cytosolic NAD^+/NADH ratio is subject to dynamic fluctuations imposed by the rate of NAD^+ reduction to NADH, which is mediated by glyceraldehyde 3-phosphate dehydrogenase, and the rate of NADH oxidization to NAD^+, which is collectively governed by lactate dehydrogenase, the glycerol phosphate shuttle, and the malate aspartate shuttle (Robergs *et al*., 2004). The mitochondrial monocarboxylate transporter, which mediates the uptake of pyruvate into mitochondria, also indirectly contributes to the regulation of the NAD^+/NADH ratio. Acute exercise of moderate to high intensity induces a redox response by increasing NADH concentration (Sahlin *et al*., 1987;Odland *et al*., 2000) or decreasing NAD^+ concentration (Graham *et al*., 1978). In addition, the lactate/pyruvate ratio increases as exercise intensity increases, shifting the lactate dehydrogenase equilibrium and decreasing the NAD^+/NADH ratio (Denis *et al*., 1991). Green et al. (1992) have shown a progressive decline in NAD^+/NADH ratio during 60 min of exercise at 67-76% VO_{2peak}. However, measurements of NAD^+/NADH ratio during exercise are subject to controversy and a number of methodological considerations (Graham & Saltin, 1989). NAD^+/NADH ratio has also been reported to increase

during submaximal and maximal exercise (Graham & Saltin, 1989). Fluctuations in NAD^+/NADH and the production of lactate are potential intracellular signals affecting skeletal muscle gene regulation (Fig. 2.6) (Hawley & Zierath, 2004).

The function of several dehydrogenases is NAD^+/NADH-dependent including Sirt1 and CtBP (sections 2.3.3 & 2.4). A decrease in the NAD^+/NADH ratio increases CtBP-dependent repression (Zhang *et al*., 2002), which in theory would mean that acute exercise would increase the repressive function of RIP140, contrary to what intuitively would be expected to occur in response to exercise. Sirt1-PGC-1α regulation (section 2.3.3) provides a means of linking energy status of the cell, sensed by Sirt1 through the NAD^+/NADH ratio, to a transcriptional output on metabolic networks regulated by PGC-1α. However, an exercise-induced decrease in the NAD^+/NADH ratio would potentially lead to a decrease in Sirt1 activity. In terms of effects on gene expression, this again is contrary to what intuitively would be expected to occur in response to exercise. Thus far there is no experimental data examining the function or expression of CtBP, RIP140 or Sirt1 in response to acute exercise. The relationships between NAD^+/NADH ratio and these transcriptional regulators may be tissue- or stimulus-specific (Freyssenet, 2007), or it may be that the NAD^+/NADH concentrations in the particular cellular compartments (i.e. cytosolic vs. mitochondrial redox) are more important in their regulation than those that have been routinely measured during exercise.

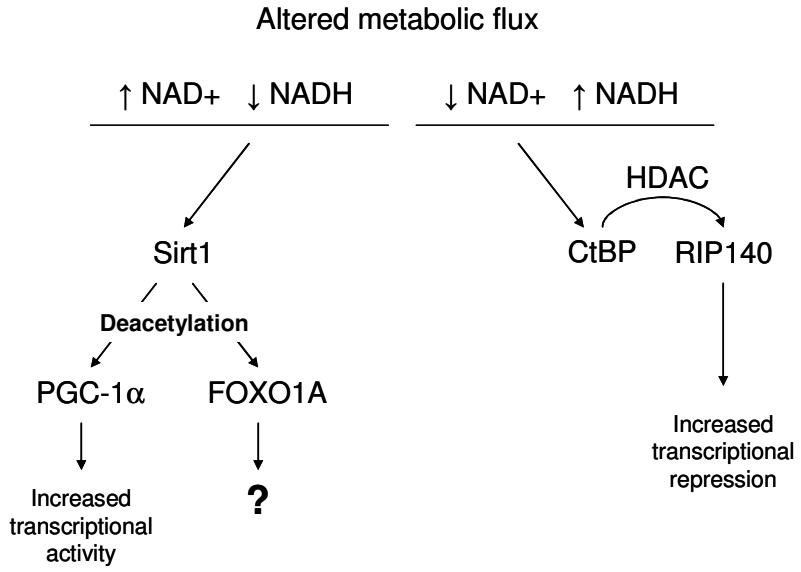

Figure 2.6 Putative regulation of transcriptional pathways by alterations in cellular redox state. See text for detailed discussion.

Finally, it is well established that acute exercise stimulates lactate production in human skeletal muscle and with increasing exercise intensity, lactate production increases proportionately and is manifested as a curvilinear increase blood lactate concentration (Robergs *et al*., 2004). Recently, lactate has been identified as a potent activator of gene transcription of a subset of genes in myotubes (Hashimoto *et al*., 2007). Incubation with physiological relevant concentrations of lactate, resulted in increased mRNA expression of the mitochondrial

monocarboxylate transporter MCT1, PGC-1α and COXIV, and increased the DNA binding of NRF-2 and CREB, both of which have binding sites in the MCT1 promoter. This program of expression is consistent with the induction of a mitochondrial lactate oxidation complex, in direct response to an increase in intracellular lactate and succinctly demonstrates the specificity of adaptation in response to a physiological stimulus. Coordination of MCT1 expression and mitochondrial biogenesis by NRF-2 and CREB is likely physiologically relevant for increasing oxidative lactate clearance capacity in skeletal muscle. In addition, lactate may serve as a second messenger in the initiation of a program of mitochondrial biogenesis in an exercise context such that elevated lactate flux and concentration signals not only lead to adaptation of pathways of lactate removal, but also signals many of the adaptations in muscle found in response to endurance training (Hashimoto *et al.*, 2007).

In summary, preliminary evidence suggests that metabolites under redox control including NAD^+, NADH and lactate are potential sensory molecules mediating contraction-induced modulation of skeletal muscle gene expression. However, the precise mechanisms of regulation and subsequent signal transduction pathways remain to be established in exercising human skeletal muscle.

2.6.6 Integrating signalling cascades/cross talk

Although the review thus far has defined discrete signalling pathways in response to contractile activity, it is unlikely that these pathways act in isolation. These cascades are likely to be highly regulated at multiple levels, with substantial crosstalk between pathways producing a highly sensitive, complex transduction network (Fig. 2.7)(Coffey & Hawley, 2007). Signalling to muscle adaptation is likely to involve more than one signal transduction pathway depending on the nature of the exercise stimulus and gene targets. Similarly, signal transduction pathways may be redundant in certain instances or only regulate part of the adaptation response. Much of the data on this aspect of signalling comes from *in vitro* pharmacological and transgenic animal models and is not established in exercising human skeletal muscle.

Regarding CaMKII and AMPK, it is tempting to consider these as distinct signalling cascades that can be preferentially activated by stimuli such as exercise or pharmacological agents (Ojuka, 2004). However, recent evidence suggests that each has common characteristics and overlapping effects on cellular metabolism. AMPK and CaMKII are closely related enzymes that belong to the same protein kinase subfamily and recognize the same amino acid consensus sequence (Hawley *et al.*, 1995). The tumor suppressor kinase LKB1 has been identified as the predominant AMPK kinase in skeletal muscle but CaMK kinases show significant homology to LKB1 (Hurley *et al.*, 2005). CaMKK has been shown to phosphorylate AMPK *in vitro* (Hawley *et al.*, 1995). Furthermore, several lines of evidence in cell and rodent models suggest that CaMKK is both an upstream AMPKK and regulates its functional activity (Hurley *et al.*, 2005;Witczak *et al.*, 2007;Jensen *et al.*, 2007). Alternatively, a CaMKII-TAK1/MAP3K7 pathway has been proposed to regulate AMPK phosphorylation directly or through LKB1 (Jensen *et al.*, 2007). Taken together, current evidence suggests that the AMPK and calcium-dependent

signalling pathways are not entirely independent. However, whether a time- or intensity-dependent switch occurs in the relative importance of each pathway (Jensen *et al*., 2007) especially with regard to functional effects during exercise *in vivo* remains to be determined. Recently, acute elevations in intracellular calcium leading to alterations in gene expression have been shown to act through p38 MAPK, secondary to the activation of CaMKII (Wright *et al*., 2007a). Inhibition of CaMKII prevented p38 MAPK phosphorylation whereas inhibition of p38 MAPK prevented the calcium-induced increases in mitochondrial gene expression. AMPK activation was also shown in response to acute elevations in intracellular calcium through both CaMKII-dependent and -independent mechanisms that in turn increase fatty acid oxidation (Raney & Turcotte, 2008). In addition, these data indicate that the calcium-independent regulation of fatty acid uptake in skeletal muscle is mediated through an ERK1/2 mechanism.

The activation of multiple signal transduction cascades in response to muscle contraction has been well established in exercising human skeletal muscle. Cell culture and animal studies have in turn demonstrated that these pathways are critical to skeletal muscle metabolism and target various nodes critical to transcriptional regulation. However, the interplay between contraction-induced signalling pathways and demonstrating continuity between pathway activation and functional consequences is perhaps too complex to be fully delineated in the milieu of exercising human skeletal muscle.

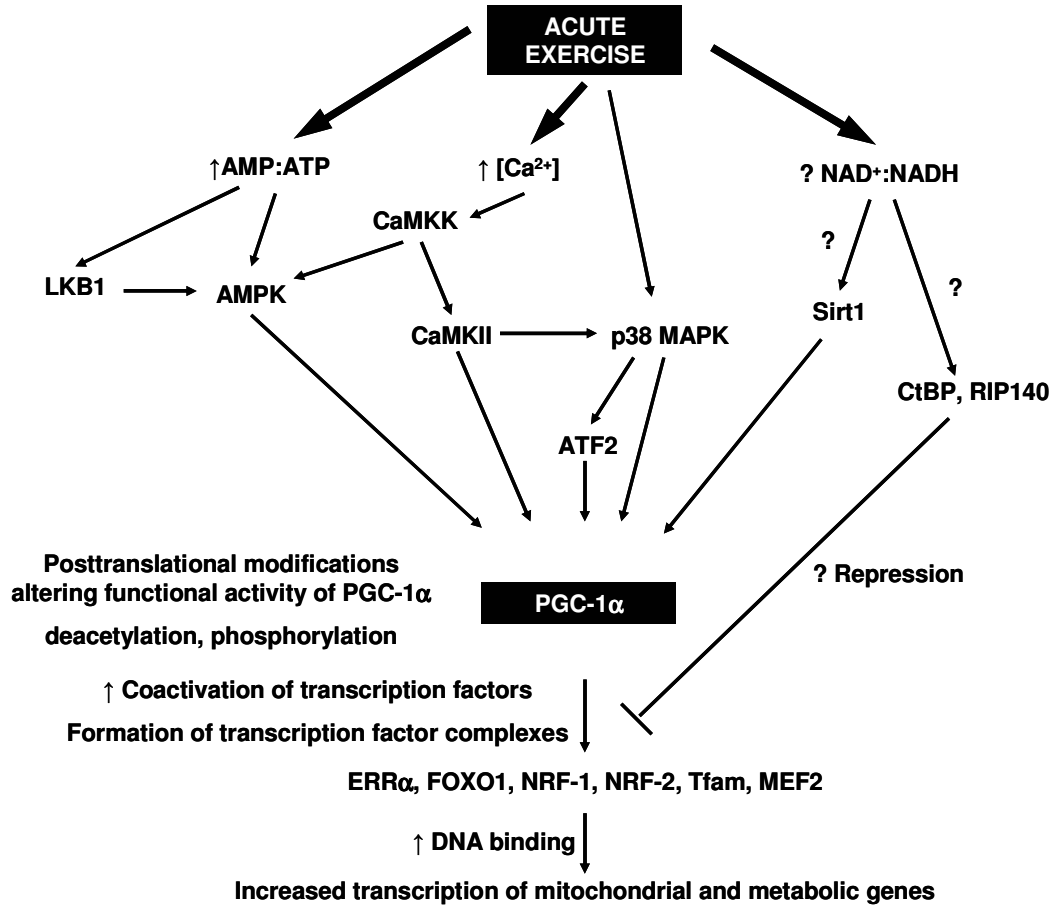

Figure 2.7 Current players in the integration of acute exercise-induced signals to alterations in skeletal muscle gene expression

2.6.6.1 Modulating the exercise-induced signal by altering exercise intensity

The intensity of exercise is well established as a determinant of substrate utilisation during exercise (Romijn *et al.*, 1993;van Loon *et al.*, 2001) as well as a critical component in determining the nature and magnitude of training adaptation (Dudley *et al.*, 1982). Given that skeletal muscle energy flux is intensity-dependent, it is unsurprising that signal transduction cascades are differentially regulated by the intensity of an acute exercise challenge (Wojtaszewski *et al.*, 2000;Widegren *et al.*, 2000;Rose *et al.*, 2006). A simple working hypothesis is that increasing intensity of exercise leads to greater activation of signal transduction pathways, which, assuming the molecular regulation of transcription by these kinases described above is conserved in exercising human skeletal muscle, would result in differential changes in gene expression of target genes compared to lower intensity exercise. For instance, the activation of p38 MAPK, AMPK, and CaMKII increases as exercise intensity increases. The regulation of PGC-1α transcriptional activity is under direct and indirect control of p38 MAPK-, AMPK- and CaMKII-dependent mechanisms. Greater activation of these kinases will potentially lead to greater PGC-1α transcriptional activity, and hence, the expression of PGC-1α-dependent transcriptional programs.

2.6.7 Alterations in signal transduction pathways after endurance exercise training

Endurance exercise training modulates the magnitude of the contraction-induced signalling response by altering the flux of signal activators, the activity of the signalling kinases themselves and the expression of the signalling components. This is ultimately reflected as a reduction in the activation of contraction-induced signalling kinases after a period of exercise training (McConell *et al.*, 2005) or when comparing trained to untrained muscle (Yu *et al.*, 2003).

A period of exercise training in rats attenuates, but does not abolish, the activation of skeletal muscle AMPK during treadmill exercise (Durante *et al.*, 2002). In addition, during 20 min of exercise at 80%VO_{2peak} in endurance-trained athletes compared with untrained individuals, there is an attenuation of the exercise-induced increases in AMPK Thr^{172} phosphorylation and ACCβ phosphorylation in the endurance-trained participants, although α_2 AMPK activity increases similarly in both groups (Nielsen *et al.*, 2003). The same pattern has been observed for AMPK phosphorylation in another study but this was coincident with a reduction in α_2 AMPK activity in the trained individuals, even though the trained group completed twice the amount of exercise (Yu *et al.*, 2003). McConell et al. (2005) found that the approximately 10-fold increase in α_2 AMPK activity observed during prolonged exercise before training was abolished during exercise at the same absolute workload following short-term exercise training in previously sedentary individuals. Increased expression of AMPK isoforms and elevated basal AMPK activity are also observed after training (Frosig *et al.*, 2004) and may explain the reduced increase in exercise-induced AMPK activation. In this context, these results illustrate the important point that AMPK activity is not a marker of total energy flux during exercise, but rather a result of the perturbations in the energy charge induced by exercise (Nielsen *et al.*, 2003). After a period of exercise training, the same cellular rate of mitochondrial oxidative

phosphorylation is attained with a lower rate of oxidative phosphorylation per mitochondrion for any absolute workload in muscle fibres with elevated mitochondrial content (Dudley *et al.*, 1987). ATP and creatine phosphate concentrations decrease to a smaller degree, and the free ADP concentration increases to a lesser extent in cells with elevated mitochondrial contents at the same absolute work rate (Dudley *et al.*, 1987;Green *et al.*, 1991). Better preservation of free ADP lowers the rate of AMP formation, ultimately decreasing the activation of AMPK and its upstream activators.

A considerably lower increase in lactate concentration is observed after endurance training when exercise is performed at the same submaximal workload, coincident with a greater contribution of fat to overall substrate utilisation at the expense of glycolytic metabolism (Holloszy & Coyle, 1984). This would possibly alter the NAD^+/NADH-dependent regulation of Sirt1 activity, which is yet to be established in exercise-mediated skeletal muscle metabolism. Muscle glycogen stores are higher at rest and are depleted less rapidly during standardized exercise in the trained state. Muscle glycogen is established as a potent regulator of muscle metabolism (Hargreaves, 2004) and alterations in muscle glycogen content can modulate both exercise-induced gene expression and contraction-responsive kinase activation (Pilegaard *et al.*, 2002;Chan *et al.*, 2004).

Exercise-induced signalling responses for p38 MAPK have been shown to be greater in untrained men, even at the same relative cycling exercise intensity, even though the trained and untrained subjects exercised at approximately the same relative intensity (approximately 85–90% of VO_{2peak})(Yu *et al.*, 2003). Thus, skeletal muscle from previously-highly trained individuals appears to require a greater stimulus to activate signal transduction via p38 MAPK, indicating an extent of training-induced adaptation along MAPK pathways. Little data is available on the adaptation of calcium signalling to exercise training. Repeated bouts of exercise have been shown to induce an adaptive response, resulting in less perturbation in calcium release and uptake, and subsequently improved calcium cycling and resistance to fatigue (Holloway *et al.*, 2006). Exercise training results in increased CaMKII mRNA expression (Timmons *et al.*, 2005b), basal CaMKII protein expression and maximal and autonomous CaMKII(Rose *et al.*, 2007b) but the CaMKII response to acute exercise after training is not established.

Taken together, these results can be interpreted in several ways. The muscle from highly trained athletes with a prolonged history of endurance training requires a greater stimulus to activate signal transduction through MAPK and AMPK pathways. The fact that signals induced by acute exercise are less pronounced after training may simply indicate that these kinases are not the sole mediators of the adaptive response or that there are finite limits to the adaptive response with repeated exercise bouts. Alternatively, the elevations in basal kinase activity and isoform expression may result in downstream targets of these pathways being more sensitive to activation by exercise, with consequent reduction in the requirement for activation. Similarly, enhanced AMPK and CaMKII expression/activity in the rest periods between training bouts may also affect muscle gene expression in a sustained rather than acute manner.

2.6.8 PGC-1α expression in response to acute exercise and exercise training

Given the remarkable similarity between the phenotype of endurance-exercise trained humans (Holloszy & Coyle, 1984) and transgenic mice overexpressing PGC-1α (Calvo *et al.*, 2008), and between the phenotype of long-term inactivity in humans (Booth & Lees, 2007) and muscle-specific PGC-1α knockout mice (Handschin *et al.*, 2007a), it is not surprising that investigations on the modulation of PGC-1α expression and function by exercise have been numerous. If exercise is indeed a panacea for metabolic dysfunction by inducing mitochondrial biogenesis, improvements in oxidative phosphorylation, and favourable changes in metabolic gene expression, then it will presumably do so through the system of transcriptional regulation described thus far. The transcriptome response to acute exercise and exercise training will be considered in more detail in section 2.6.9, but firstly consideration will be given to the effect of exercise on PGC-1α and the possible role in mediating exercise-induced alterations in skeletal muscle metabolism.

Goto et al. (2000) first demonstrated an exercise effect on PGC-1α mRNA abundance in rodent skeletal muscle. An increase in PGC-1 mRNA by ~50% and two-fold above control after three and seven days, respectively, of 2 h/day swim training was observed in the epitrochlearis muscle (Goto *et al.*, 2000). The same investigators later demonstrated a 7-fold increase in PGC-1α mRNA above control immediately after exercise in response to 6 h of low intensity swimming (Terada *et al.*, 2002). This finding was also observed by another research group who showed a two-fold increase in PGC-1α protein content 18 h after the cessation of exercise (Baar *et al.*, 2002). However, the first study on human skeletal muscle showed no affect of either acute exercise (60 min at 63% VO_{2peak}) or nine days of exercise training on PGC-1 mRNA, despite measurable changes in mRNA abundance of lipid metabolism genes such as CPT1 (Tunstall *et al.*, 2002).

In human skeletal muscle, Pilegaard et al. (2003) were the first to demonstrate a rapid increase in PGC-1α mRNA in response to a single bout exercise. Using a nuclear run-on technique to measure the rate of transcription, exercise (3 h of knee extensor exercise) was shown to transiently increase the rate of PGC-1α gene transcription which was manifested as an elevation in mRNA content at 2 and 6 h after exercise. Interestingly, four weeks of training resulted in a dramatic increase in the rate of PGC-1α gene transcription at rest and after exercise and a larger increase in PGC-1α mRNA during recovery when compared to the untrained response. This suggested that the factors regulating PGC-1α expression become more 'sensitive' with training (Pilegaard *et al.*, 2003), a finding that has recently been confirmed (Mortensen *et al.*, 2007).

Numerous studies since have shown an increase in PGC-1α mRNA expression in response to acute exercise, peaking at 0-3 h after exercise, and returning to baseline later than 6 h after exercise (Norrbom *et al.*, 2004;Watt *et al.*, 2004;Vissing *et al.*, 2005;Russell *et al.*, 2005;Cartoni *et al.*, 2005;Cluberton *et al.*, 2005;Mathai *et al.*, 2008). In short, when exercise is performed at a sufficient intensity, the magnitude of the increase above baseline or control has been reported

between 50% up to 12-fold. This induction is entirely as a result of contractile activity. In the absence of exercise, PGC-1α mRNA abundance did not change in response to repeated biopsy or dietary intervention (Vissing *et al.*, 2005). The continuity between an elevation in mRNA abundance and subsequent increase in PGC-1α protein content after a single bout of exercise is equivocal. In rodent experiments, a 50% up to two-fold increase in PGC-1α protein content is seen at various time points up to 18 h after exercise (Baar *et al.*, 2002;Terada & Tabata, 2004;Terada *et al.*, 2005;Akimoto *et al.*, 2005;Wright *et al.*, 2007b). In human studies, PGC-1α protein content has generally gone unreported although one report showed a tendency for an increase (70%) immediately after exercise (Watt *et al.*, 2004). Recently, Mathai et al. (2008) have reported elevated PGC-1α protein content (~23%) during the first two hours of recovery from ~120 min of exhaustive exercise at 65% VO_{2peak}. After 24 h of recovery, PGC-1α protein remained elevated by 16% above baseline values. These changes in protein content were preceded by 2- to 6-fold increases in mRNA abundance in the first two hours of recovery but returned to pre-exercise values after 24 h (Mathai *et al.*, 2008).

The rapid upregulation of PGC-1α mRNA and protein during recovery from exercise is consistent with a role for the coactivator in the regulation of exercise-induced gene expression as part of the adaptive response to exercise. However, the necessity for an increase in PGC-1α protein content to mediate alterations in gene expression in response to an acute bout of exercise and exercise training is debated (Wright *et al.*, 2007b;Leick *et al.*, 2008). If accumulation of increments in mRNA abundance and alterations in protein content are indeed the molecular basis of training adaptation, a measurable increase in PGC-1α expression is expected after a period of endurance training. This, in fact, has been observed in human skeletal muscle in several experiments both in terms of mRNA and protein content (Russell *et al.*, 2003;Short *et al.*, 2003;Kuhl *et al.*, 2006;Burgomaster *et al.*, 2008), where two- to three-fold changes in PGC-1α protein content have been observed. In these studies, this has been coincident with increases in mitochondrial enzyme activity and mitochondrial gene expression (Russell *et al.*, 2003;Burgomaster *et al.*, 2008). Similar findings in terms of the acute (Atherton *et al.*, 2005) and chronic (Irrcher *et al.*, 2003) responses to contraction have been demonstrated in electrical stimulation models. A strong correlation ($r = 0.74$) was established between COX activity and PGC-1α protein expression, indicating that over 50% of the variance in mitochondrial content, as reflected by COX activity, can be attributed to variations in PGC-1α protein level (Irrcher *et al.*, 2003). However, causation by PGC-1α in the adaptation to training has not been conclusively established.

A link between PGC-1α and exercise training adaptations is illustrated in mice with muscle-specific overexpression of PGC-1α who demonstrate marked improvements in exercise performance both during submaximal exercise intensities and during graded exercise to exhaustion (Calvo *et al.*, 2008). The mice exhibited lower RER values during submaximal exercise reflecting that the molecular modifications induced by high PGC-1α levels increase fat oxidation and thus likely provide a carbohydrate-sparing effect relative to wild-type mice. This is coincident with an increase in mRNA data for genes encoding enzymes in oxidative

phosphorylation and fat metabolism, and citrate synthase activity, indicating an increased muscle oxidative capacity. Together these findings show that PGC-1α-induced changes in metabolic protein expression profile can indeed change performance and metabolism during exercise in mice.

Muscle-specific PGC-1α knockout mice have reduced resting protein content of mitochondrial proteins such as cytochrome c and COXI and reduced exercise tolerance phenotype (Leick *et al.*, 2008). Despite this, no difference was observed in the magnitude of change in cytochrome c or COXI mRNA and protein content in the adaptive response to five weeks of endurance training. PGC-1α is therefore not likely to be mandatory for exercise-training induced adaptations in skeletal muscle mitochondrial proteins (Leick *et al.*, 2008), despite the fact that PGC-1α is necessary to maintain normal resting levels of the same mRNA and proteins (Lin *et al.*, 2002;Leick *et al.*, 2008). However, the induction of cytochrome c mRNA after a single exercise bout is attenuated in skeletal muscle of PGC-1α knockout mice compared to wild-type mice (Leick *et al.*, 2008), and suggests that PGC-1α protein does modulate the transcriptional response to acute exercise. Consistent with the role for PGC-1α in muscle refuelling (Wende *et al.*, 2007), reduced muscle glycogen content at rest and impaired glycogen resynthesis during recovery was reported in the knockout mice, although the molecular mechanism responsible was not established (Leick *et al.*, 2008).

Therefore, PGC-1α is a key factor in regulating resting levels of mitochondrial gene expression, adaptive responses to acute endurance exercise leading to enhanced oxidative capacity of skeletal muscle and, thus, increased capacity for both fat and carbohydrate utilization, but is not the sole mediator of the adaptive response to exercise training. Other, as of yet unknown, compensatory factors or mechanisms may take over when PGC-1α is absent (Leick *et al.*, 2008). Conversely, the activation or stabilisation of existing PGC-1α protein may mediate the effects of acute exercise in modulating skeletal muscle gene expression (Wright *et al.*, 2007b) in concert with an increase in transcription factor functional activity such as DNA binding (Baar *et al.*, 2002;Wright *et al.*, 2007b). Critical to delineating the molecular mechanisms in the transcriptome response is resolving the key exercise-induced signal transduction pathways that lead to these effects on transcriptional regulation.

2.6.9 Exercise-induced alterations in skeletal muscle gene expression

In addition to mitochondrial biogenesis, increased gene expression of metabolic proteins following endurance exercise contributes to promoting the enhanced endurance phenotype (Coffey & Hawley, 2007). An important characteristic of many metabolic genes is that they are transcribed at a basal level, yet can be readily activated or repressed in relation to the nutritional or physiological state (White *et al.*, 2008). Contraction generates transient increases in the abundance of mRNA for a multitude of genes that typically peaks 3-12 h after cessation of exercise and returns to basal levels within 24 hours (Pilegaard *et al.*, 2000;Mahoney *et al.*, 2005;Yang *et al.*, 2005a;Schmutz *et al.*, 2006). Increments in mRNA are associated with the same directional change in their encoded protein to what is considered a new steady-state level

(Booth & Baldwin, 1996). Training-induced muscle adjustments are by definition the consequence of repetition of single exercise stimuli (Fluck, 2006). Thus, as stated earlier, long term adaptation to training is due to the cumulative effects of each acute exercise bout leading to a new steady-state of mRNA and protein expression and a new functional threshold.

The adaptation to increased contractile activity is thought to be confined to the recovery phase from each fatiguing bout of exercise. This allows an overshoot of cellular adaptations that support the accumulation of incremental remodelling responses after each session, and with the repetition of the single exercise stimuli, this would support the enhanced endurance performance (Fig. 2.4)(Fluck, 2006). The molecular phenomenon underlying exercise-induced increase in mRNA abundance is an increased rate of gene transcription for the target gene (Pilegaard *et al.*, 2000;Hildebrandt *et al.*, 2003), consistent with a mechanism in which contraction-induced signals activate pathways leading enhanced transcriptional activation through a subset of activated transcriptional regulators.

2.6.9.1 Gene transcripts elevated during recovery from a single bout of endurance exercise

Gene-specific transcriptional activation during and after a bout of exercise is involved, on some level, in reestablishing homeostasis in skeletal muscle during recovery, as well as contributing to the skeletal muscle adaptations that occur in response to exercise training (Mahoney & Tarnopolsky, 2005). In human skeletal muscle, a single bout of endurance exercise has been shown to increase mRNA abundance of a growing number of genes, including immediate early genes (c-fos, c-jun), myogenic genes (myogenin, MRF4, myoD), antioxidant factors (metallothionein II), calcium signalling (MCIP1), carbohydrate metabolism (HKII, PDK4, GLUT4, glycogenin), lipid mobilisation, transport and oxidation (LPL, CPT1, β-HAD), mitochondrial metabolism (UCP3, COXIV), mitochondrial biogenesis (PGC-1α, NRF-1, NRF-2, Mfn2, ERRα) and transcriptional regulators (PPARα, PPARδ, MEF2, GEF, FOXO1A) (Puntschart *et al.*, 1998;Pilegaard *et al.*, 2000;Keller *et al.*, 2001;Pilegaard *et al.*, 2002;Pilegaard *et al.*, 2003;Norrbom *et al.*, 2004;Watt *et al.*, 2004;Vissing *et al.*, 2005;Russell *et al.*, 2005;Cartoni *et al.*, 2005;Cluberton *et al.*, 2005;Civitarese *et al.*, 2005;Pilegaard *et al.*, 2005;Mahoney *et al.*, 2005;Penkowa *et al.*, 2005;Yang *et al.*, 2005a;Kraniou *et al.*, 2006;Schmutz *et al.*, 2006;Coffey *et al.*, 2006a;Tunstall *et al.*, 2007). The induction of mRNA expression is generally several-fold above resting values for each target gene, but the magnitude of this increase is specific to the gene and the exercise challenge. In addition, the temporal pattern of induction is specific to the gene of interest with some genes (e.g. PGC-1α) consistently shown to be elevated in the first 6 h of recovery from exercise, whereas others (e.g. COXIV) are elevated much later in recovery (Pilegaard *et al.*, 2003;Cartoni *et al.*, 2005).

In response to acute exercise stress, the recovery period in skeletal muscle after the exercise bout is now thought to be characterized by two major phases: the first is homeostatic recovery, and the second is the cellular contributions to adaptation (Mahoney & Tarnopolsky, 2005). Homeostatic recovery is the process whereby skeletal muscle recovers from the stress imposed by the exercise bout and reestablishes homeostasis in the immediate hours after exercise. The metabolic consequences are characterized by glucose sparing, elevated fat oxidation, glycogen

resynthesis (Kiens & Richter, 1998;Kimber *et al.*, 2003). The molecular adaptation that underpins endurance training described above leads to an enhanced ability to maintain muscle homeostasis during exercise, enhancing the resistance to fatigue. The temporal pattern of induction of genes involved in substrate metabolism in the immediate recovery period after exercise, followed latterly by genes involved in mitochondrial metabolism supports this contention. The induction of mRNA of broad range of transcriptional regulators during recovery is consistent with their transcriptional activation by exercise-mediated processes and their subsequent role in the induction of expression of target genes involved in cellular metabolism. However, continuity between the induction of mRNA and the expression of the encoded protein has not always been demonstrated.

Expression of muscle mRNA may not accurately reflect the protein abundance and can give no information regarding their posttranslational modifications (Hojlund *et al.*, 2008). This indicates a need to correlate the results from global gene expression experiments and measurements of protein abundance. The half-lives of many mRNAs are relatively short compared with their target proteins, with transcription gene activation sometimes occurring before sustained and measurable increases in protein (Hargrove et al., 1991). Although the extent to which a protein might be modified in response to an adaptive stimulus cannot be predicted from an increase in mRNA, it has been reported that increased abundance of genes encoding for substrate metabolism is accompanied by a concomitant increase in the cellular content of the transcribed protein (Peters *et al.*, 2001;Cameron-Smith *et al.*, 2003;Tsintzas *et al.*, 2006). Discrepancies in the magnitude or temporal pattern of mRNA induction from different experiments is attributed to specifics of the research design including nature of exercise protocol, timing of muscle sampling, training status of the muscle, probe and primer design, kinetics of the specific gene target, and nutritional control.

2.6.9.2 Modulation of exercise-induced gene expression by nutritional intervention and altering metabolic stress

The specific but malleable nature of the transcriptome response to acute exercise is illustrated by experiments where muscle glycogen content (Pilegaard *et al.*, 2002;Pilegaard *et al.*, 2005), blood glucose availability (Russell *et al.*, 2005;Cluberton *et al.*, 2005) and circulating FFA concentration (Watt *et al.*, 2004;Tunstall *et al.*, 2007) have been manipulated. Presumably the modulation of the transcriptional response reflects an alteration in the nature of the metabolic stress of exercise and thus affecting either the contraction-induced signal or the activity of transcription factors in some manner.

The mechanisms linking alterations in muscle glycogen content and modulation of gene expression are not established. However, low muscle glycogen content has been associated with the potentiation of exercise-induced gene expression. Exercise with lowered muscle glycogen concentration increases the magnitude of exercise-induced elevations in mRNA abundance for HKII, PDK4, UCP3 and IL-6 (Keller *et al.*, 2001;Pilegaard *et al.*, 2002). In a similar fashion, low carbohydrate feeding during recovery elicits a prolonged and/or secondary activation of select exercise-responsive metabolic genes (PDK4, UCP3, LPL, CPT1, FAT/CD36,

FOXO1A), whereas high carbohydrate feeding elicits a reversal of the exercise-induced activation of these genes (Pilegaard *et al.*, 2005). PGC-1α mRNA expression increased to a similar extent in both trials up to 5 h of recovery but after 8 h expression returned to baseline with high carbohydrate feeding but remained elevated in the low carbohydrate diet. Therefore, replenishment of muscle glycogen reserves may be linked to the regulation of metabolic gene expression during recovery from exercise. Alternatively, signalling mechanisms affected by differences in circulating substrate availability (i.e. glucose and/or FFA, insulin, counterregulatory hormones) may be primary factors contributing to the transcriptional regulation of metabolic genes during recovery from exercise (Pilegaard *et al.*, 2005). Evidence for this hypothesis is equivocal. Feeding carbohydrate during exercise has been shown to have no measurable effect on exercise-induced gene expression of transcription factors or coregulators (Russell *et al.*, 2005). Conversely, carbohydrate feeding blunts GLUT4 and PDK4 mRNA induction and downregulates UCP3 and CPT1 mRNA expression compared to fasted, placebo-fed exercise (Civitarese *et al.*, 2005). Similarly, carbohydrate feeding in the recovery period after exercise suppresses exercise-induced increases in PDK4 and UCP3 mRNA but does not alter the pattern of PGC-1α mRNA induction (Cluberton *et al.*, 2005).

Carbohydrate feeding is associated with suppression of lipolysis and a reduction in circulating FFA concentration. To test whether alterations in FFA concentrations are responsible for the observed effects of carbohydrate feeding, lipolysis and circulating FFA levels have been suppressed by pharmacological means prior to exercise (Watt *et al.*, 2004;Tunstall *et al.*, 2007). This did not affect the exercise-induced response in mRNA induction, although elevated PGC-1α mRNA at rest in the suppressed FFA trial is associated with elevated epinephrine (Watt *et al.*, 2004). This effect may be mediated through β-adrenergic receptor activation, which, in part, is responsible for the induction of PGC-1α (Puigserver *et al.*, 1998;Miura *et al.*, 2007). Finally, Vissing et al. (2005) have demonstrated that certain genes such as PDK4 are more responsive to feeding and fasting than exercise, whereas PGC-1α is indeed responsive to exercise.

The studies outlined above raise two important considerations. Firstly, in experiments examining exercise-induced modulation of gene expression, dietary intake preceding and during recovery from exercise must be standardised for all participants. In addition, results must be considered in light of any potential influence of the dietary intake pattern i.e. meal timing, fasting duration. Secondly, the exercise-induced modulation of selected genes is highly malleable in response to alterations in whole body metabolism, as evidenced by altering nutritional state. The molecular mechanism is thought to involve alterations in signal transduction, possibly through AMPK (Hargreaves, 2004), which is consistent with the idea that the transcriptome response is proportional to the metabolic stress imposed (Hoppeler *et al.*, 2007). This raises the interesting possibility that by modifying the metabolic stressor (i.e. the nature of the imposed exercise demand), alterations in signal transduction may be reflected by concomitant alterations in gene expression. For example, comparing the response to hypoxic versus normoxic exercise has shown that key transcripts of carbohydrate metabolism and mitochondrial biogenesis are upregulated to a greater extent when exercise is carried out under hypoxic stress (Zoll *et al.*,

2006). This is potentially mediated by elevated AMPK signalling observed during hypoxic exercise (Wadley *et al.*, 2006). Similarly, during exercise at identical power outputs, the induction of PGC-1α mRNA is higher when ischemic stress is applied compared to unrestricted blood flow (Norrbom *et al.*, 2004). Thus, by modulating the metabolic stress of exercise, it may be possible to alter activation of signalling pathways and consequent transcriptional activation to explore *in vivo* integration of kinase activation and gene activation.

An attractive variable to utilise here would be the intensity of exercise, which is well established as an important regulator of substrate utilisation (Romijn *et al.*, 1993), adaptation to exercise (Dudley *et al.*, 1982) and signal transduction (Wojtaszewski *et al.*, 2002;Rose *et al.*, 2006). Surprisingly, the effect of intensity of exercise on skeletal muscle gene expression is poorly described. In human skeletal muscle, GLUT4 mRNA induction by exercise is similar whether 400 kcal of exercise was performed at either 40% or 80% VO_{2peak} (Kraniou *et al.*, 2006). However, Sriwijitkamol et al. (2007) have shown a tendency for higher PGC-1α and NRF-1 mRNA after 40 min at 70% VO_{2peak} compared to 50% VO_{2peak}. These results could be potentially confounded by the differences in total work completed. In addition, data from animal studies (Hildebrandt *et al.*, 2003;Terada *et al.*, 2005) has shown a tendency for an intensity-dependent effect on mRNA and protein expression. Both the intensity and duration of exercise influence the magnitude of the increase in HKII, PDK4, UCP3 and LPL transcription during recovery (Hildebrandt *et al.*, 2003). In terms of PGC-1α protein content, Terada et al. (2005) compared responses to high (14x20 s with 14% body mass attached) and low (2x3 h with 45 min rest) intensity swimming exercise in rats. Although actual energy expenditure during exercise was not controlled for or quantified, at 18 h after exercise PGC-1α protein content was increased by 67% in LO, whereas in the HI trial, it increased by 126%. However, this trend did not result in a statistically significant difference between the trials, so it was concluded that the intensity of exercise does not affect the subsequent protein content of PGC-1α. Clearly the effect of modulating metabolic stress through exercise intensity and the molecular mechanisms underlying altered gene expression requires further investigation.

2.6.9.3 Effect of exercise mode and exercise training on the acute transcriptional response

Like the induction of intracellular signal transduction pathways, the transcriptome response to a single exercise bout is specific to the stimulus and can be modulated by the training status of the muscle. Using microarray analysis of global mRNA, Mahoney et al. have shown that the induction of gene transcripts in response to endurance exercise is very much distinct to gene transcripts induced by acute eccentric exercise (Mahoney *et al.*, 2005;Mahoney *et al.*, 2008). Endurance exercise is associated with elevations in mRNA encoding proteins involved in substrate metabolism and mitochondrial biogenesis, the oxidant stress response and electrolyte transport across membranes, whereas eccentric contractions induce elevations in mRNA encoding proteins involved in cholesterol and lipid synthesis and/or modification, skeletal muscle growth, remodelling, and stress management. The divergent transcriptome response is thought to reflect both the different homeostatic perturbation and the nature of the adaptive response to endurance versus eccentric resistance exercise (Mahoney & Tarnopolsky, 2005). In

addition, direct comparison of the two gene sets revealed thirty-six genes that responded in a similar time course and magnitude to the two distinct exercise challenges. These genes may belong to a class of ‘‘exercise-responsive’’ genes, which respond to common signals and stresses inherent in both exercise modes, as evidenced by the fact that many of the genes are immediate early genes or genes involved in the cell stress response (Mahoney & Tarnopolsky, 2005). The number of common genes probably would decrease throughout a period of training, as the muscle fine tunes the transcriptional response to meet the needs of the specific stresses placed on it more accurately. Coffey et al. (2006a) have shown, using well-trained resistance and endurance-trained athletes, that a single endurance exercise bout produced comparatively similar transcriptional responses in metabolic genes whereas training history markedly altered the response of myogenic genes. A single bout of resistance exercise produced a markedly greater transcriptional response in endurance-trained subjects but few differences were evident in the overall response to resistance exercise between groups. This suggests that prior training history can modify the acute gene responses in skeletal muscle to subsequent exercise.

That training may alter the transcriptional response to exercise is illustrated by the fact that IL-6 mRNA induction after 3 h of acute exercise is reduced to approximately 25% of pre-training values after ten weeks of training (1 h/d, 5 d/wk), even though the amount of work done was 44% higher in the post-training bout (Fischer *et al*., 2004). Similarly, short-term training has been shown to modulate the transcriptional response to a single bout of exercise of genes including PDK4, HKII, Tfam, PPARα, CPT1 (Tunstall *et al*., 2002;Pilegaard *et al*., 2003;Nordsborg *et al*., 2003). Studies using gene-by-gene analysis are said to be inadequate in predicting long-term adaptations in skeletal muscle phenotype to endurance training (Keller *et al*., 2001). Thus, transcriptomics or genome-wide assessments of the transcriptional response to exercise have been employed as a more powerful tool (Mahoney *et al*., 2005;Timmons *et al*., 2005b). Detailed studies on the modulation the transcriptome response to a single bout of exercise by a period of exercise training are few but indicate (i) exercise training results in an increase in steady-state mRNA, (ii) exercise training attenuates the transcriptome response to acute exercise, and (iii) the induction of certain genes prior to training may not necessarily be involved in the adaptive response to repeated exercise but merely reflect the nature of the stresses placed on the muscle (Choi *et al*., 2005;Timmons *et al*., 2005b;Schmutz *et al*., 2006;Keller *et al*., 2007). This hypothesis is supported by a microarray study in mice, in which a single bout of endurance exercise elicited the expression of many genes involved in myocyte proliferation, a stress-related paradigm more commonly observed after resistance exercise, a response that was not observed after a period of endurance training (Choi *et al*., 2005). In humans, Schmutz et al. (2006) have used custom-designed arrays to examine the transcriptome response to acute exercise before and after six weeks of endurance training. An approximately two-fold enhancement of steady-state levels of mRNAs encoding proteins for fatty acid transport and mitochondrial respiratory function was observed after training, consistent with the theory that increments in mRNA underpin phenotypic adaptation. However, the vast majority of these transcripts were not measurably increased by a single bout of exercise before or after training. In addition, the transcriptional response, i.e. transient

upregulation of many genes, to a matched exercise bout before and after training was blunted. Functional and structural adaptations are attenuated with ongoing training with the same relative intensity, a phenomenon that may be explained in part by the attenuated transcriptional response in the trained state (Schmutz *et al.*, 2006). Modulation of the transcriptome response may be due to increased steady-state mRNA abundance resulting in a more sensitive transcriptome in which large changes in mRNA are no longer required. Similarly, it may be that the response to exercise becomes fine-tuned and as such the induction of certain mRNA targets is no longer evident. Finally, it has been suggested that some acutely responsive genes are induced to programme the restoration of homeostasis, but if not involved in the molecular basis of adaptation are not responsive to exercise training (Mahoney *et al.*, 2005). Clearly, further investigation of the effect of training on the transcriptional response to exercise and the time course of alterations in steady-state mRNA in response to training is warranted.

In summary, an intense bout of exercise induces a rapid but transient transcriptome response in untrained muscle. This results in an increase in the rate of gene transcription and mRNA abundance during recovery from the exercise bout. These induced transcripts encode proteins involved in many aspects of skeletal muscle metabolism including substrate transport and utilisation, mitochondrial metabolism and mitochondrial biogenesis. However, continuity between the induced transcript and a change in protein function has not always been established. These transcriptional alterations are thought to be induced to affect a biphasic response to acute exercise resulting first in restoration of homeostasis and latterly cellular adaptation to exercise. Repeated exercise leads to increments in steady-state levels of mRNA abundance as a consequence of these transient pulses in mRNA. This is thought to be the molecular basis for exercise training-induced alteration of skeletal muscle phenotype.

2.7 General summary

The regulation of mitochondrial biogenesis and skeletal muscle metabolism is governed by a well-defined subset of transcription factors and transcriptional coregulators. Several signalling pathways involving cytoplasmic protein kinases, transcription factors and transcriptional coregulators are recognised as regulators by which activation transduces physiological stimuli into transcriptional adaptations (Fluck, 2004). Regulation of transcriptional activity occurs via change in the protein content or activity (e.g. posttranslational modifications) of transcriptional regulators (Spiegelman & Heinrich, 2004). Therefore, transcription factors, nuclear receptors and their transcriptional coregulators are key players in integrating signals from physiological stimuli into adaptive tissue responses to coordinate metabolic processes (Spiegelman & Heinrich, 2004;Desvergne *et al.*, 2006;Feige & Auwerx, 2007). Skeletal muscle contraction is one such physiological stimulus and initiates a cascade that results in the activation and/or repression of specific signalling pathways regulating exercise-induced gene expression (Coffey & Hawley, 2007).

PGC-1α overexpression, both *in vivo* and *in vitro*, is associated with an increase in the induction of mitochondrial gene expression program including increased respiratory chain subunit

expression, mitochondrial density and mitochondrial enzyme activity (Wende *et al.*, 2007;Benton *et al.*, 2008). The synergistic effect of these phenotypical changes is responsible for the improvement in exercise capacity and decreased reliance on glucose metabolism during acute exercise seen in transgenic mice overexpressing PGC-1α (Calvo *et al.*, 2008), mirroring the phenotype seen in endurance-trained humans (Holloszy & Coyle, 1984). Endurance-type exercise training induces an increase in mitochondrial enzymatic activity and mitochondrial volume density in the recruited muscle (Holloszy, 1967;Hoppeler *et al.*, 1973), and is one of the more dramatic phenotypic alterations in response to contractile activity.

The most accepted hypothesis is that adaptation is mediated at the level of transcription (Mahoney & Tarnopolsky, 2005). That the activation of intracellular signalling pathways and the expression and/or function of transcriptional regulators can be markedly and rapidly induced by acute exercise is critical to the molecular regulation of skeletal muscle plasticity in an exercise context. Based on data from microarray experiments, a single bout of exercise induces a robust, but transient, increase in transcript abundance of hundreds of genes encoding proteins involved in a variety of processes critical to cellular metabolism (Mahoney *et al.*, 2005;Schmutz *et al.*, 2006). A single exercise stimulus carries a molecular signature that is typical both for the type of stimulus as well as the actual condition of the muscle tissue (Hoppeler *et al.*, 2007). Transcriptional activation of certain genes is thought to play a role in immediate homeostatic recovery, whereas the transcriptional activation of others is thought to be linked to the adaptation to exercise training (Mahoney & Tarnopolsky, 2005). Therefore, the accumulation of specific gene transcripts in skeletal muscle due to repetitive action of an exercise training stimulus may contribute to functional improvements that are typical of the endurance-trained state (Hood *et al.*, 2006;Fluck, 2006). The series of experiments reported herein have attempted to examine these contentions in *in vivo* human exercise models using exercise intensity, training status and short-term endurance training as investigative tools.

Chapter III General methodology

Detailed descriptions of the experimental design and procedures for each experiment are described in the respective Chapters IV, V and VI. This chapter outlines the design of each experiment and describes the measurements and protocols common to all three experiments.

3.1 Experimental design overviews

Experiment I: Contraction-induced signalling and gene expression of metabolic genes and transcriptional regulators in human skeletal muscle: influence of exercise intensity

Eight healthy, sedentary males performed two isocaloric (400 kcal) cycle exercise trials, once each at either 40% or 80% VO_{2peak}. Skeletal muscle biopsies from the *m. vastus lateralis* were taken at rest and at +0 h, +3 h and +19 h after exercise.

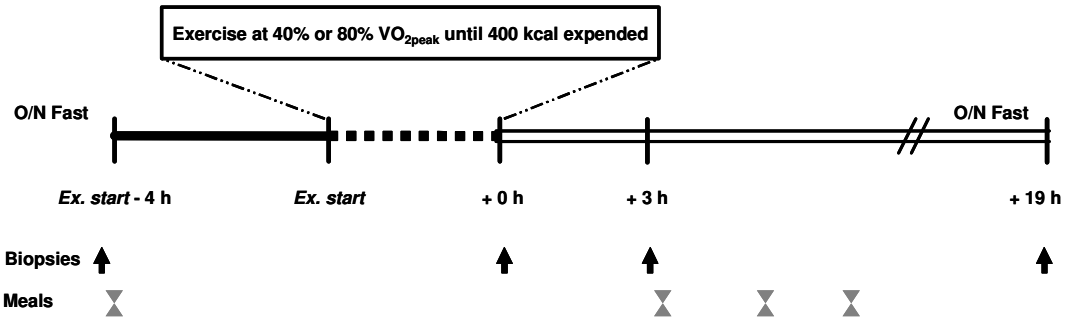

Figure 3.1 Schematic of experimental design for experiment I

Experiment II: The effect of glycogen-depleting exercise on contraction-induced signalling and gene expression of metabolic genes and transcriptional regulators in well-trained human skeletal muscle

Eight healthy well-trained males cycled for 90 min at 73% VO_{2peak}. Skeletal muscle biopsies from the *m. vastus lateralis* were taken at rest and at +0 h and +1 h after exercise.

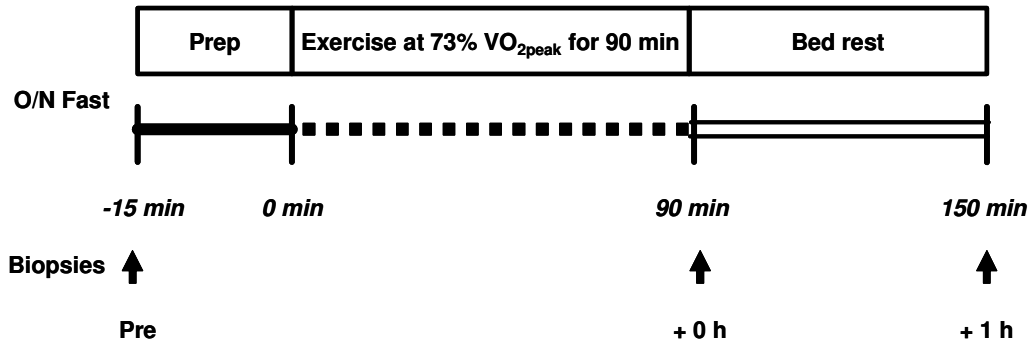

Figure 3.2 Schematic of experimental design for experiment II

Experiment III: The effect of fourteen consecutive days of exercise training on the expression of metabolic and mitochondrial genes and their transcriptional regulators in skeletal muscle of previously untrained men

Eight healthy, sedentary males performed 60 min of exercise at 80% VO_{2peak}, once per day for fourteen consecutive days. Skeletal muscle biopsies from the *m. vastus lateralis* were taken prior to the start of training, and on the morning after the 1st, 3rd, 7th, 10th and 14th training session.

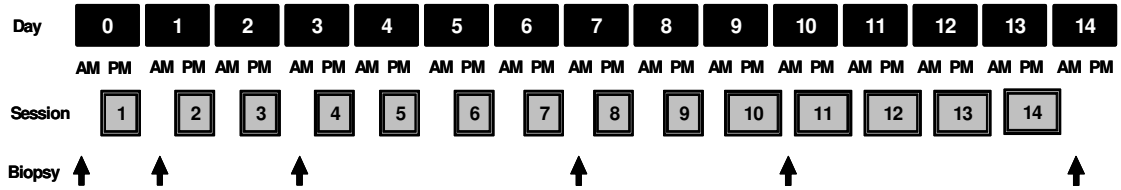

Figure 3.3 Schematic of experimental design for experiment III

3.2 Participant recruitment

Participants for each experiment were recruited by email from the undergraduate, postgraduate and staff population at Dublin City University. A plain language statement was given to those expressing an interest in the study and written informed consent was provided by individuals wishing to participate. Each experiment was approved by the Dublin City University Research Ethics Committee and conformed to the Declaration of Helsinki. Finally, each participant completed a health history questionnaire underwent a medical screening examination and electrocardiogram to confirm their suitability for the experiment.

3.3 Determination of maximal oxygen uptake

All exercise tests took place under standard laboratory conditions (19-21°C, 40-55% relative humidity). In each experiment, prior to any experimental testing participants performed an incremental exercise test to volitional fatigue on an electronically braked cycle ergometer (Ergoline 900, SensorMedics, Yorba Linda, CA) to determine peak oxygen consumption (VO_{2peak}). Briefly, in experiments one and two, participants began cycling at 100 W for five minutes warm-up and the power output was increased by 50 W every two minutes thereafter until volitional fatigue. In experiment III, participants began cycling at 80 W for five minutes warm-up and the power output was increased by 40 W every two minutes thereafter until volitional fatigue. Oxygen uptake was considered to have peaked if two of the following criteria were met: (i) a levelling off of VO_2 with increasing power output (increase of less than 2 $mL.kg^{-1}.min^{-1}$), (ii) a HR within 10 beats of the age predicted HR_{max} (220 bpm – age in years), (iii) a RER greater than 1.10. Expired air was collected continuously throughout exercise and analysed using the Vmax 29C gas analysis system (SensorMedics, Yorba Linda, CA). Heart

rate was continuously monitored during exercise by telemetry (Polar Vantage NVTM; Polar, Port Washington, NY).

3.4 Verification of submaximal exercise intensities

The power output required to elicit a given percentage of VO_{2peak} was interpolated based on the linear relationship between oxygen uptake (*y*-axis) and power output (*x*-axis). For a given percentage of VO_{2peak}, the corresponding power output is estimated by solving for *x* using the linear function, $y=mx+c$, where *y* is VO_2, *x* is power output, *m* is the slope of the relationship between VO_2 and power output and *c* is the y-axis intercept. Between four and seven days after maximal oxygen uptake test, each participant returned to the laboratory to perform a test to verify the power outputs required to elicit the requisite VO_{2peak} for each experimental trial: experiment I, 40% and 80% VO_{2peak}; experiment II, 75% VO_{2peak}; experiment III, 80% VO_{2peak}. Participants cycled for 10 min at each of three different power outputs: 15 W below the predicted power output, at the predicted power output and 15 W above the predicted power output. Pedal cadence was required to be kept between 75 and 80 rpm for all exercise tests and experimental trials

3.5 Assessment of body composition

Body density was calculated by the method of Jackson & Pollock (1978) based on the sum of seven skinfolds (tricep, subscapular, mid-axillary, pectoral, suprailiac, abdominal, thigh) measured with Harpendon skinfold callipers (Jackson & Pollock, 1978). Percentage body fat was calculated using the Siri equation (Siri, 1961).

3.6 Muscle biopsy procedure

Skeletal muscle specimens were taken by muscle biopsy from the *m. vastus lateralis* under local anaesthesia. An area of skin, subcutaneous tissue, and fascia was anaesthetized with 2% w/v Lidocaine HCl and a small (0.5 cm) incision made. The biopsy needle was inserted into the muscle and approximately 100 mg of tissue removed (200 mg in experiment III) using the percutaneous muscle biopsy technique with suction applied. A fresh incision was made for each biopsy, at least 2 cm from a previous biopsy site. Muscle samples were snap-frozen in liquid nitrogen and stored at –80°C until analysis. Specifics of timing, location and number of biopsies are described in the respective experiment chapters.

3.7 Calculations of substrate utilisation

Values for VO_2, VCO_2, RER, $V_{E\ (STPD)}$, and F_EO_2 were recorded from expired air using 60 s averages and used to calculate the rate of energy expenditure (Weir, 1949), rates of carbohydrate and fat oxidation (Zuntz, 1901), and the percentage contribution of each substrate to total energy production (Kuo *et al.*, 2005).

3.8 Determination of muscle glycogen concentration

Frozen muscle samples (~10 mg) were lyophilised, dissected free of connective tissue, weighed and hydrolysed with 1 M HCl by incubation at 100°C for 2 h and then neutralised with 0.67 M NaOH. Glycogen concentrations were determined by a standard enzymatic technique with fluorometric detection (Passonneau & Lauderdale, 1974).

3.9 RNA extraction from biopsy samples

Total RNA was isolated from ~20 mg crude muscle tissue based on the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi, 1987) using TRI reagent (T9424; Sigma-Aldrich, UK) as per the manufacturer's instructions. Muscle tissue was lysed using a motorised pestle in 1 ml of TRI reagent (4°C) per 50 mg of tissue, followed by drawing through a 25G needle with a 1 ml syringe. Total RNA concentration was determined spectrophotometrically at an absorbance of 260 nm (NanoDrop ND-1000 Spectrophotometer, ThermoFisher Scientific, Waltham, MA). The integrity of each RNA sample was verified by gel electrophoresis (RNA 6000 Nano Lab Chip & 2100 Bioanalyzer, Agilent Technologies, Palo Alto, CA) and by measuring the spectrophotometric A260/A280 (>1.8) and A260/A230 (>1.5) ratios. RNA (1 µg) was reverse transcribed to cDNA using the Reverse Transcription System (A3500; Promega, Madison, WI) primed with oligo-$dT_{(15)}$ as per the manufacturer's instructions. The cDNA template was stored at -20°C until subsequent analysis.

3.10 Quantitative real-time PCR

qPCR was performed using the ABI Prism 7500 Sequence Detection System and software package (version 1.1; Applied Biosystems, Foster City, CA) using Assay-on-Demand pre-designed gene-specific primer and probe sequences (P/N 4331182; Taqman® Gene Expression Assays, Applied Biosystems). The PCR reaction mix in each well consisted of 30 ng cDNA template (6 ng/µl), Taqman probe, forward and reverse primer set, Taqman Universal Master Mix (Applied Biosystems) and nuclease-free water in a 20 µl reaction. Gene targets for qPCR are reported in Table 3.1. The PCR profile for all genes consisted of one cycle at 50°C for 2 min, followed by a denaturing cycle at 95°C for 10 min, followed by 40 cycles of denaturing at 95°C for 15 sec and annealing and elongation at 60°C for 1 min. Each sample was run in duplicate. mRNA content was calculated from a corresponding standard curve (critical threshold cycle number vs. log dilution) run together with the samples. The standard curve was constructed using serial dilutions of an RNA sample pooled from the entire sample set and included in the qPCR reaction. The average critical threshold cycle (C_T) value of the unknown samples was converted to relative expression data using the appropriate standard curve. mRNA data is expressed as the ratio between the gene of interest and a housekeeper gene, allowing normalisation of mRNA expression to a stable mRNA.

According to Mahoney et al. (2004), the selection of housekeeping gene as an endogenous control requires several considerations: (i) expression of the gene must stay constant

throughout the given intervention; (ii) amplification efficiency of the gene should be similar to that of the gene of interest; (iii) abundance of housekeeping gene should be similar to that of genes of interest. In general, housekeeping genes should be constitutively expressed and minimally regulated (Mahoney *et al.*, 2004). We explored the suitability of several proposed housekeeping genes for each experimental design. These included GAPDH (4333764F, Applied Biosystems), cyclophilin A (4333763F, Applied Biosystems), β2-microglobulin (4333766F, Applied Biosystems) and 18S rRNA (4331182: Hs99999901_s1, Applied Biosystems). Our analysis showed that GAPDH was an appropriate housekeeping gene for experiments I and III, whereas 18S ribosomal RNA was appropriate for experiment II.

Table 3.1. Gene targets for qPCR

Target	Gene ID	Assay ID
COXIV	1327	Hs00266371_m1
CPT1	1375	Hs00189258_m1
CREB	1385	Hs00231713_m1
ERRα	2101	Hs01067166_g1
FOXO1A	2308	Hs00231106_m1
GEF	56731	Hs00219920_m1
GLUT4	6517	Hs00168966_m1
MEF2A	4205	Hs00271535_m1
MEF2D	4209	Hs00232237_m1
NRF-1	4899	Hs00192316_m1
NRF-2	2551	Hs00745591_s1
PDK4	5166	Hs00176875_m1
PGC1α	10891	Hs00173304_m1
PPARδ	5467	Hs00602622_m1
RIP140	8204	Hs00534035_s1
Sirt1	23411	Hs01009006_m1
UCP3	7352	Hs00243297_m1

Assay ID corresponding to the TaqMan® Gene Expression Assay ID (P/N 4331182; Applied Biosystems). Probe and primer sequences are proprietary information.

3.11 SDS-PAGE and immunoblot analysis

Muscle specimens were homogenised in ice-cold homogenisation buffer (20 mM Tris [pH 7.8], 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1% Triton X-100, 10% [wt/vol] glycerol, 10 mM NaF, 1 mM EDTA, 5 mM sodium pyrophosphate, 0.5 mM Na_3VO_4, 1 µg/ml leupeptin, 0.2 mM phenylmethyl sulfonyl fluoride, 1 µg/ml aprotinin, 1 mM dithiothreitol, 1 mM benzamidine, and 1 µM microcystin) using a motorised pestle. Homogenates were rotated end over end for 60 min at 4°C. Samples were subjected to centrifugation (12,000 *g* for 15 min at 4°C), and protein concentration of the supernatant was determined using a commercially available detergent-compatible colorimetric assay based on the Lowry method (Lowry *et al.*, 1951) (Bio-Rad Laboratories, Hercules, CA; 500-0116). In experiment I, crude muscle samples were

homogenised in 1 ml of buffer per 25 mg wet weight of tissue. In experiments II and III, muscle samples were lyophilised and dissected free of connective tissue and homogenised in 1 ml of buffer per 5 mg dry weight of tissue.

An aliquot of muscle homogenate (50 µg protein) was mixed with Laemmli buffer containing 20% v/v β-mercaptoethanol (protein concentration, 2 µg/µl) and subjected to SDS-PAGE. Samples for each subject from the respective exercise trials were compared in parallel, and representative blots of one such subject are shown in each "Results" section. Proteins were separated by SDS-PAGE (6, 8 or 10% resolving gel), transferred to PVDF membranes (Immobilon P-IPVH00010; Millipore, Billerica, MA) and blocked with Tris-buffered saline (pH 7.6) with 0.02% Tween 20 (TBS-t) containing 5% non-fat dried milk protein for 2 h at room temperature. Membranes were incubated overnight at 4°C with primary antibodies for respective protein targets detailed in Table 3.2. Membranes were washed in TBS-t and incubated with appropriate secondary horseradish peroxidase-conjugated antibodies (1:20000; Bio-Rad Laboratories, Hercules, CA). Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences, Arlington Heights, IL) and quantified by densitometry.

Table 3.2. Antibodies directed against target proteins for immunoblot analysis

Target	Supplier	Product code	Concentration
COXIV	Cell Signaling Technology	4844	1:1000
CPT1	Santa Cruz Biotechnology	sc-20670	1:500
ERRα	Santa Cruz Biotechnology	sc-32972	1:500
FOXO1A	Cell Signaling Technology	9454	1:1000
GAPDH	Santa Cruz Biotechnology	sc-25778	1:500
HKII	Cell Signaling Technology	2106	1:1000
PDK4	Abgent	AP7041b	1:500
PGC-1α	Santa Cruz Biotechnology	sc-13067	1:500
phospho-Ser^{133}-CREB	Cell Signaling Technology	9191	1:1000
phospho-Ser^{79}-ACC	Cell Signaling Technology	3661	1:500
phospho-Thr^{172}-AMPK	Cell Signaling Technology	2531	1:1000
phospho-Thr^{286}-CaMKII	Cell Signaling Technology	3361	1:1000

3.12 Data analysis

Experimental data are presented as mean ± SE. Data were evaluated using the SPSS for Windows v14.0 software package (SPSS, Inc., Chicago, IL).

Chapter IV Contraction-induced signalling and gene expression of metabolic genes and transcriptional regulators in human skeletal muscle: influence of exercise intensity

4.1 Introduction

Skeletal muscle shows a remarkable malleability to remodel its phenotype and to adapt functionally in response to contractile stimuli (Hood *et al.*, 2006;Fluck, 2006). Exercise-induced phenotypes are accepted to involve the sensation and transduction of contraction-induced stimuli through intracellular signal transduction pathways, their integration by downstream targets including transcription factors and transcriptional regulators (Fluck, 2004;Hood *et al.*, 2006;Freyssenet, 2007). The consequent alterations in mRNA abundance and protein accretion result in functional and structural adaptations localised to the exercised muscle.

In human skeletal muscle, ATP turnover, calcium flux and mechanical stress are thought to be the prominent contraction-induced signals resulting in altered metabolic gene expression in response to exercise (Ojuka, 2004;Long *et al.*, 2004;Chin, 2005;Jorgensen *et al.*, 2006). Transduction of these signals through the activation of AMPK, CaMKII and p38 MAPK pathways modulates PGC-1α-dependent transcriptional regulation of skeletal muscle metabolism (Akimoto *et al.*, 2005;Jager *et al.*, 2007;Wright *et al.*, 2007a). Pharmacological activation of AMPK (Jorgensen *et al.*, 2005), calcium flux (Ojuka *et al.*, 2003) and p38 MAPK (Akimoto *et al.*, 2005) induces PGC-1α mRNA expression and the induction of several transcription factors and exercise-responsive genes. Given that skeletal muscle energy flux during contraction is intensity-dependent, it is unsurprising that signal transduction cascades are differentially regulated by the intensity of an acute exercise challenge (Wojtaszewski *et al.*, 2000;Widegren *et al.*, 2000;Rose *et al.*, 2006). The intensity of exercise is well established as a determinant of substrate utilisation during exercise (Romijn *et al.*, 1993;van Loon *et al.*, 2001) as well as a critical component in determining the nature and magnitude of training adaptation (Dudley *et al.*, 1982;Gibala *et al.*, 2006). However, it is less well understood how the intensity or duration of a single bout of exercise regulates skeletal muscle gene expression. Whether differential activation of these signalling cascades could lead to intensity-dependent regulation of PGC-1α expression is unknown.

Sustained contraction generates transient increases in mRNA abundance of a multitude of genes, which typically peaks 3-12 h after cessation of exercise and returns to basal levels within 24 hours (Pilegaard *et al.*, 2000;Mahoney *et al.*, 2005;Yang *et al.*, 2005a;Schmutz *et al.*, 2006). In human skeletal muscle, PGC-1α mRNA is transiently increased following a single bout of exercise (Pilegaard *et al.*, 2003;Norrbom *et al.*, 2004;Russell *et al.*, 2005;Cartoni *et al.*, 2005). PGC-1α coactivation of the orphan nuclear receptor ERRα is required for PGC-1α-mediated mitochondrial biogenesis (Schreiber *et al.*, 2004). In addition, regulators of substrate utilization such as FOXO1A and PDK4 are also transiently increased following a single bout of exercise (Pilegaard *et al.*, 2003;Mahoney *et al.*, 2005). However, ERRα expression is also regulated by RIP140, a transcription factor corepressor that serves mutually antagonistic functions to PGC-1α in the regulation of mitochondrial activity and fibre type composition (Seth *et al.*, 2007). The regulation of metabolic gene networks may be determined by the relative levels or activation of PGC-1α and RIP140 (Christian *et al.*, 2006;Seth *et al.*, 2007). RIP140 is relatively highly

expressed in skeletal muscle (Seth *et al*., 2007) and but to date, the effect of exercise on RIP140 expression is unknown.

The purpose of this experiment was to determine the impact of exercise intensity on contraction-mediated signalling cascades that regulate PGC-1α expression and nuclear-encoded mitochondrial genes in human skeletal muscle. We hypothesised that a single bout of high intensity exercise would result in greater activation of the AMPK and calcium signalling cascades, leading to a differential regulation of PGC-1α-mediated gene expression, when compared to a single bout of isocaloric low intensity exercise.

4.2 Materials and methods

4.2.1 Experimental design

Eight healthy, sedentary, males volunteered to participate in the study (Table 4.1). Each participant had a preliminary screening, an assessment of peak oxygen uptake and a verification of the power output required to elicit the desired exercise intensities as described in Chapter III. The first experimental trial took place at least seven days after the verification test. The experimental design (Fig. 4.1) required the participants to complete two main experimental trials consisting of cycle exercise on a stationary ergometer at either high (80% VO_{2peak}, HI) or low (40% VO_{2peak}, LO) intensity, in random order. Experimental trials were separated by exactly seven or fourteen days depending on facility and participant availability. The exercise bouts were isocaloric and required each participant to expend 400 kcal *a priori*.

Experimental set up and protocol for the each trial was identical in every aspect except for the intensity, and consequently the duration, of the respective exercise challenge (Table 4.2). To preclude the influence of circadian variation on any experimental variable, each experimental trial began between 0730 and 0930. Participants reported the Metabolic Physiology Research Unit after an overnight (>8 h) fast and were requested to rest quietly in a supine position for approximately ten minutes. A resting muscle biopsy was taken (#1). Subjects then consumed a high CHO breakfast (see “Dietary control”) and remained in the laboratory with minimal ambulation until the commencement of the exercise bout. Exercise began 4 h after the consumption of the morning meal. Expired air was collected continuously throughout exercise and analysed using the Vmax 29C gas analysis system (SensorMedics, Yorba Linda, CA). Values for VO_2 and VCO_2 were used to monitor the exercise intensity, and calculate the rate of energy expenditure (Weir, 1949) and cumulative energy expenditure on a minute-by-minute basis. Participants were required to maintain the cycle cadence at between 75 and 80 rpm. A second muscle biopsy (#2) was taken immediately after the termination of exercise and another (#3) was taken 3 h after the termination of exercise. During this 3 h recovery, participants remained in the laboratory with minimal ambulation and were permitted to consume only water *ad libitum*. After the third biopsy, a standardised afternoon meal was consumed after which participants were free to leave the laboratory. In addition, a standardised evening meal and snack were provided and water was allowed *ad libitum*. The following morning participants

reported to the laboratory after an overnight fast equivalent to the previous night's and were requested to rest quietly for approximately fifteen minutes. A fourth muscle biopsy (#4) was taken 19 h after the termination of exercise.

Table 4.1. Physical characteristics of participants

	n=8
Age (yr)	24±1
Height (m)	1.79±0.02
Mass (kg)	80.3±2.2
BMI ($kg \cdot m^{-2}$)	25.1±1.2
Body fat (%)	16.0±3.3
VO_{2peak} ($L \cdot min^{-1}$)	3.23±0.18

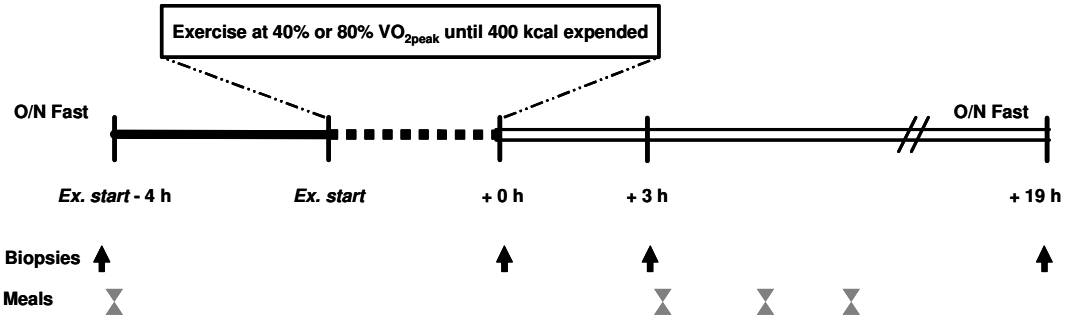

Figure 4.1. Schematic of experimental design

4.2.2 Muscle biopsies

Biopsies #1 and #4 were taken from the right leg; biopsies #2 and #3 were taken from the left leg. A fresh incision was made for each biopsy and the site of each biopsy was at least 2 cm distal or proximal to any previous biopsy site. In the case of biopsy #2, local anaesthetic was administered immediately prior to exercise during HI. During LO, exercise was interrupted briefly at approximately fifteen minutes prior to the estimated exercise termination time and the anaesthetic was administered. Biopsies immediately after exercise were excised within 90 seconds of the termination of exercise.

4.2.3 Dietary control

Pre-exercise preparation was the same for each exercise test. Subjects were asked to abstain from caffeine and alcohol and refrain from physical activity of any nature for 24 h prior to testing. Subjects were asked to keep a one day food diary on the day prior to the first experimental trial and asked to repeat the content and pattern of intake on the day preceding the second experimental trial.

Dietary intake during each trial was standardised in terms of total energy intake and macronutrient composition for each participant. Total energy intake was based on an estimate of daily energy expenditure using the Harris-Benedict equation

BMR (kcal) = (13.75 * body mass in kg) + (5 * height in cm) - (6.76 * age in yr) + 66

(Harris & Benedict, 1919)

multiplied by an appropriate activity factor (1.4) (Durnin, 1996), plus 400 kcal added for calories expended during the exercise challenge; i.e. (1.4 * Harris-Benedict) + 400. This caloric intake was provided in the form of three main meals and one snack. Each of the main meals provided 30% of the total caloric intake with the remaining 10% provided by the evening snack. Total energy, CHO, fat and protein intake was 36 kcal•kg^{-1} BM, 6.0 g•kg^{-1} BM, 0.8 g•kg^{-1} BM, and 12 g•kg^{-1} BM, respectively. Hence, the percentage contribution of each macronutrient was 67% CHO, 20% fat and 13% protein. No other food or beverage other than water was allowed.

4.2.4 Statistical analysis

Two-way (trial x time) repeated measures ANOVA with pair-wise comparisons was used to determine differences between the two intensities of exercise for variables with serial measurements. Where a main effect was found, Tukey's post-hoc test was used to determine where the difference existed. Paired t-tests were used to determine differences between the trials for variables with single measurements.

4.3 Results

4.3.1 Energy expenditure and substrate utilization

Total energy expenditure was similar between trials despite the difference in exercise intensity and total exercise time ($p<0.05$) (Table 4.2). The difference in the rate of energy expenditure ($p<0.05$) resulted in a greater reliance on the relative ($p<0.05$) and absolute ($p<0.05$) contribution of carbohydrate oxidation during the high intensity trial. As expected the contribution of fat oxidation to total energy expenditure was lower during the high intensity trial ($p<0.05$).

Muscle glycogen content was similar at baseline (259±17 vs. 249±19 mmol•kg^{-1} dw for the LO and HI trials, respectively) and decreased following both exercise trials (176±22 vs. 128±34

mmol•kg^{-1} dw for the LO and HI trials, respectively; $p<0.05$), but returned to baseline the following morning. The net rate of glycogen utilization was higher during the high intensity trial (1.3±0.2 vs. 3.1±1.0 mmol•kg^{-1} dw•min^{-1}, for the LO and HI trials respectively, $p<0.05$). Plasma lactate concentration was unchanged from baseline during LO, but increased to 7.23±1.07 mM at the end of HI ($p<0.001$ compared to both baseline and LO).

Table 4.2. Energy expenditure and substrate utilization during isocaloric low and high intensity exercise trials. Values are mean±SE. ** significantly different compared to low intensity trial ($p<0.05$).

	LO	HI
Total EE (kcal)	412±11	403±1
Rate of EE (kcal·min^{-1})	6.0±0.3	11.5±0.7**
Exercise intensity (%VO_{2peak})	38.8±0.4	79.4±1.5**
Exercise time (min)	69.9±4.0	36.0±2.2**
RER	0.90±0.01	0.98±0.01**
CHO oxidation rate (g•min^{-1})	0.9±0.1	2.5±0.2**
Total carbohydrate oxidized (g)	64±2	89±3**
Rate of fat oxidation (g•min^{-1})	0.23±0.02	0.12±0.04**
Total fat oxidized (g)	15±1	4±1**
Rate of glycogen utilization (mmol•kg^{-1} dw•min^{-1})	1.3±0.2	3.1±1.0**
Plasma lactate at termination (mM)	1.22±0.11	7.23±1.07**

4.3.2 Contraction-activated signalling cascades

AMPK phosphorylation (Fig. 4.2A) was similar at baseline, but increased 4.1-fold following exercise in the high ($p<0.05$), but not low intensity trial, resulting a difference between trials ($p<0.05$). ACCβ phosphorylation (Fig. 4.2B) increased 3.6- and 7.9-fold immediately following exercise for the low and high intensity trials, respectively ($p<0.05$), resulting in a difference between trials at this time point ($p<0.05$).

Total CaMKII phosphorylation [summation of β_M and γ/δ isoforms (Rose *et al.*, 2006;Rose *et al.*, 2007a)] increased immediately following the high (42%, $p<0.05$), but not low intensity trial, with a difference between trials (Fig. 4.2C; $p<0.05$). Phosphorylation of CREB (Fig. 4.2D) was unaltered immediately or 3 h after exercise, but tended to increase after 19 h of recovery. Phosphorylation of the CaMKII substrates phospholamban and SRF was unaltered either immediately after exercise or in the recovery period (data not shown).

4.3.3 Gene expression

PGC-1α mRNA expression increased in response to the exercise protocols in a manner analogous to the intracellular signalling cascades (Fig. 4.3A). PGC-1α mRNA was elevated by 3.8- and 10.2-fold 3 h after LO and HI, respectively ($p<0.05$), with an effect of exercise intensity noted between trials ($p<0.05$). ERRα mRNA expression was unaltered at any time point (Fig. 4.3E). Expression of FOXO1A mRNA (Fig. 4.3B) increased immediately and 3 h after both the LO and HI trials ($p<0.05$). FOXO1A mRNA was greater 3 h following the high intensity compared to the low intensity trial ($p<0.05$). Expression of PDK4 mRNA (Fig. 4.3C) was increased 3 h after exercise ($p<0.05$), with a similar effect observed between trials.

Exercise did not change the expression of a broad range of transcription factors (NRF-1 and NRF-2), nuclear-encoded mitochondrial genes (COXIV, CPT1, UCP3) or genes related to glucose transporter expression (GLUT4, MEF2A, MEF2D, GEF; Table 4.3). However, an intensity-dependent difference in the expression of PPARδ mRNA was observed 3 h following exercise. Interestingly, exercise increased the expression of RIP140 mRNA, both immediately ($p<0.05$) and 3 h after exercise ($p<0.05$). There was no effect of exercise intensity on this pattern of expression (Fig. 4.3D).

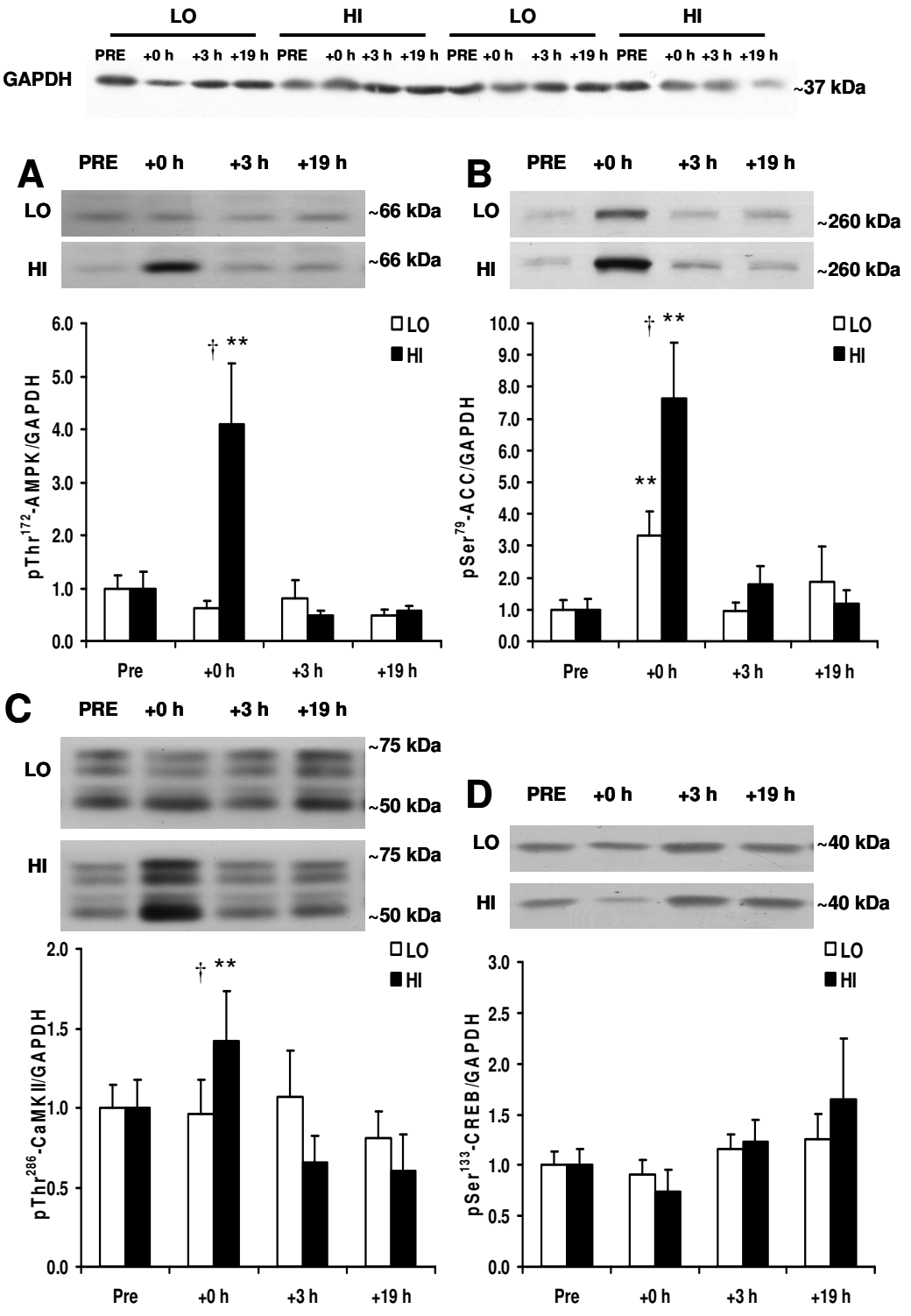

Figure 4.2. Protein phosphorylation of (A) AMPK, (B) ACCβ, (C) CaMKII and (D) CREB, normalised to GAPDH protein content. Representative blots for each protein are included. Values are mean±SE. ** significantly different to Pre ($p<0.05$); † significant difference between trials ($p<0.05$).

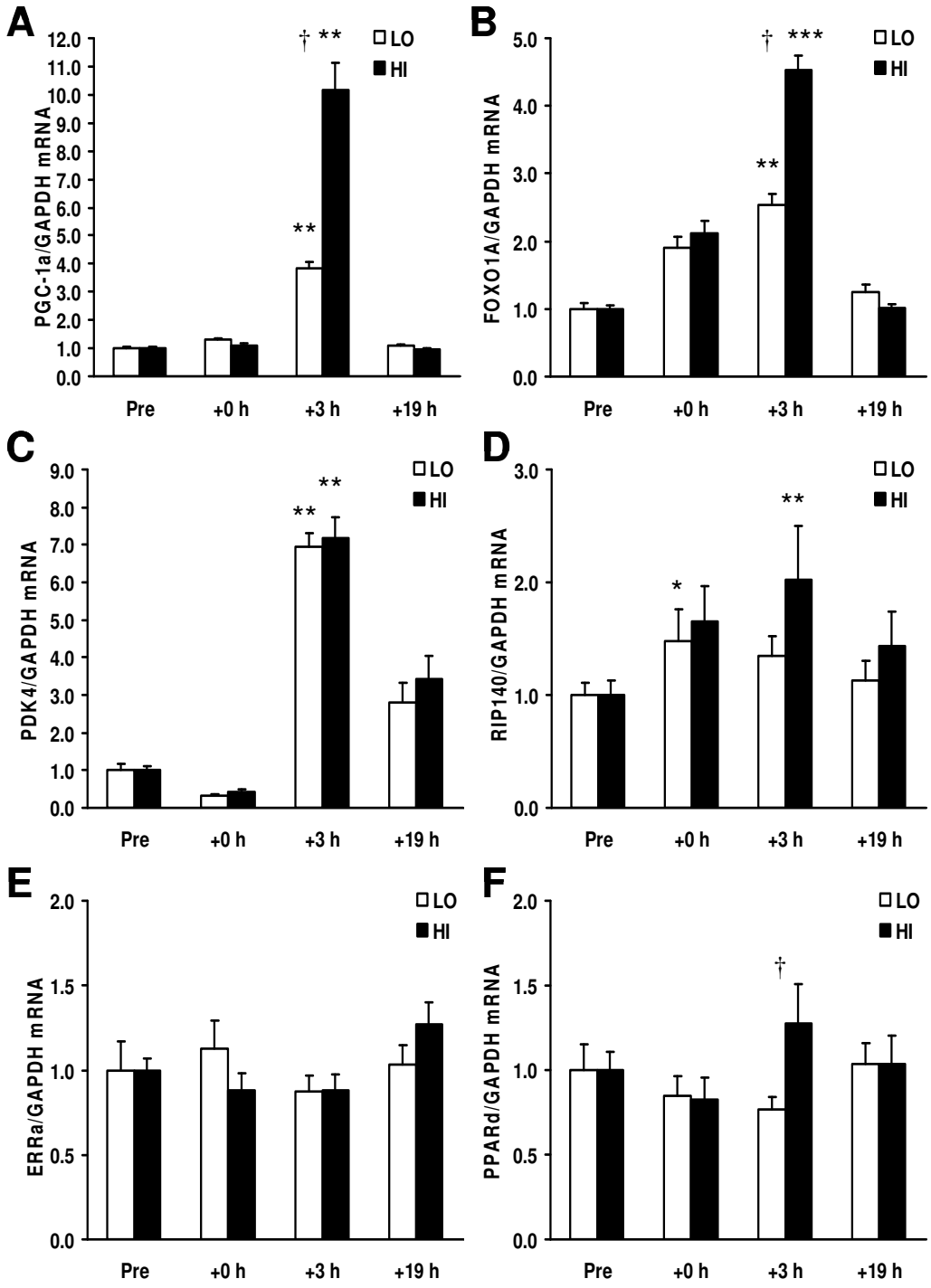

Figure 4.3. mRNA expression of selected transcriptional regulators and metabolic genes, (A) PGC-1α, (B) FOXO1A, (C) PDK4, (D) RIP140, (E) ERRα, and (F) PPARδ. Values are mean±SE, normalised to GAPDH mRNA. *** significantly different to Pre ($p<0.001$); ** significantly different to Pre ($p<0.05$); * significantly different to Pre ($p<0.1$); † significant difference between trials ($p<0.05$).

Table 4.3. mRNA expression of selected transcription factors, nuclear-encoded mitochondrial genes and metabolic genes unaltered by exercise. Values are mean±SE, normalised to the housekeeper gene GAPDH.

	Pre		+ 0 h		+ 3 h		+ 19 h	
	LO	*HI*	*LO*	*HI*	*LO*	*HI*	*LO*	*HI*
NRF-1	1.21±0.30	1.22±0.24	0.99±0.10	1.37±0.21	1.09±0.11	1.40±0.12	0.98±0.06	1.72±0.19
NRF-2	0.97±0.07	1.12±0.05	0.90±0.08	1.29±0.17	0.90±0.04	1.32±0.18	1.23±0.19	1.17±0.12
COXIV	1.17±0.19	1.26±0.15	1.16±0.14	1.03±0.20	1.08±0.12	1.10±0.22	1.20±0.18	1.18±0.20
CPT1	1.23±0.26	1.21±0.13	1.14±0.16	0.98±0.15	0.99±0.16	0.93±0.20	1.11±0.20	1.16±0.24
UCP3	1.01±0.20	0.98±0.21	0.61±0.09	0.57±0.13	1.12±0.14	1.34±0.29	1.19±0.16	1.23±0.15
GLUT4	0.96±0.11	1.13±0.16	1.15±0.14	1.04±0.16	0.95±0.11	0.98±0.15	1.19±0.20	1.23±0.21
MEF2A	0.89±0.14	1.12±0.14	1.01±0.16	1.30±0.29	1.08±0.19	1.18±0.20	1.26±0.17	0.84±0.13
MEF2D	1.02±0.10	1.08±0.08	1.32±0.09	1.14±0.11	0.99±0.06	1.00±0.09	1.14±0.12	0.99±0.13
GEF	1.23±0.14	1.06±0.13	1.20±0.10	1.05±0.19	0.92±0.06	0.89±0.14	0.86±0.10	1.08±0.10

4.4 Discussion

We tested the hypothesis that altering the intensity of exercise can engage distinct signalling cascades in active skeletal muscle, which may in turn differentially regulate gene expression in response to an isocaloric exercise bout. We show that PGC-1α expression is increased in an intensity-dependent manner following acute exercise. This may be partly mediated by an exercise intensity-dependent differential activation of AMPK and calcium-dependent signalling cascades. The increase in FOXO1A and PDK4 mRNA during recovery from exercise is consistent with a previous report (Mahoney *et al*., 2005) and may be part of a transcriptional response initiated with the goal of reestablishing metabolic homeostasis through increased glucose sparing and promoting a shift toward lipid oxidation. We also report the novel finding that the mRNA expression of RIP140, a transcription factor corepressor that serves mutually antagonistic functions to PGC-1α in the regulation of mitochondrial activity and fibre type composition (Seth *et al*., 2007), is increased immediately after acute exercise.

AMPK phosphorylation increased following exercise at 80% VO_{2peak}, but not at 40% VO_{2peak} (Fig. 4.2A). Low intensity exercise increases ATP turnover, but not necessarily the AMP/ATP ratio (Howlett *et al*., 1998), unlike high intensity exercise where the rates of energy expenditure and glycogen utilization were higher (Table 4.2) and the AMP/ATP ratio is expected to increase. However, low intensity exercise may transiently activate AMPK, as ACCβ phosphorylation was increased following both exercise trials (Fig 4.2B). ACCβ phosphorylation is considered a sensitive indicator of skeletal muscle AMPK activity *in vivo* (Park *et al*., 2002;Chen *et al*., 2003). An increase in ACC phosphorylation in the absence of an increase in AMPK phosphorylation,

as seen in the low intensity trial, has been previously reported (Chen *et al.*, 2003;Sriwijitkamol *et al.*, 2007). Despite the absence of an increase in AMPK phosphorylation in LO, ACC phosphorylation was increased 3.6-fold above resting values suggesting that there may have been transient activation of AMPK during LO that wasn't measurable at the cessation of exercise. Consequently, the increase in ACCβ phosphorylation was intensity-dependent (Fig. 4.2B). ACCβ phosphorylation suppresses malonyl CoA formation and promotes lipid oxidation (Hardie & Hawley, 2001), but the precise role for AMPK in contraction-mediated regulation of metabolic gene expression is incompletely understood. AMPKα_2 knockout mice have a similar exercise-induced increase in PGC-1α, FOXO1A and PDK4 mRNA to wild-type littermates, but this effect is suppressed in the knockout mice following AICAR stimulation (Jorgensen *et al.*, 2005), suggesting AMPK is not the sole mediator of exercise-induced gene transcription.

Calcium flux is rapid and transient during muscle contraction, leading to signal transduction via activation of a calcium-dependent cascade believed to act through CaMKII in human skeletal muscle (Rose & Hargreaves, 2003;Chin, 2005). CaMKII activation during exercise is rapid and sustained (Rose & Hargreaves, 2003); it increases in an intensity-dependent manner and correlates with autonomous activity (Rose & Hargreaves, 2003;Rose *et al.*, 2006;Rose *et al.*, 2007a). Our results demonstrate a similar intensity-dependent effect on CaMKII phosphorylation (Fig. 4.2C). However, the rapid and transient calcium oscillations during repeated contraction limit the delineation of this signalling cascade in exercising human skeletal muscle despite its putative role in metabolism (Wright *et al.*, 2004) and gene transcription (Ojuka, 2004;Chin, 2005). However, the pattern of CaMKII phosphorylation, autonomous activity, and phosphorylation of downstream substrates, is not always consistent (Rose *et al.*, 2006;Rose *et al.*, 2007b). Additionally, we did not observe an increase in phosphorylation of the putative CaMKII targets CREB (Fig. 4.2D), phospholamban and SRF (data not shown), which have been reported to increase during exercise (Rose *et al.*, 2006).

PGC-1α expression increased in an intensity-dependent manner following isocaloric exercise (Fig. 4.3A). We propose a permissive role for AMPK and CaMKII signalling cascades in this phenomenon. The importance of these signalling pathways in muscle metabolism (Wright *et al.*, 2004) and initiating PGC-1α-dependent transcriptional programmes is well established (Wu *et al.*, 2002;Zong *et al.*, 2002). The transcriptional response to a single bout of exercise is linked to metabolic recovery and adaptation such as increased lipid oxidation, glucose sparing and glycogen synthesis during recovery from exercise (Mahoney *et al.*, 2005). FOXO1A positively regulates fat metabolism and glycogen sparing through induction of LPL and PDK4 expression (Kamei *et al.*, 2003;Furuyama *et al.*, 2003), and influences PGC-1α expression (Kamei *et al.*, 2004). In transgenic mice overexpressing muscle-specific FOXO1A, PGC-1α mRNA levels are increased at rest suggesting that FOXO1A promotes PGC-1α gene expression in muscle (Kamei *et al.*, 2004). The observed increase in PDK4 and FOXO1A expression following an acute bout of exercise is supported by other studies (Pilegaard *et al.*, 2003;Russell *et al.*, 2005;Mahoney *et al.*, 2005). FOXO1A expression was increased in an intensity-dependent manner immediately and 3 h after exercise similarly to the temporal pattern of PGC-1α and

PDK4 expression. Thus, FOXO1A may have important roles in the regulation of substrate utilization and the selected expression of PGC-1α target genes in an exercise context.

Surprisingly, the effect of exercise intensity on skeletal muscle gene expression is poorly described. In human skeletal muscle, Kraniou et al. (2006) have shown no difference between GLUT4 mRNA induction whether 400 kcal of exercise was performed at either 40% or 80% VO_{2peak}. However, Sriwijitkamol et al. (2007) have shown a tendency for higher PGC-1α and NRF-1 mRNA after 40 min at 70% VO_{2peak} compared to 50% VO_{2peak}. These results could be potentially confounded by the differences in total work completed. In addition, data from animal studies (Hildebrandt *et al.*, 2003;Terada *et al.*, 2005) has shown a tendency for an intensity-dependent effect on mRNA and protein expression. Both the intensity and duration of exercise influence the magnitude of the increase in HKII, PDK4, UCP3 and LPL transcription during recovery (Hildebrandt *et al.*, 2003). In terms of PGC-1α protein expression, Terada et al. (2005) compared responses to high (14x20 s with 14% BM attached) and low (2x3 h with 45 min rest) intensity swimming exercise in rats. Although actual energy expenditure during exercise was not controlled for or quantified, at 18 h after exercise PGC-1α protein content was increased by 67% in LO, whereas in the HI trial protein content increased by 126%. However, this trend did not result in a statistically significant difference between the trials, so it was concluded that the intensity of exercise does not affect the subsequent protein content of PGC-1α. Clearly the effect of modulating metabolic stress through exercise intensity and the molecular mechanisms underlying altered gene expression warrants further investigation.

Given the intensity-dependent effects on PGC-1α and FOXO1A, both of which target the PDK4 gene (Furuyama *et al.*, 2003;Wende *et al.*, 2005;Araki & Motojima, 2006), the lack of an intensity-dependent response for PDK4 expression was unexpected. The regulation of PDK4 mRNA in response to exercise has been linked to muscle glycogen content (Pilegaard *et al.*, 2005) and the faster rate of glycogen utilization during high intensity exercise would have been expected to elicit a greater response. PDK4 expression is increased during an 8 h fast with or without a bout of exercise (Vissing *et al.*, 2005), and this effect is diminished when carbohydrate is provided during recovery (Pilegaard *et al.*, 2005). In addition, in humans fasted for 48 h, FOXO1A mRNA, total protein expression and Ser^{256} phosphorylation were unaltered despite marked changes in whole body metabolism and the expression of PDK4 (Tsintzas *et al.*, 2006), which suggests that the role of FOXO1A in regulating PDK4 is transient or lacks importance in the context of human skeletal muscle.

Alternatively, the expression of the orphan nuclear receptor ERRα is regulated by PGC-1α (Schreiber *et al.*, 2003) and is necessary for PDK4 expression (Wende *et al.*, 2005;Araki & Motojima, 2006), in addition to PGC-1α-induced mitochondrial biogenesis (Schreiber *et al.*, 2003;Schreiber *et al.*, 2004). ERRα is particularly important for the expression of genes regulating oxidative phosphorylation (Schreiber *et al.*, 2003), fatty acid oxidation (Huss *et al.*, 2004) and mitochondrial DNA expression (Schreiber *et al.*, 2004). ERRα may be a more important regulator of PDK4 expression than PGC-1α and the existing ERRα protein content

may be rate limiting. Moreover, PDK4 expression may be dependent on energy balance, rather than exercise itself, as the total energy expenditure was similar between trials.

Despite the 10.2-fold increase in PGC-1α mRNA expression, the mRNA expression of transcription factors, including NRF-1, NRF-2 and ERRα, or the nuclear-encoded mitochondrial proteins COXIV, CPT1 and UCP3 targeted by PGC-1α were unaltered. The exercise challenge may not have been sufficient to activate transcription of these genes as previous studies have generally used a higher intensity (Pilegaard *et al.*, 2005;Mahoney *et al.*, 2005) or longer duration of exercise (Russell *et al.*, 2005) resulting in greater total energy expenditure compared to the present study. Another possible explanation for the lack of observed change in PGC-1α-associated gene targets may be that the signal transduction pathways activated by exercise resulted in increases rates of PGC-1α transcription or mRNA stability but did not necessarily affect PGC-1α protein content or function. Although it has generally been assumed that the exercise-induced increase in PGC-1α expression mediates an increase in mitochondrial biogenesis, Wright et al. (2007b) hypothesise that the initial phase of the adaptive increase in mitochondrial biogenesis induced by exercise is mediated by activation of existing PGC-1α protein rather than by the increase in PGC-1α expression. DNA binding of NRF-1 and NRF-2 to response elements on the cytochrome c and COXIV promoters, respectively, was markedly increased in response to exercise and occurred prior to an increase in PGC-1α protein (Wright *et al.*, 2007b). NRF-1 and NRF-2 DNA binding activities are regulated by coactivation by PGC-1α (Wu *et al.*, 1999;Mootha *et al.*, 2004). In addition, mRNA and protein levels of a number of mitochondrial constituents were increased prior to the increase in PGC-1α protein. Therefore, rather than an increase in PGC-1α mRNA being indicative of enhanced PGC-1α activity, measurement of the functional activity of existing PGC-1α protein and its associated transcription factors is necessary. Thus, while AMPK activation and increased calcium-dependent signalling are known to acutely upregulate PGC-1α mRNA expression (Jager *et al.*, 2007;Wright *et al.*, 2007a), their effects on its function as a transcriptional coactivator *in vivo* in the context of exercising skeletal muscle are not conclusively established. Together with the absence of an increase in ERRα, NRF-1 and NRF-2 mRNA, the nature of the present exercise challenge may have been insufficient to stimulate changes in the functional activity of these transcription factors. This would explain why changes in the expression of nuclear-encoded mitochondrial proteins were not observed.

A novel observation of this study was the exercise-induced increase in RIP140 mRNA abundance. This corepressor specifically targets metabolic gene networks and has common nuclear receptor targets as PGC-1α, including ERRα (Powelka *et al.*, 2006;Seth *et al.*, 2007). RIP140 deletion is associated with an increase in TCA enzymes, oxidative phosphorylation and mitochondrial biogenesis (Powelka *et al.*, 2006;Seth *et al.*, 2007). Despite the increase in PGC-1α expression, RIP140 may have repressed ERRα or other nuclear receptors and prevented the expression of related genes. We observed an exercise- but not intensity-dependent change in the expression of RIP140 mRNA (Fig. 4.3D), which suggests that RIP140 may play some role in the recovery or adaptation to a single bout of exercise. RIP140 has a repressor domain with

CtBP interaction motifs (White *et al.*, 2008). CtBP is an NAD^+ and NADH binding dehydrogenase, whose repressor function is increased during hypoxia by decreasing the NAD^+/NADH ratio (Zhang *et al.*, 2002). Exercise induces a similar redox response by increasing NADH (Sahlin *et al.*, 1987) or decreasing NAD^+ (Graham *et al.*, 1978) and would therefore be a plausible stimulus for RIP140-mediated corepression. The subsequent increase in CtBP interaction with RIP140 would increase the repression of ERRα and mitochondrial biogenesis (Christian *et al.*, 2006). The balance between PGC-1α coactivation and RIP140 corepression on gene expression and mitochondrial biogenesis during recovery from exercise remains to be determined.

In conclusion, we report an exercise intensity-dependent increase in PGC-1α mRNA abundance that follows the activation pattern of the AMPK and CaMKII signalling cascades in human skeletal muscle. Despite the increase in PGC-1α mRNA abundance, PGC-1α target genes encoding mitochondrial proteins were unaltered. Increased RIP140 expression after exercise suggests that this corepressor potentially plays some role in exercise-induced alterations in gene expression during recovery. Exercise-induced increases in PDK4 and FOXO1A mRNA abundance may form part of a transcriptional response that contributes to exercise-induced alterations in skeletal muscle metabolism including glucose sparing and increased fat oxidation. Our results suggest that the early adaptive transcriptional response to a single bout of exercise is modulated by the intensity of the preceding exercise bout.

Chapter V The effect of glycogen-depleting exercise on contraction-induced signalling and gene expression of metabolic genes and transcriptional regulators in well-trained human skeletal muscle

5.1 Introduction

The transcriptome response to a single bout exercise includes an upregulation of mRNAs involved in carbohydrate and lipid metabolism, oxidative phosphorylation and mitochondrial biogenesis (Pilegaard *et al*., 2000;Pilegaard *et al*., 2003;Russell *et al*., 2005;Mahoney *et al*., 2005). Enhanced steady-state levels of gene transcripts due to repeated exercise bouts support the synthesis of protein components and provoke structural remodelling and functional adjustments in the long term (Fluck, 2006). For example, endurance-trained athletes with approximately two-fold higher VO_{2peak} and muscle mitochondrial volume compared to sedentary individuals have elevated mRNA concentrations for genes encoding enzymes involved in oxidative phosphorylation (Puntschart *et al*., 1995). These elevations are in direct proportion to the higher mitochondrial content of the muscle. Therefore, regulation of gene expression at the transcriptional level is accepted as a key process in understanding the overall adaptive response to exercise.

Training history is known to modulate the expression of mRNA in response to a single bout of exercise. Recently, it has been shown that short-term training elevates the resting steady-state level of mRNA for certain gene targets (Tunstall *et al*., 2002;Schmutz *et al*., 2006). In addition, there is accumulating evidence that the transcriptional response of metabolic factors may be different in trained compared to untrained muscle (Tunstall *et al*., 2002;Pilegaard *et al*., 2003;Fischer *et al*., 2004;Lundby *et al*., 2006;Schmutz *et al*., 2006) or depending on the nature of exercise challenge relative to the training history (Coffey *et al*., 2006a). After a period of training, attenuated responsiveness of transcript levels to a single bout of endurance exercise has been reported (Fischer *et al*., 2004;Lundby *et al*., 2006;Schmutz *et al*., 2006). Both functional and structural adaptations are attenuated with ongoing training with the same relative intensity, which may be attributed to the attenuated transcriptional response in the trained state (Schmutz *et al*., 2006). However, the induction of certain genes during acute exercise prior to training may not necessarily be involved in the adaptive response to repeated exercise but merely reflect the nature of the stresses placed on the muscle (Choi *et al*., 2005;Keller *et al*., 2007). Similarly, it has been suggested that some genes are involved in the restoration of homeostasis and therefore are induced during recovery from exercise, whereas other acutely responsive genes are not involved in the molecular basis of adaptation and hence are not responsive to exercise training (Mahoney *et al*., 2005).

The metabolic and mitochondrial adaptations to regular exercise are under the control of an increasingly well-defined subset of transcription factors and their coregulators. As described in earlier chapters, transcriptional regulators of metabolism in skeletal muscle include PGC-1α, NRF-1, NRF-2, ERRα, FOXO1A, and RIP140, which subsequently coordinate of alterations the expression of genes involved in carbohydrate and lipid metabolism, oxidative phosphorylation and mitochondrial biogenesis (Handschin & Spiegelman, 2006;Freyssenet, 2007;Feige & Auwerx, 2007). These factors, apart from being crucial to the regulation of gene expression in response to cellular signals, can actually be primary targets for signal transduction pathways

(Gerhart-Hines *et al*., 2007;Jager *et al*., 2007). The contraction-induced activation of signal transduction pathways regulating skeletal muscle gene expression is modulated after a period of regular exercise training (McConell *et al*., 2005;Rose *et al*., 2007b) or depending on training status of the muscle (Yu *et al*., 2003). In general terms, at the same relative intensity of exercise, the activation of contraction-induced signalling pathways is reduced in response to training (Yu *et al*., 2003;McConell *et al*., 2005). This may be mediated by a reduction in the metabolic stress of subsequent exercise and/or an increase in the sensitivity of the energy sensing molecules to variations in energy homeostasis (Freyssenet, 2007). A blunted contraction-induced signalling response to acute exercise may in part explain the reduced transcriptional response to acute exercise observed in trained skeletal muscle.

The purpose of this study was to examine the effect of acute exercise on the mRNA abundance of transcription factors and transcriptional coregulators in well-trained human skeletal muscle. As it has been reported that the signalling response to acute exercise in trained muscle may require a greater stimulus to elicit similar activation (Yu *et al*., 2003) and the transcriptional response is greater when muscle glycogen is lower (Pilegaard *et al*., 2002;Pilegaard *et al*., 2005), we chose a 90 minute bout of cycle exercise expected to deplete muscle glycogen. Given the severity of the exercise stimulus, we hypothesised that the induced mRNA expression of selected gene targets would be qualitatively similar to those reported in untrained muscle.

5.2 Materials and methods

5.2.1 Experimental design

Eight healthy, well-trained males volunteered to participate in the study (Table 5.1). Preliminary screening, the test for maximal oxygen uptake and verification of the power output required to elicit the desired exercise intensity were performed as described in chapter three. The experimental trial (Figure 5.1) took place at least seven days after the verification test and consisted of 90 min of cycling at 75% VO_{2peak}, followed by one hour of recovery with minimal ambulation i.e. lying supine. To preclude the influence of circadian variation on any experimental variable, all experimental trials began between 0730 and 0930. Subjects were asked to abstain from caffeine and alcohol and refrain from physical activity of any nature for 24 h prior to testing. Sweetened water (15 ml per kg BM) was provided immediately after exercise. On the day of the experimental trial, participants arrived at the Metabolic Physiology Research Unit after an overnight fast (>8 h) were requested to rest quietly in a supine position for approximately ten minutes. A resting muscle biopsy was taken (#1). Exercise began 15 minutes after the first biopsy. Cadence was maintained between 75-80 rpm throughout. Expired air was analysed for the first 15 min of exercise, between 35-45 min and again between 75-85 min to verify exercise intensity. Approximately 20 min before the end of exercise, subjects briefly paused cycling while local anaesthetic was administered into the m. *vastus lateralis*. Exercise was continued within 2 min. Immediately at the end of exercise, a muscle biopsy (#2) was obtained from the anaesthetized area. A final muscle biopsy (#3) was taken 1 h after exercise.

Each biopsy was taken from the same leg but each site was at least 3 cm distal or proximal to the previous biopsy site.

Table 5.1. Physical characteristics of participants

	n=8
Age (yr)	23.4±1.7
Height (m)	1.84±2.4
Mass (kg)	78.8±2.8
BMI ($kg \cdot m^{-2}$)	23.2±0.5
Body fat (%)	9.4±1.1
VO_{2peak} ($L \cdot min^{-1}$)	4.57±0.10

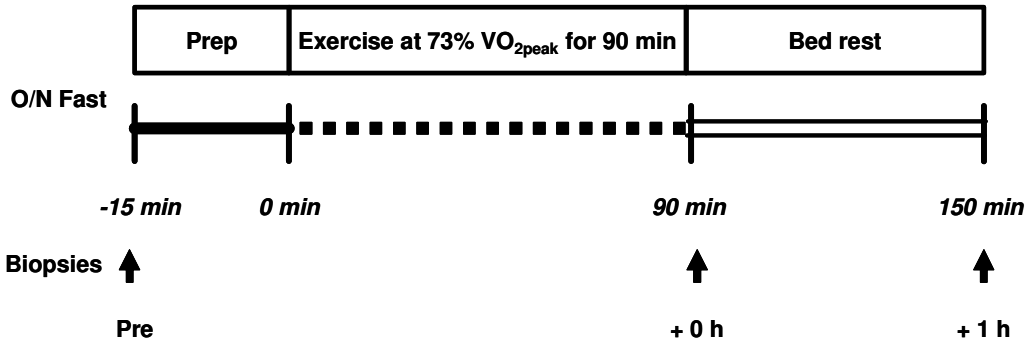

Figure 5.1. Schematic of experimental design

5.2.2 Statistical analysis

One-way ANOVA, with post-hoc pair-wise comparisons using Fisher's least significant difference, was used determine the effect of exercise on the variables of interest.

5.3 Results

5.3.1 Energy expenditure and substrate utilisation

Average total energy expenditure for 90 min of exercise was 1439 ± 33 kcal (Table 5.2). The percentage contribution of CHO and fat to total energy expenditure during exercise was 73.0 ±

2.8% and 27.0 ± 2.8%, respectively. Muscle glycogen content was decreased by exercise by 76% (149.6 ± 10.9 vs. 36.3 ± 12.1 mmol•kg dry weight^{-1}, $p<0.05$; Fig. 5.2).

Table 5.2. Energy expenditure, substrate utilization and metabolic variables during exercise

Total EE (kcal)	1439±33
Rate of EE (kcal•min^{-1})	16.0±0.4
Exercise intensity (%VO_{2peak})	72.5±1.8
Exercise time (min)	90
RER	0.92±0.01
CHO oxidation rate (g•min^{-1})	2.9±0.1
Total carbohydrate oxidized (g)	261±9
Rate of fat oxidation (g•min^{-1})	0.49±0.05
Total fat oxidized (g)	44.1±4.5
Rate of glycogen utilization (mmol•kg^{-1} dw•min^{-1})	1.26±0.12
Plasma lactate at termination (mM)	2.92±0.38

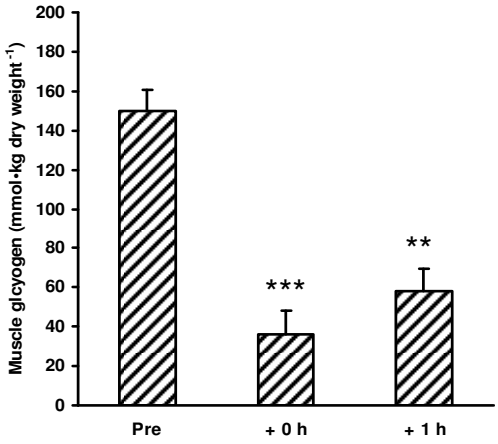

Figure 5.2 Muscle glycogen concentrations. Values are mean±SE. *** significantly different from Pre ($p<0.001$); ** significantly different from Pre ($p<0.05$).

5.3.2 Contraction-activated signalling cascades

ACCβ phosphorylation, as a surrogate marker of AMPK activity, was increased ($p<0.001$) both immediately after exercise and after 1 h of recovery (Fig. 5.3). Exercise resulted in a 5.8- and 6.4-fold elevation in phosphorylated ACCβ protein immediately after and 1 h after exercise, respectively.

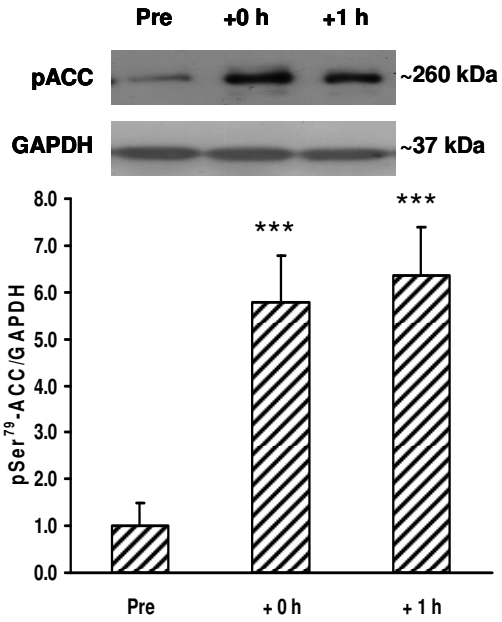

Figure 5.3 Phosphorylated ACCβ protein, normalised to GAPDH protein content. Representative blots for each protein are included. Values are mean±SE. *** significantly different from Pre ($p<0.001$).

5.3.3 Gene expression

No increase in mRNA expression for any gene target was observed immediately after exercise (Fig. 5.4). Expression of ERRα mRNA was reduced by 34% ($p<0.05$) immediately after exercise but returned to a value similar to pre-exercise values after 1 h of recovery (Fig. 5.4E). PGC-1α mRNA expression in response to exercise increased by 4.0-fold after 1 h of recovery ($p<0.001$; Fig. 5.4A). Similarly, FOXO1A mRNA was elevated 3.9-fold 1 h after exercise ($p<0.05$; Fig. 5.4B). The expression of PDK4 mRNA was increased by 11.1-fold at the same time point ($p<0.05$; Fig. 5.4C). Sirt1 mRNA was increased by 55% ($p<0.05$), whereas RIP140 tended to increase (49%, $p<0.1$), both at 1 h after cessation of exercise (Fig. 5.4F & D, respectively). The expression of NRF-1, NRF-2, CREB and GLUT4 mRNA was unaffected by exercise at the time points investigated (Table 5.3).

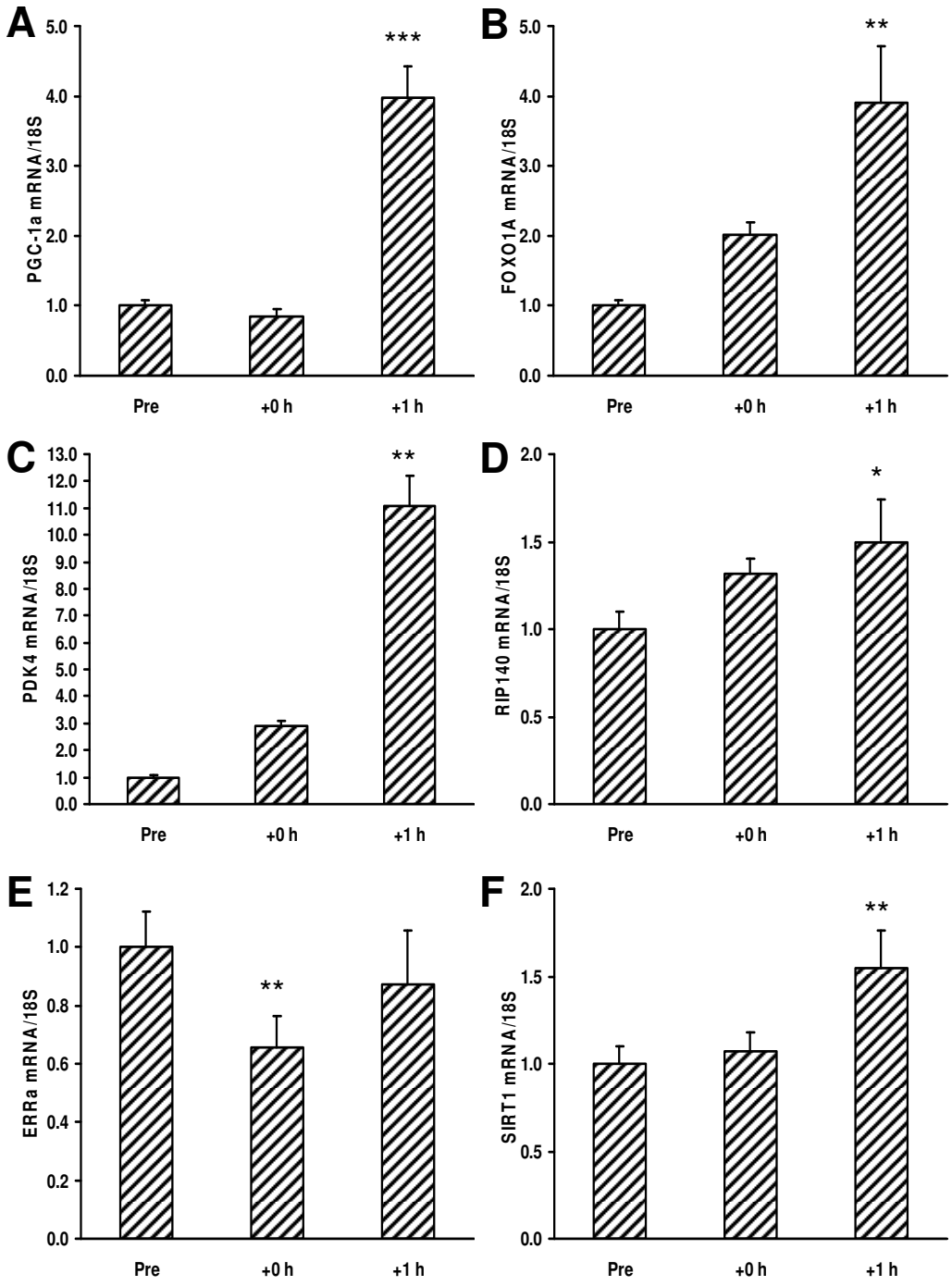

Figure 5.4. mRNA expression of selected transcriptional regulators and metabolic genes, (A) PGC-1α, (B) FOXO1A, (C) PDK4, (D) RIP140, (E) ERRα, and (F) Sirt1. Values are mean±SE, normalised to 18S rRNA. *** significantly different to Pre ($p<0.001$); ** significantly different to Pre ($p<0.05$); * significantly different to Pre ($p<0.1$).

Table 5.3. mRNA expression of selected gene targets unaltered by exercise. Values are mean±SE, normalised to the housekeeper gene 18S.

	Pre	+ 0 h	+ 1 h
NRF-1	0.94±0.15	0.71±0.08	1.11±0.28
NRF-2	0.74±0.12	0.89±0.11	0.87±0.23
CREB	0.87±0.14	0.76±0.10	1.08±0.26
GLUT4	1.00±0.21	0.67±0.13	1.03±0.23

5.4 Discussion

Activation of contraction-induced signal transduction pathways and elevations in mRNA of selected genes after a single bout of exercise are reduced after a period of regular exercise training (McConell *et al*., 2005;Schmutz *et al*., 2006). We demonstrate that acute exercise of a sufficient stimulus induces qualitatively similar signalling and transcriptional responses to those observed in untrained muscle. Acute exercise-induced activation of AMPK-dependent signalling and increased mRNA expression of transcriptional (PGC-1α, FOXO1A) and metabolic (PDK4) regulators is conserved in muscle of men with a prolonged history of training. In addition, as we have previously shown in untrained muscle, acute exercise induces an increase in mRNA expression of the transcriptional corepressor RIP140. Finally, we are the first to report that the mRNA abundance of Sirt1, a class III HDAC and known regulator of PGC-1α-associated transcriptional regulation, is increased during recovery from exercise.

AMPK is a key regulator of contraction-induced alterations in gene expression (Jorgensen *et al*., 2006). Pharmacological activation of AMPK induces similar effects to chronic exercise, resulting in increased expression of genes encoding mitochondrial and metabolic proteins and promoting mitochondrial biogenesis through PGC-1α-dependent pathways (Bergeron *et al*., 2001;Zong *et al*., 2002). Due to technical limitations, we were unable to measure AMPK phosphorylation in this experiment. However, exercise resulted in an approximately six-fold increase ACCβ phosphorylation that was present immediately after exercise and remained up to 1 h into recovery (Fig 5.3). We conclude that the magnitude of this increase represents a significant increase in AMPK activity based on several lines of evidence. Firstly, although small (less than two-fold) but significant increases in ACCβ phosphorylation have been previously reported coincident with unaltered AMPK activity, this has been attributed to transient fluctuations in AMPK activity and the fact the ACCβ phosphorylation is an extremely sensitive indicator of relative AMPK activity (Park *et al*., 2002;Chen *et al*., 2003;Sriwijitkamol *et al*., 2007). Secondly, AMPK activity has been reported to be ablated in response to acute exercise after a period of training when exercise is conducted at the same absolute exercise intensity as prior to training (McConell *et al*., 2005). However, when trained and untrained men exercise at approximately

the same relative intensity, AMPK activity is attenuated in trained muscle compared to untrained but is still significantly elevated above basal values (Yu *et al*., 2003;Nielsen *et al*., 2003). Finally, the reduction in AMPK activity in trained muscle is in part explained by a reduction in the degree of muscle energy imbalance (McConell *et al*., 2005). After a period of exercise training, the same cellular rate of mitochondrial oxidative phosphorylation is attained with a lower rate of oxidative phosphorylation per mitochondrion for any absolute workload in muscle fibres with elevated mitochondrial content (Dudley *et al*., 1987). ATP and creatine phosphate concentrations decrease to a smaller degree, and the free ADP concentration increases to a lesser extent in cells with elevated mitochondrial contents at the same absolute work rate (Dudley *et al*., 1987;Green *et al*., 1991). Better preservation of free ADP lowers the rate of AMP formation, ultimately decreasing the activation of AMPK and its upstream activators. However, in well-trained men, a similar, but shorter (60 min), exercise protocol has previously been shown to activate AMPK despite PCr and ADP concentrations and the AMP/ATP ratio being unchanged (Wojtaszewski *et al*., 2003).

A single bout of exercise is known to transiently activate transcription and increase the mRNA abundance of many metabolic genes and transcription factors during recovery (Pilegaard *et al*., 2003;Russell *et al*., 2005). The transcriptional response to a single bout of exercise in the untrained state is linked to metabolic recovery and adaptation such as increased lipid oxidation, glucose sparing and glycogen synthesis during recovery from exercise (Mahoney *et al*., 2005). Exercise training-induced skeletal muscle adaptation is mediated by the accumulation of repeated transient elevations in mRNA (Fluck, 2004). However, the transcriptional response to a single bout of exercise in the trained state is less well described. In the present study, the increased expression of selected gene targets was qualitatively similar to those reported in untrained muscle in experiment I. mRNA abundance of PGC-1α, FOXO1A and PDK4 was markedly and rapidly elevated in response to a single bout of exercise (Fig. 5.4). The induction of FOXO1A and PDK4 is consistent with the initiation of a transcriptional response encoding proteins that may serve to reestablish metabolic homeostasis through coordinated changes in substrate metabolism (Mahoney *et al*., 2005). FOXO1A is known to positively regulate fat metabolism and glucose sparing through the induction of LPL and PDK4 gene transcription (Kamei *et al*., 2003;Furuyama *et al*., 2003) and fat transport by enhancing FAT/CD36 expression in the plasma membrane (Bastie *et al*., 2005). FOXO1A binds directly to the PDK4 promoter (Kwon *et al*., 2004) and overexpression of inducible FOXO1A in C_2C_{12} cells increases PDK4 expression, leading to decreased pyruvate dehydrogenase activity and glycolytic flux (Bastie *et al*., 2005). The pyruvate dehydrogenase complex is the key step regulating the complete oxidation of glucose proceeding from glycolysis to the Kreb cycle. Inactivation of this complex by PDK4 shuts down the conversion of pyruvate to acetyl CoA, resulting in allosteric inhibition of glycolysis and suppression of glucose oxidation leading to the diversion of imported glucose to storage (Sugden & Holness, 2006). PGC-1α-associated induction of PDK4 expression is mediated by the activity of ERRα and FOXO1A (Furuyama *et al*., 2003;Wende *et al*., 2005;Araki & Motojima, 2006). Induction of ERRα and subsequent binding to the PDK4

gene promoter in a complex with PGC-1α is induced by PGC-1α and results in increased PDK4 expression, a pathway in which ERRα is indispensable (Wende *et al.*, 2005). Therefore it is likely that the upregulation of transcription of these genes by acute exercise is induced to initiate these processes.

Recently the relationship between the acute transcriptional response and nature of training adaptation has been questioned (Mahoney & Tarnopolsky, 2005;Timmons *et al.*, 2005b). Direct comparison between 'acute' and 'chronic' transcriptomes does not demonstrate a link between acute exercise responses and the chronic phenotype shift associated with endurance exercise training (Keller *et al.*, 2007). In addition, repeated exercise training reduces the magnitude of the increase in mRNA of certain genes in response to an acute exercise bout (Fischer *et al.*, 2004;Lundby *et al.*, 2006;Schmutz *et al.*, 2006). This has been attributed to elevated resting mRNA levels and increased sensitivity of the response (Schmutz *et al.*, 2006). However, Timmons et al. (2005) have reported that the cluster of mitochondrial and metabolic regulatory genes that are activated after a single bout of exercise are not elevated after training. The basal mRNA abundance of COXIV and LPL was increased indicating that training did result in quantitative adaptations in mitochondrial and lipid metabolism (Timmons *et al.*, 2005b). Moreover, the steady-state mRNA abundance of PDK4 and UCP3 was downregulated by training, despite the fact that these genes are robustly increased after a single bout of exercise (Pilegaard *et al.*, 2000;Schrauwen *et al.*, 2002;Pilegaard *et al.*, 2003;Mahoney *et al.*, 2005). Taken together, acutely responsive genes are involved in the restoration of homeostasis but, if not involved in the molecular basis of adaptation, are not responsive to exercise training (Mahoney & Tarnopolsky, 2005).

Support for this hypothesis has been shown by Cartoni et al. (2005), who have found marked increases in mRNA encoding regulators of mitochondrial biogenesis (PGC-1α, ERRα, NRF-2, COXIV) in the muscle of trained cyclists during recovery from a 10 km time-trial. This suggests that even in muscle with highly adapted mitochondrial phenotype (Wittwer *et al.*, 2004), a sufficient exercise stimulus can induce a transcriptional response designed to coordinate further alterations in mitochondrial phenotype. We have not observed an increase in mRNA for ERRα, NRF-1 or NRF-2, but it cannot be ruled out that an increase occurred outside of the time frame measured. ERRα and NRF-2 mRNA abundance has been previously shown to be elevated after 2 and 24 h of recovery, respectively (Cartoni *et al.*, 2005). In addition, it is not possible to ascertain the molecular mechanisms regulating this transcriptional response (e.g. DNA binding, posttranslational modifications), nor is it possible to definitively conclude that these alterations in mRNA are coupled to a functional metabolic response.

Recent evidence has implicated the nuclear receptor corepressor RIP140 in the regulation of metabolic homeostasis by acting on specific gene targets also regulated by the transcriptional coactivator PGC-1α (Christian *et al.*, 2006). Advances in the understanding of the regulation of PGC-1α in skeletal muscle metabolism have recently identified the class III HDAC, Sirt1, as a key regulator of PGC-1α functional activity by deacetylating PGC-1α protein (Lagouge *et al.*, 2006;Gerhart-Hines *et al.*, 2007). The effect of acute exercise on Sirt1 or RIP140 is unknown. In

the present study, a single bout of exercise resulted in an increase in both RIP140 and Sirt1 mRNA abundance after 1 h of recovery (Fig. 5.4). The functional implications for this finding are unknown at present but suggest that muscle contraction regulates Sirt1 and RIP140 expression at a transcriptional level, which may be manifested as an alteration in protein content with repeated bouts of training. The subsequent role in skeletal muscle metabolism or adaptation to acute exercise or chronic training remains to be investigated.

We have previously shown that the expression of RIP140 is increased immediately and 3 h after exercise in untrained men (experiment I). This corepressor specifically targets metabolic gene networks and has common nuclear receptor targets as PGC-1α, including ERRα (Powelka *et al*., 2006;Seth *et al*., 2007). RIP140 deletion is associated with an increase in TCA enzymes, oxidative phosphorylation and mitochondrial biogenesis (Powelka *et al*., 2006;Seth *et al*., 2007). Control of RIP140 repressive activity is largely under the control of posttranslational modifications including phosphorylation and acetylation (White *et al*., 2008). Phosphorylation of RIP140 results in greater HDAC recruitment and enhanced repression (Gupta *et al*., 2005), whereas acetylation of RIP140 prevents CtBP recruitment and relieves transcriptional repression (Vo *et al*., 2001). Therefore it is likely that cellular signal transduction cascades can regulate its activity but acute exercise-induced effects are entirely unexplored at this time.

Sirt1 is capable of direct physical interaction with PGC-1α, deacetylating the PGC-1α protein and increasing its transcriptional activity (Nemoto *et al*., 2005;Gerhart-Hines *et al*., 2007). Activation of Sirt1 by resveratrol treatment enhances both the whole-body aerobic capacity and the endurance running performance, with a concomitant increase in the mitochondrial oxidative capacity of the skeletal muscle (Lagouge *et al*., 2006). Ectopic PGC-1α expression increases ERRα expression and increases mitochondrial gene expression, including respiratory chain (cytochrome c, COXV) and lipid metabolism (MCAD, CPT1 and PDK4) genes, an effect that was largely prevented by knocking down Sirt1 (Gerhart-Hines *et al*., 2007). Given the importance of Sirt1 to PGC-1α-dependent transcriptional programmes, intuitively Sirt1 may play a role in exercise-mediated transcriptional regulation. This has yet to be confirmed, but we show that Sirt1 mRNA abundance is elevated during recovery from acute exercise. Only one previous study has examined the regulation of Sirt1 by exercise. In rodent muscle, acute exercise increased Sirt1 protein content by 18% 2 h after cessation of exercise, an effect that was not observed at 0, 1, 18 or 24 h after exercise (Suwa *et al*., 2008). In addition, two weeks of endurance training in the same animals increased Sirt1 protein content by 19% at rest. Although the effect of exercise on Sirt1 functional activity remain to be examined, this data and our results suggests that exercise modulates the expression of Sirt1, which may in turn play a role in exercise-mediated metabolic adaptations (Suwa *et al*., 2008).

The regulation of both Sirt1 and RIP140 activity is influenced by the cellular redox state via the NAD^+/NADH ratio (Rodgers *et al*., 2008;White *et al*., 2008). Fluctuations in this ratio are known to occur during exercise (Robergs *et al*., 2004) and are potential contraction-induced metabolic signals (Hawley & Zierath, 2004). This suggests that these factors can be modulated by contraction-induced mechanisms with possible downstream effects on gene expression

(Freyssenet, 2007). The repressive function of RIP140 is mediated by the presence of four repression domains which recruit HDACs leading to increased repression (Wei *et al.*, 2000). The recruitment of HDACs is mediated by the binding of CtBP, an NAD^+/NADH-dependent dehydrogenase. Therefore changes in cellular redox potential may affect RIP140 repressive activity mediated by CtBP acting as a sensor of redox state (White *et al.*, 2008). Sirt1 enzymatic activity is directly affected by fluctuations in NAD^+ as well as the ratio of NAD^+/NADH. Thus, the redox state of the cell, controlled by the activity of different metabolic pathways, modulates Sirt1 activity (Rodgers *et al.*, 2008). NAD^+/NADH ratio has also been reported to increase during submaximal and maximal exercise (Graham & Saltin, 1989), whereas Green et al. (1992) have shown a progressive decline in NAD^+/NADH ratio during 60 min of exercise at 67-76% VO_{2peak}.

A decrease in the NAD^+/NADH ratio increases CtBP-dependent repression (Zhang *et al.*, 2002), which suggesting that acute exercise would increase the repressive function of RIP140. Conversely, an increase in the NAD^+/NADH ratio increases Sirt1-mediated PGC-1α deacetylation (Lagouge *et al.*, 2006), which in theory would mean that acute exercise would increase the transcriptional activity of PGC-1α.The relationships between NAD^+/NADH ratio and these transcriptional regulators may be tissue- or stimulus-specific (Freyssenet, 2007). In addition, it may be that the NAD^+/NADH concentrations in the particular cellular compartments (i.e. cytosolic vs. mitochondrial redox) are more important in their regulation than those that have been routinely measured during exercise. Clearly, several lines of investigation are required to fully elucidate the potential influence of these novel regulators on exercise-mediated metabolic adaptation.

In summary, given a sufficient stimulus, the activation of AMPK and increase in mRNA abundance of exercise-responsive genes is conserved in well-trained skeletal muscle in response to a single bout of exercise. The exercise-induced increases in PGC-1α, FOXO1A and PDK4 are qualitatively similar to those seen in untrained muscle. This coordinated induction of several genes may form part of a transcriptional response that contributes to exercise-induced alterations in skeletal muscle metabolism including glucose sparing and increased fat oxidation in the post-exercise period (Kimber *et al.*, 2003). This is consistent with the coupling of the induction of a transcriptional response to exercise specifically to a functional metabolic demand. We also report that the mRNA abundance of the novel transcriptional regulators, RIP140 and Sirt1, is increased during recovery from exercise suggesting that these factors are exercise-responsive and may play a role in the acute and/or adaptive response to exercise.

Chapter VI The effect of fourteen consecutive days of exercise training on the expression of metabolic and mitochondrial genes and their transcriptional regulators in skeletal muscle of previously untrained men

6.1 Introduction

A single bout of exercise generates transient increases in mRNA abundance for a multitude of genes that typically peaks 3-12 h after cessation of exercise but generally returns to basal levels within 24 hours (Pilegaard *et al.*, 2000;Pilegaard *et al.*, 2003;Mahoney *et al.*, 2005;Yang *et al.*, 2005a;Schmutz *et al.*, 2006). These include coordinated induction of the expression of genes involved in carbohydrate metabolism (HKII, PDK4, GLUT4), lipid metabolism (CPT1, MCAD, LPL), oxidative phosphorylation (cytochrome c, COXIV) and mitochondrial biogenesis (Tfam). Accumulation of these transcripts with repeated exercise sessions may be the molecular mechanism underlying the functional adaptations to exercise training including proteins involved in mitochondrial ATP production (Holloszy, 1967), mobilisation, transport and oxidation of fatty acids (Bonen *et al.*, 1999), glucose transport and glycogen synthesis (Houmard *et al.*, 1995).

Comparison of highly-trained and sedentary individuals (Puntschart *et al.*, 1995;Schmitt *et al.*, 2003;Wittwer *et al.*, 2004), or longitudinal studies of previously untrained individuals commencing training (Tunstall *et al.*, 2002;Russell *et al.*, 2003;Short *et al.*, 2003;Schmutz *et al.*, 2006) support this contention. In trained athletes, elevations in transcript expression of proteins involved in lipid and mitochondrial metabolism are in proportion to the augmented mitochondrial volume density and elevated VO_{2peak} compared to untrained controls (Puntschart *et al.*, 1995;Schmitt *et al.*, 2003). Similarly, exercise training elevates steady-state mRNA abundance of genes encoding proteins involved in lipid transport and oxidation and mitochondrial metabolism when compared to pre-training levels (Tunstall *et al.*, 2002;Schmutz *et al.*, 2006).

Exercise training for as few as fourteen days results in qualitatively similar adaptations to those observed after more prolonged training. These include an increase in maximal oxygen uptake, and when exercise was performed at the same absolute intensity after training, smaller decreases in high-energy phosphates, a reduction in exercising RER, muscle glycogen utilisation and blood lactate accumulation, and concomitant increase in lipid utilisation was observed (Green *et al.*, 1991;Green *et al.*, 1992;Mendenhall *et al.*, 1994;Phillips *et al.*, 1996a). Spina et al. (1996) have shown that seven to ten days of training for 2 h/d at 60-70% VO_{2peak} is sufficient to induce mitochondrial adaptation, as measured by citrate synthase and β-HAD activity coincident with an increase in maximal oxygen consumption. In addition, molecular events underlying these training adaptations in this time frame are observed at mitochondrial (Spina *et al.*, 1996), metabolic (Green *et al.*, 1991), intracellular signalling (McConell *et al.*, 2005) and transcriptional (Pilegaard *et al.*, 2003) levels. The transient but robust increase in mRNA expression of transcription factors and transcriptional coregulators (e.g. PGC-1α, NRF-2, ERRα, FOXO1A) after acute exercise suggests a role in the adaptation to chronic exercise (Pilegaard *et al.*, 2003;Cartoni *et al.*, 2005;Mahoney *et al.*, 2005). However, the effect of exercise training on the expression of these factors during short-term training is not clearly defined. Six weeks of endurance training leads to steady-state elevations of mRNA encoding PGC-1α and PPARα (Russell *et al.*, 2003), an effect that was not present after nine days of training (Tunstall *et al.*, 2002). The transcriptional response to acute exercise therefore is likely

to stimulate mitochondrial biogenesis (Wright *et al.*, 2007b), but the accumulation of these transcripts or the time course of these changes remains to be confirmed.

The purpose of this study was firstly to examine whether proposed accumulation of mRNA underlying muscular adaptation after repeated bouts of exercise training (Fluck, 2006) is evident after fourteen days of training. We hypothesised that an increase in steady-state mRNA content of selected genes would be consistent with those that are reported to be transiently elevated after a single bout of exercise. The second purpose of this study was to examine the continuity between alterations in mRNA and translational changes in protein content during training. The final purpose of this study was to examine the time course of adaptation at both the transcriptional and translational level over the course of fourteen consecutive days of training.

6.2 Materials and methods

6.2.1 Experimental design

Eight healthy, sedentary males volunteered to participate in the study (Table 6.1). Preliminary screening, the test for maximal oxygen uptake and verification of the power output required to elicit the desired training intensity were performed as described in chapter three. Seven days after the test to verify the power output required for training, the training phase commenced. The experimental design (Fig. 6.1) consisted of participants cycling for 60 min per session at ~80%VO_{2peak} on fourteen consecutive days. Muscle biopsies were taken on the morning prior to the first training session, and on five other mornings throughout the training period. A test for maximal oxygen uptake was performed 48-72 hours after the last training session to measure training-induced changes in VO_{2peak}.

Table 6.1. Physical characteristics of participants. Values are mean±SE. *** significant difference to Pre-training ($p<0.001$)

	n=8
Age (yr)	23±2
Height (m)	1.79±0.03
Mass (kg)	75.3±3.0
BMI ($kg \cdot m^{-2}$)	23.6±0.9
Body fat (%)	13.3±2.2
Pre-training VO_{2peak} ($L \cdot min^{-1}$)	2.81±0.15
Post-training VO_{2peak} ($L \cdot min^{-1}$)	3.28±0.12***
Δ VO_{2peak} (%)	17.5±3.8

6.2.2 Training programme

The training phase consisted of fourteen exercise training sessions performed on fourteen consecutive days, beginning on day 0 (Fig. 6.1). Each training session was supervised and performed in the Human Performance Laboratory at Dublin City University. Each training session was 60 min in duration at an intensity equivalent to 80% of pre-training VO_{2peak}. Plain water was allowed for consumption *ad libitum* throughout each session. Training sessions for each individual took place at the same time of day ± 1 h to preclude any influence of circadian variation on the response to individual training sessions. Expired air was collected for 5 min on three occasions during each session; 15-20 min, 35-40 min, 55-60 min. From this, VO_2 was monitored as the measure of exercise intensity and the power output was adjusted accordingly to elicit the target exercise intensity. Heart rate (Polar Vantage NVTM; Polar, Port Washington, NY) and rating of perceived exertion (Borg, 1998) were recorded at 5 min intervals during exercise. The target oxygen uptake was increased by 10% after seven sessions to ensure the training stimulus increased commensurate with the expected increase in VO_{2peak}. Using similar protocols, Gulve & Spina (1995) and Spina et al. (1996) have shown 9-10% increases in VO_{2peak} after seven to ten days of cycle ergometer training.

6.2.3 Muscle biopsies

Six muscle biopsies were taken throughout the training programme. On the morning of day 0, participants reported the Metabolic Physiology Research Unit after an overnight (>8 h) fast and were requested to rest quietly in a supine position for approximately 15 min. A resting muscle biopsy was taken (#1, baseline, day 0). On the morning of day 1, participants reported again after an overnight fast and rested quietly as before. A second muscle biopsy was taken (#2, day 1). The biopsy was taken 16 h after the cessation of the last exercise training session. This pattern was repeated for biopsies #3, #4, #5 and #6, which were taken on the morning of days 3, 7, 10 and 14 respectively, i.e. 16 h after training sessions 3, 7, 10 and 14. Biopsies #1, #2 and #6 were taken from the left leg; biopsies #3, #4 and #5 were taken from the right leg. A fresh incision was made for each biopsy and was 3 cm proximal or distal to a previous biopsy site.

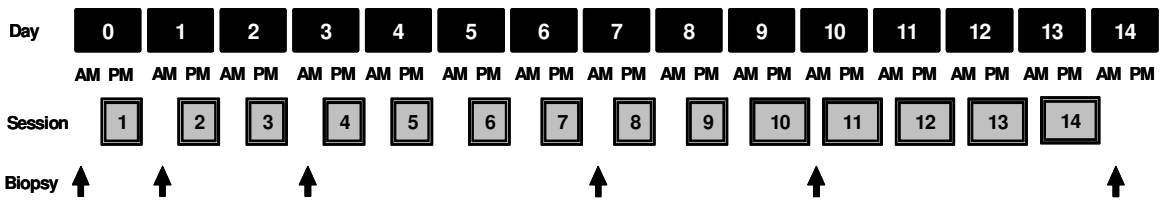

Figure 6.1. Schematic of the experimental design

6.2.4 Dietary control

For the three days prior to day 0, participants were required to keep a record of all food and fluid consumed. Participants were then asked to repeat this pattern of intake throughout the training phase to reduce the variability in metabolic responses that have been due to day-to-day variation in dietary intake.

Participants were weighed to the nearest 0.1 kg in minimal clothing prior to and immediately after each training session to determine fluid loss induced by each session. Each participant was then provided with a volume of plain water equivalent to 150 % of the difference in body mass i.e. for every kg of body mass lost during the session, 1.5 L of water was provided. At this time, each participant was given two cereal bars (Nutrigrain, Kellogg's, UK) for consumption with the bolus of fluid. This provided 50 g of CHO, 3 g of protein and 7 g of fat immediately after each session.

6.2.5 Statistical analysis

One-way ANOVA with post-hoc pair-wise comparisons using Fisher's least significant difference, was used determine the effect of exercise training on variables of interest with serial measurements. A paired t-test was used to determine the differences between pre- and post-training VO_{2peak} scores.

6.3 Results

6.3.1 Training log and aerobic capacity

Each participant completed his full complement of fourteen days of exercise training as described, indicating 100% compliance. The specifics of exercise intensity and energy expenditure are described in the training log (Table 6.2).

Exercise training resulted in 17.8±3.5% increase in VO_{2peak} compared to pre-training values (Table 6.1). Average exercise intensity of the fourteen training sessions was 80.5±1.9% of the pre-training VO_{2peak}, which represents 68.8±2.3% of the post-training VO_{2peak}.

Table 6.2. Training log

Session	*1*	*2*	*3*	*4*	*5*	*6*	*7*
VO_2 ($L \cdot min^{-1}$)	2.17±0.08	2.20±0.08	2.24±0.11	2.21±0.12	2.18±0.13	2.15±0.07	2.20±0.10
EE (kcal)	650±24	661±25	672±34	662±39	653±38	646±20	661±30
% PRE VO_{2peak}	77.5±1.8	78.9±2.2	79.8±2.0	78.4±1.8	77.3±1.3	77.2±1.9	78.6±1.2
%POST VO_{2peak}	66.1±1.3	67.4±2.0	68.4±2.4	67.2±2.6	66.4±2.8	65.9±1.2	67.2±1.6

Session	*8*	*9*	*10*	*11*	*12*	*13*	*14*
VO_2 ($L \cdot min^{-1}$)	2.20±0.14	2.27±0.13	2.38±0.13	2.35±0.14	2.33±0.11	2.39±0.08	2.35±0.13
EE (kcal)	659±41	681±38	715±39	706±43	699±33	717±25	705±38
% PRE VO_{2peak}	77.9±1.6	80.1±1.9	84.8±2.2	83.4±1.1	83.2±2.9	85.7±2.6	83.7±1.9
%POST VO_{2peak}	66.8±2.7	67.4±3.1	72.7±2.9	71.6±2.8	71.1±2.5	73.1±1.9	71.6±2.1

	Sessions 1-7	*Sessions 8-14*	*Sessions 1-14*
VO_2 ($L \cdot min^{-1}$)	2.19±0.10	2.32±0.12	2.26±0.11
EE (kcal)	658±0.30	697±37	677±33
% PRE VO_{2peak}	78.2±1.7	82.7±2.0	80.5±1.9
%POST VO_{2peak}	66.9±2.0	70.6±2.6	68.8±2.3

6.3.2 Gene expression

The expression of genes encoding proteins involved in mitochondrial metabolism and transcriptional control of mitochondrial biogenesis is shown in Figure 6.2. Steady-state expression of PGC-1α mRNA increased above pre-training values at days 3 and 7 (48%, $p<0.05$; 31%, $p<0.1$) but returned to values similar to day 0 thereafter (Fig. 6.2A). ERRα mRNA expression was elevated at day 3 ($p<0.05$) and remained elevated up to day 14 (44-63%, $p<0.05$; Fig. 6.2B). COXIV mRNA tended to be elevated after 1 and 3 days (~40%, $p<0.1$) and continued to be elevated up to day 14 (60-85%, $p<0.05$; Fig. 6.2E). NRF-1 and PPARδ mRNA were elevated after 1 day of training (51% and 68%, respectively; $p<0.05$) but returned to values similar to pre-training values thereafter (Fig. 6.2C & D). No change was observed in UCP3 mRNA, although a tendency ($p=0.108$) existed for elevated expression after 1 day of training (Fig. 6.2F).

The expression of genes encoding proteins involved substrate selection and metabolism is shown in Figure 6.3. Steady-state mRNA expression of FOXO1A (2.7-fold, $p<0.05$) and PDK4 (8.7-fold, $p<0.001$) was elevated after 1 day of training but were not different to pre-training values at any time point (Fig. 6.3A & B). CPT1 mRNA expression was highest after 3 days of training (2.0-fold greater than day 0, $p<0.05$), tended to remain above pre-training values at days 7 and 10 (both 54%, $p<0.1$) and was still elevated by 75% of pre-training values at day 14 ($p<0.05$; Fig. 6.3C). GLUT4 mRNA expression was markedly reduced after one training session (-51%, $p<0.05$) but returned to pre-training levels after day 3 and remained unchanged until day 14.

One training session induced an increase in mRNA expression for RIP140 (2.1-fold, $p<0.001$), CREB (43%, $p<0.05$) and Sirt1 (2.1-fold, $p<0.001$), after which values returned to pre-training levels for the remainder of the training period (Fig. 6.4A, B & C). However, CREB mRNA tended to remain elevated and after 14 days of training remained 30% above pre-training levels ($p<0.1$; Fig. 6.4C).

6.3.3 Protein content

No significantly measurable change was observed in protein content of PGC-1α (Fig 6.5A), FOXO1A (Fig.6.5C), PDK4 (Fig. 6.6B) or CPT1 (Fig. 6.6C). ERRα tended to be elevated after days 10 and 14 compared to pre-training protein content (~70%, $p<0.1$; Fig. 6.5B). HKII protein content (Fig. 6.6B) was elevated by day 7 (2.9-fold, $p<0.05$), peaked at day 10 (4.1-fold, $p<0.001$) and remained elevated at day 14 (3.1-fold, $p<0.05$). COXIV protein content tended to be elevated at days 3, 7 and 10 (~27%, all $p<0.1$) and was highest after 14 days of training (36%, $p<0.05$).

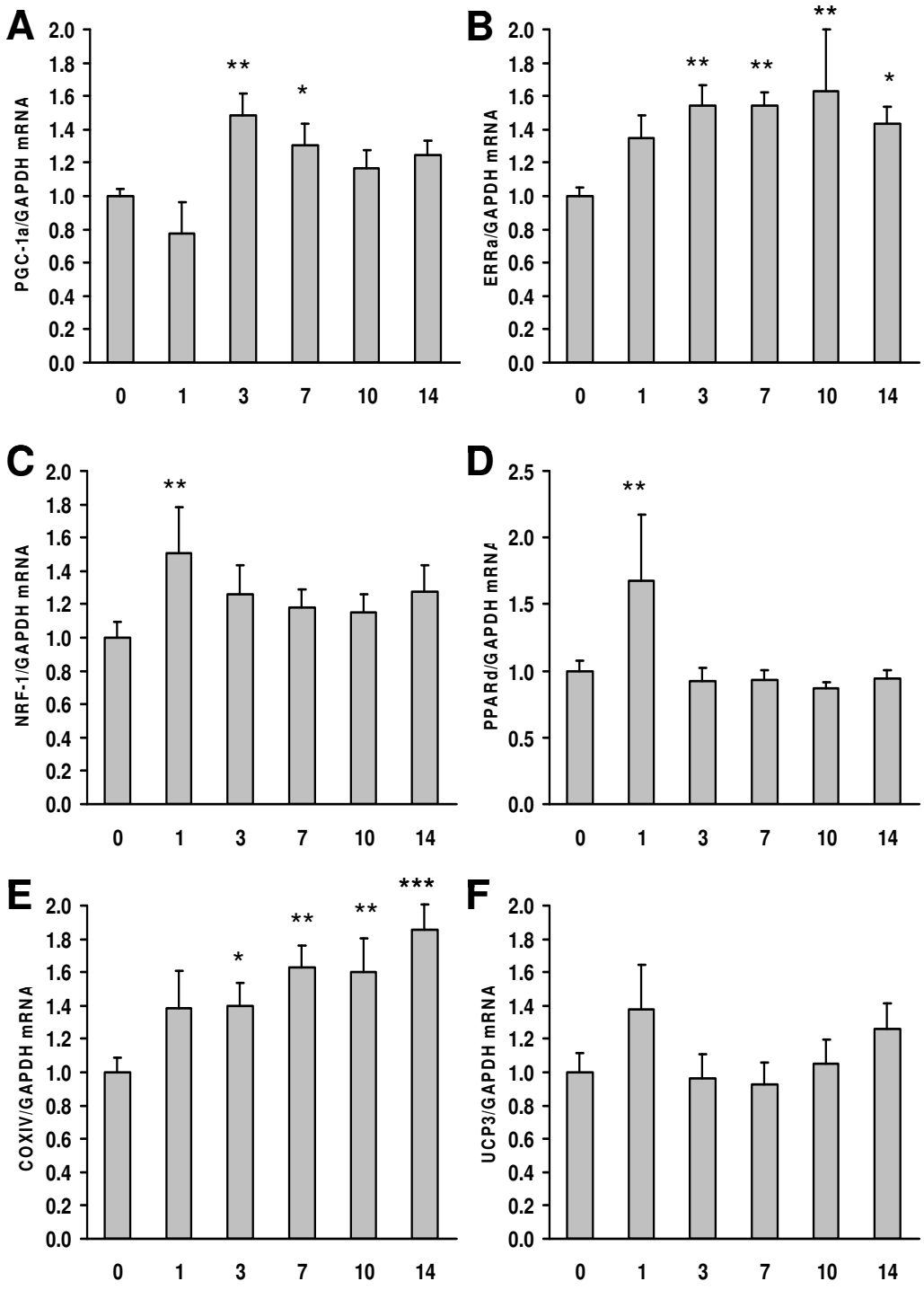

Figure 6.2. mRNA expression of genes encoding proteins involved in mitochondrial metabolism and transcriptional regulation of mitochondrial biogenesis, (A) PGC-1α, (B) ERRα, (C) NRF-1, (D) PPARδ, (E) COXIV, and (F) UCP3. Values are mean±SE, normalised to GAPDH mRNA. *** significantly different to Day 0 ($p<0.001$); ** significantly different to Day 0 ($p<0.05$); * significantly different to Day 0 ($p<0.1$).

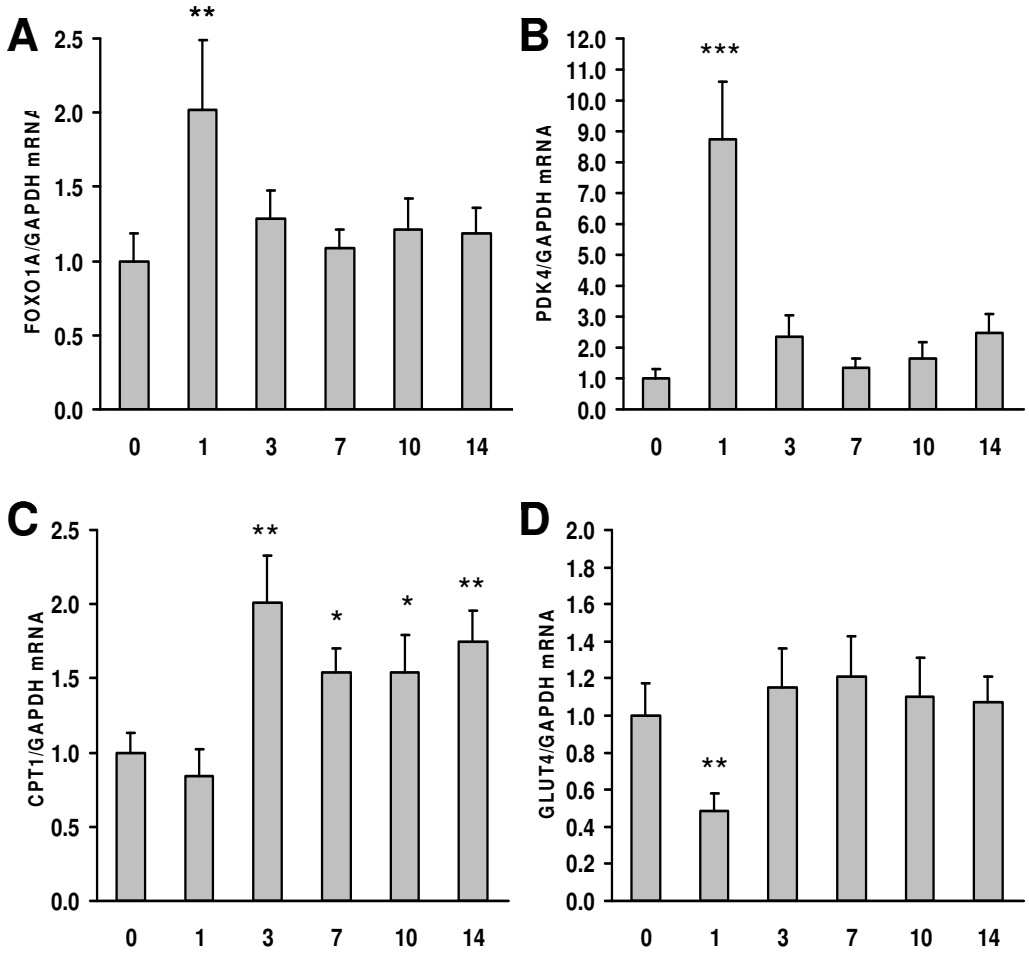

Figure 6.3. mRNA expression of genes encoding proteins involved in substrate selection and metabolism, (A) FOXO1A, (B) PDK4, (C) CPT1, (D) GLUT4. Values are mean±SE, normalised to GAPDH mRNA. *** significantly different to Day 0 ($p<0.001$); ** significantly different to Day 0 ($p<0.05$); * significantly different to Day 0 ($p<0.1$).

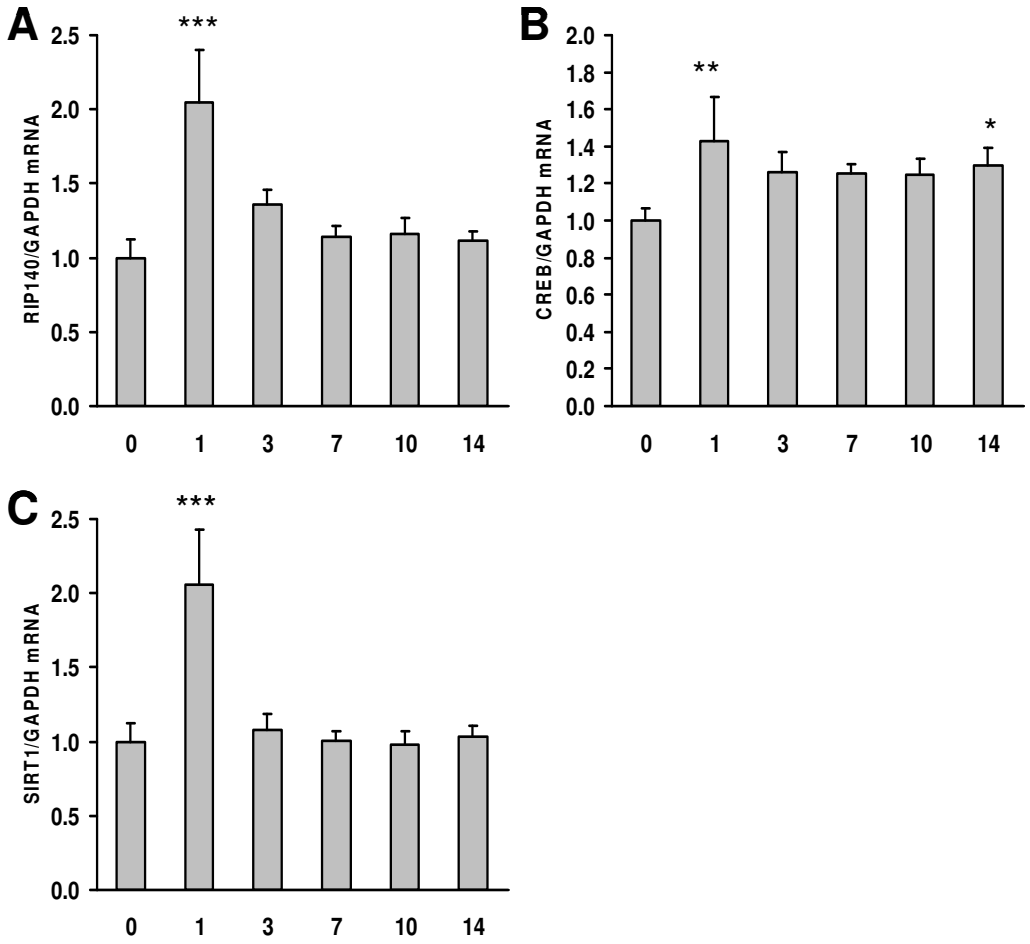

Figure 6.4. mRNA expression of genes encoding proteins involved in regulation of PGC-1α expression and corepression of transcription (A) RIP140, (B) CREB, (C) Sirt1. Values are mean±SE, normalised to GAPDH mRNA. *** significantly different to Day 0 ($p<0.001$); ** significantly different to Day 0 ($p<0.05$); * significantly different to Day 0 ($p<0.1$).

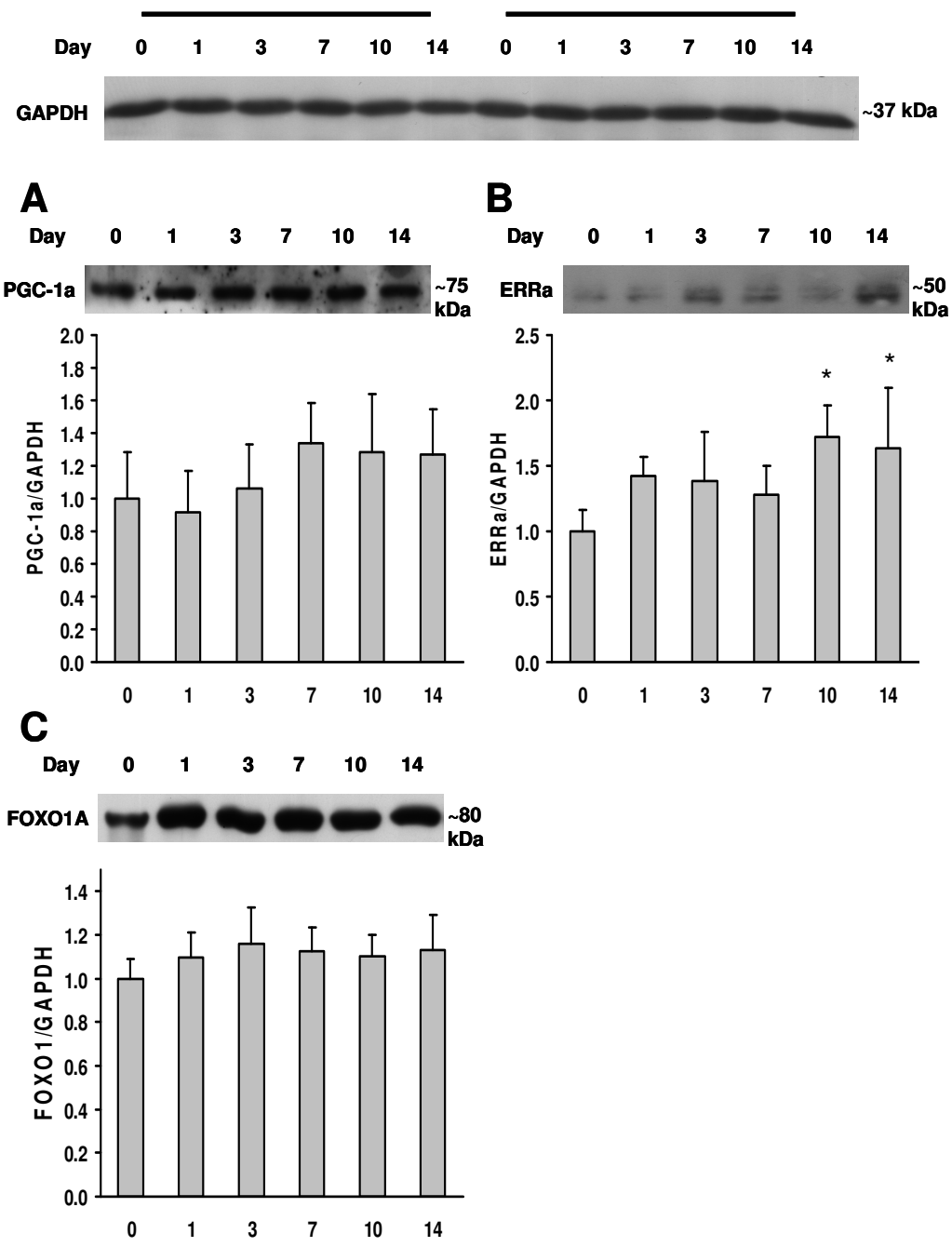

Figure 6.5. Protein content of (A) PGC-1α, (B) ERRα, and (C) FOXO1A, normalised to GAPDH protein content. Representative blots for each protein are included. Values are mean±SE. * significantly different to Day 0 ($p<0.1$).

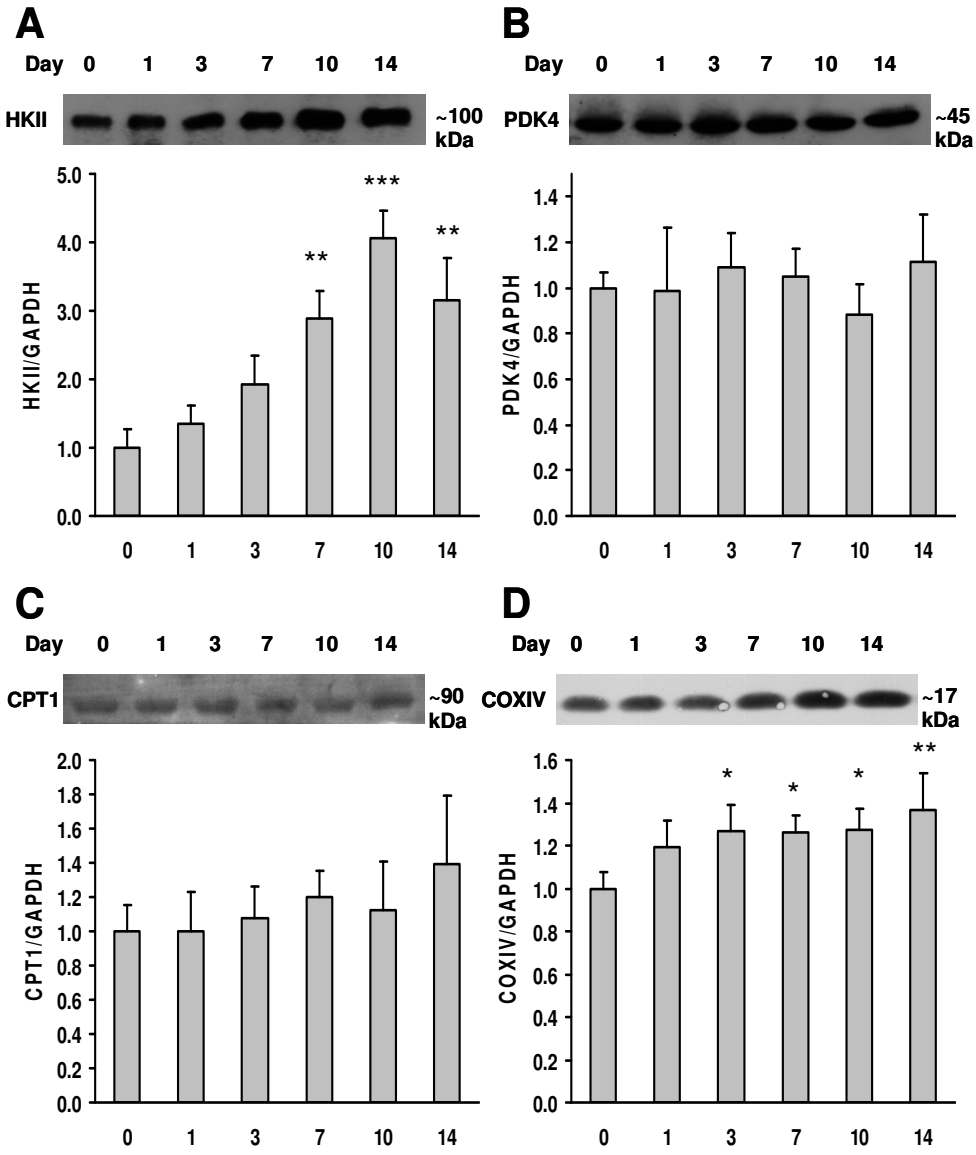

Figure 6.6. Protein content of (A) HKII, (B) PDK4, (C) CPT1, and (D) COXIV, normalised to GAPDH protein content. Representative blots for each protein are included. Values are mean±SE. *** significantly different to Day 0 ($p<0.001$); ** significantly different to Day 0 ($p<0.05$); * significantly different to Day 0 ($p<0.1$).

6.4 Discussion

A single bout of exercise generates a robust but transient increase in the quantity of mRNA for a multitude of genes, which are thought to contribute to the recovery from and adaptation to acute exercise (Pilegaard *et al.*, 2003;Cartoni *et al.*, 2005;Mahoney *et al.*, 2005). A widely accepted hypothesis is that training adaptation is mediated largely by these pulses of elevated mRNA abundance after individual exercise bouts within a training program leading to long-term increases in protein abundance that culminate in physiological adaptations (Mahoney & Tarnopolsky, 2005;Fluck, 2006). In the present study, we tested this contention by examining the time course of alterations in mRNA and protein abundance in response to short-term

training. However, the results are equivocal in their support. With regard to alterations in mRNA abundance of selected genes, there are three broad patterns to emerge: (i) no increase in mRNA, (ii) an increase after one exercise session but a return to baseline levels thereafter, or (iii) an increase after three exercise sessions that is sustained or increases further throughout the training period. Similarly for protein content, two distinct patterns are evident: (i) no change in protein content during training, (ii) an increase in protein content evident after seven days of training that is sustained until the end of the training period.

An incremental accumulation of mRNA of genes encoding regulators of mitochondrial biogenesis (PGC-1α, ERRα), lipid transport (CPT1) and mitochondrial metabolism (COXIV) was observed during the fourteen day training program. This was evident as an increase above baseline values after three sessions (Figs 6.2A, B & E, 6.3C). These genes have been previously shown to robustly increase after a single bout of exercise (Pilegaard *et al.*, 2000;Cartoni *et al.*, 2005), and our results indicate an accumulation effect with repeated exercise bouts. This suggests that transcriptional activation of these genes plays some role in the adaptation to exercise. The regulation of mitochondrial biogenesis and metabolic gene expression in skeletal muscle is under control of a well-defined set of factors including PGC-1α and ERRα (Schreiber *et al.*, 2003;Huss *et al.*, 2004;Wende *et al.*, 2005;Araki & Motojima, 2006). PGC-1α acts as a transcriptional coactivator for a large number of transcription factors regulating the expression of metabolic and mitochondrial genes (Handschin & Spiegelman, 2006). Altering the transcriptional activity of PGC-1α affects physiological responses that equip the cell to meet the energy demands of a changing environment including augmentation of mitochondrial biogenesis, cellular respiration rates and energy substrate uptake and utilisation (Wu *et al.*, 1999;Michael *et al.*, 2001;Lin *et al.*, 2002;St-Pierre *et al.*, 2003;Wende *et al.*, 2005;Rohas *et al.*, 2007;Wende *et al.*, 2007). Overexpression of PGC-1α in rodent skeletal muscle produces a phenotype remarkably similar to endurance trained muscle and improves exercise performance (Wende *et al.*, 2007;Calvo *et al.*, 2008). The expression of the orphan nuclear receptor ERRα is regulated by PGC-1α (Schreiber *et al.*, 2003) and is necessary for PGC-1α-induced mitochondrial biogenesis (Schreiber *et al.*, 2004). ERRα is particularly important for the expression of genes regulating oxidative phosphorylation (Schreiber *et al.*, 2003), fatty acid oxidation (Huss *et al.*, 2004) and mitochondrial DNA expression (Schreiber *et al.*, 2004). Therefore, this pathway in association with other factors such as the NRFs is proposed as critical to exercise-induced skeletal muscle adaptations (Baar, 2004;Hood *et al.*, 2006;Arany *et al.*, 2008).

Despite the accumulation of PGC-1α mRNA, no measurable change was observed in PGC-1α protein content whereas an increase in ERRα mRNA levels preceded an elevation in ERRα protein content. The discrepancies are difficult to explain. The half-life of PGC-1α protein is approximately 2.3 h (Puigserver *et al.*, 2001) and it is possible that the sampling times for biopsies missed any increases in protein content induced by exercise. Alternatively, it may be that the existing PGC-1α protein content is sufficient and exercise-induced PGC-1α-dependent adaptations are regulated by posttranslational modification of existing PGC-1α (Wright *et al.*,

2007b). However, PGC-1α protein content has recently been shown to be increased by ~16-23% in human skeletal muscle during the first 24 h of recovery from an exhaustive bout of exercise (Mathai *et al.*, 2008). In addition, PGC-1α mRNA and protein content have been previously shown to be elevated by six weeks of endurance training (Russell *et al.*, 2003;Burgomaster *et al.*, 2008), which suggests that the steady-state increase in PGC-1α protein content during endurance training may occur after the fourteen days monitored in this experiment. The increase in both ERRα mRNA and protein content suggests that ERRα is critical to exercise-induced adaptations in skeletal muscle. To our knowledge, this is the first experiment to report such results in the context of exercise training. Given the established function of ERRα, this regulation is likely to be at the level of the expression of genes regulating oxidative phosphorylation, lipid metabolism and angiogenesis (Schreiber *et al.*, 2003;Huss *et al.*, 2004;Arany *et al.*, 2008).

The increases in CPT1 and COXIV expression are supportive evidence for ERRα-related adaptation in the present study. PGC-1α- and ERRα-dependent regulation of CPT1 expression has been established (Huss *et al.*, 2004;Gerhart-Hines *et al.*, 2007). Regulation of fatty acyl-CoA entry into the mitochondria by CPT1 has been identified as a rate-limiting step in the oxidation of fatty acids (McGarry & Brown, 1997). The activity of CPT1 is higher in muscle from trained compared to untrained humans (Berthon *et al.*, 1998;Jong-Yeon *et al.*, 2002) and this was strongly correlated with citrate synthase activity and VO_{2peak} (Berthon *et al.*, 1998). In addition, a period of training in previously sedentary individuals results in an increase in CPT1 activity (Bruce *et al.*, 2006). An increase in CPT1 mRNA after short- and long-term training has also been observed (Tunstall *et al.*, 2002;Russell *et al.*, 2003), and in endurance-trained compared to sedentary muscle (Schmitt *et al.*, 2003). The relationship between an increase in CPT1 mRNA and enzymatic activity after exercise training is not established, but in rodent liver under pathological stress, alterations in CPT1 mRNA are reflected by changes in enzymatic activity (Mynatt *et al.*, 1994). Evidence for possible PGC-1α/ERRα-associated mitochondrial adaptation is supported by the progressive increases in both COXIV mRNA and protein content (Figs. 6.2E & 6.6D). COXIV is a nuclear-encoded subunit of the cytochrome c oxidase complex, which is the terminal enzyme of the respiratory chain responsible for establishing a transmembrane proton gradient that is used to produce ATP (Scarpulla, 2008). COX assembly and activity is dependent on COXIV (Li *et al.*, 2006), and COXIV expression is often used as a representative marker of mitochondrial biogenesis (Hood, 2001). Exercise training-induced changes in COX activity and COXIV protein are reported (Carter *et al.*, 2001;Short *et al.*, 2003;Gibala *et al.*, 2006). At the mRNA level, COXIV mRNA is elevated (~85%) in endurance-trained compared to sedentary individuals (Puntschart *et al.*, 1995), whereas endurance training resulted in a ~70% increase in COXIV mRNA after six or sixteen weeks (Russell *et al.*, 2003;Short *et al.*, 2003). *In vitro* results data have shown that COXIV induction by PGC-1α depends, at least partly, on ERRα function (Schreiber *et al.*, 2004). In response to physical activity or inactivity, COXIV expression is well correlated with PGC-1α (Short *et al.*, 2003;Timmons *et al.*, 2005a). PGC-1α-dependent regulation of COXIV expression is likely to be

mediated by coactivation of NRF-2 (Wu *et al.*, 1999;Wright *et al.*, 2007b), by virtue of the presence of an NRF-2 binding site in the COXIV promoter region (Scarpulla, 2008). Acute exercise increases NRF-2 DNA binding to COXIV (Baar *et al.*, 2002;Wright *et al.*, 2007b). In addition, an exercise-induced PGC-1α-associated increase in COXIV protein illustrates the effective coordination of transcriptional regulation of both the nuclear- and mitochondrial genomes described by Hood (2001). However, it has recently been shown in muscle-specific PGC-1α knockout mice that exercise training-induced increases in nuclear- (δ-ALAS) and mitochondrial-encoded (COXI) proteins are not dependent on PGC-1α, despite an apparent regulation of basal protein expression of these targets by PGC-1α (Leick *et al.*, 2008). This is interpreted as either PGC-1α is not involved in the training-induced adaptive response in skeletal muscle, or that as of yet undetermined compensatory factors or mechanisms modulate the response when PGC-1α is absent (Leick *et al.*, 2008). Similarly, in the present study it is not possible to ascertain whether the training-induced increase in COXIV is due to a PGC-1α coactivation of ERRα and NRF-2 or a combination of other factors in the present experiment. As such, the current data can only demonstrate coincidence, but not causality.

A cluster of genes, which have previously shown to be upregulated by an acute bout of exercise (experiments I and II) (Pilegaard *et al.*, 2003;Russell *et al.*, 2005;Cartoni *et al.*, 2005), were increased after one exercise session but returned to and remained at basal levels thereafter (Figs 6.2, 6.3, 6.4). These included mRNA of genes encoding transcription factors (NRF-1, FOXO1A, CREB, PPARδ), the transcriptional corepressor RIP140, Sirt1 and PDK4. This suggests that that these genes are not involved in adaptation and respond to exercise for different reasons (Mahoney & Tarnopolsky, 2005). For example, PDK4 mRNA has consistently been shown to increase robustly during recovery from acute exercise (Pilegaard *et al.*, 2005;Mahoney *et al.*, 2005;Yang *et al.*, 2005a). However, using microarray analysis, Timmons et al. (2005) reported that steady-state levels of PDK4 mRNA are robustly downregulated after six weeks of endurance training. Moreover, Keller et al. (2007) have compared microarray data from acute and chronic exercise studies and concluded that little direct connection is evident between acute upregulation of transcripts and chronic accumulation of transcripts. This suggests that acutely responsive genes are involved in restoring muscle cell homeostasis during the recovery period after exercise or are part of a generalized stress response that is triggered in “uninitiated skeletal muscle” before a more specific response can be “fine-tuned” throughout the training period (Mahoney & Tarnopolsky, 2005). However, even in well-trained muscle, we have shown that acute exercise can transiently increase the expression of FOXO1A, PDK4 and RIP140 among others (experiment II) suggesting that the acute response of these particular genes is conserved but does not result in transcript accumulation. This is supported by data from Nordsborg et al. (2003) who showed an apparent downregulation of PDK4 mRNA expression at rest after six weeks of high intensity training. However, acute exercise still induced an increase, albeit blunted, in PDK4 mRNA after training. Therefore, it is likely that (i) genes that are not elevated after exercise training may still be involved in adaptation and/or homeostatic recovery, and (ii) acutely responsive genes do not necessarily

participate in training adaptation. Regarding transcription factor expression, without any measurement of protein function it is not possible to rule out a role for these factors in the adaptation to exercise. Acute exercise studies demonstrate that the transcript expression of these genes is responsive to exercise. However, it may be that the expression (mRNA and/or protein) is not as important as functional activity of these targets in the regulation of exercise-induced alterations in skeletal muscle metabolism. The well established roles for NRF-1 in mitochondrial gene regulation (Wu *et al.*, 1999), FOXO1A in substrate metabolism (Bastie *et al.*, 2005) and Sirt1 in PGC-1α-associated metabolic regulation (Gerhart-Hines *et al.*, 2007) suggest that these factors are likely to have a role in the shift in skeletal muscle phenotype after exercise training.

Given the lack of change in mRNA abundance, the protein content for FOXO1A and PDK4 was, as might be expected, unaltered by exercise training (Fig. 6.5C & 6.6B). Conversely, mRNA does not always need to accumulate to affect a training-induced increase in its protein product. Rather, repeated transient pulses of mRNA can lead to an increase in protein product. Kraniou et al. (2004) have shown a 3.6-fold increase in GLUT4 protein content after seven consecutive days of training, in the absence of any change in mRNA abundance. This is despite the fact that GLUT4 transcription and mRNA is elevated during recovery from acute exercise (Neufer & Dohm, 1993;Kraniou *et al.*, 2000;Kraniou *et al.*, 2006). In the present study, we did not observe a change in GLUT4 mRNA during training, despite the fact that similar training has been previously shown to result in elevations in GLUT4 protein content (Houmard *et al.*, 1995;Kraniou *et al.*, 2004). Although we did not measure HKII mRNA in the present study, HKII protein was markedly elevated after seven days of training and remained elevated thereafter (Fig. 6.6A). Acute endurance exercise leads to a transient increase in HKII transcription and mRNA levels in human skeletal muscle (Pilegaard *et al.*, 2000;Pilegaard *et al.*, 2002), whereas HKII activity increases after a period of exercise training (Baldwin *et al.*, 1973;Green *et al.*, 1983;Spina *et al.*, 1996;Phillips *et al.*, 1996b). In addition, increases in HKII mRNA levels correlate well with an increase in HKII protein content and activity (Osawa *et al.*, 1995;Koval *et al.*, 1998). The increased HKII protein content may reflect the need for an increased glucose phosphorylation, both during and after exercise, thus ensuring substrate availability for glycolysis during exercise and for glycogen synthesis in the recovery period (Connett & Sahlin, 1996). An increase in the ability and capacity to store glycogen in muscle is robust adaptation to exercise training (Holloszy & Coyle, 1984).

In summary, adaptations to chronic exercise training are well established (Holloszy & Coyle, 1984). However, few studies have focussed on the transcriptional regulation of these adaptations despite the widely held view that transcriptional mechanisms underlie the remarkable plasticity of skeletal muscle (Booth & Baldwin, 1996;Williams & Neufer, 1996;Hood *et al.*, 2006;Fluck, 2006). We have demonstrated divergent effects of exercise training on mRNA and protein expression for a range of transcriptional regulators and metabolic and mitochondrial targets. These findings suggest that the regulation of gene expression by exercise is gene-specific. Improvements in mitochondrial function require an increase in the proteins that

participate in the generation of a dynamic mitochondrial network. We show an increase in the mRNA abundance of the transcriptional coactivator PGC-1α and the PGC-1α partner ERRα. Notably, this is the first study to report changes in ERRα protein levels following exercise training. Furthermore, there is an increase in COXIV mRNA and protein, a PGC-1α target gene and marker of mitochondrial biogenesis. These data are consistent with the concept that initial signalling events associated with acute exercise induce transient pulses of increased mRNA abundance that eventually lead to an increase in protein content and enzyme activity, and that with longer term training there are increases in mRNA abundance for some, but notably not all, mitochondrial and metabolic proteins (Hood, 2001;Mahoney & Tarnopolsky, 2005;Fluck, 2006).

Chapter VII General discussion

The goal of this body of work was to investigate alterations in skeletal muscle gene expression under various experimental conditions. In the series of experiments reported, the findings were as follows

(i) divergent exercise intensities during acute isocaloric bouts of exercise modulate the magnitude of the transcriptional response to exercise such that coincident with greater kinase activation during exercise, larger increases in mRNA abundance occur during recovery from high intensity exercise

(ii) the induction of mRNA after acute exercise in trained muscle is qualitatively similar to untrained muscle and may induce a transcriptional programme coupled to a functional metabolic response in the post-exercise period

(iii) the transcriptional response to acute exercise is linked to either the restoration of metabolic homeostasis or adaptation to repeated exercise bouts

(iv) these findings are recapitulated during exercise training, where accumulation of mRNA and corresponding protein are present for some, but not all, acutely responsive genes

The initial course of signalling events and their interactions with regulators of the transcriptional response remain elusive. However, the ensuing transcriptional response in skeletal muscle is specific in terms of its character and time course is response to acute exercise and exercise training. Our results illustrate the well-described phenomenology of skeletal muscle plasticity and suggest that transcript level adjustments underlie modulation of skeletal muscle metabolism and phenotype by regular exercise.

7.1 Contraction-mediated regulation of skeletal muscle gene expression

7.1.1 Modulation of the exercise-induced signal modulates gene expression during recovery

The activation of AMPK, CaMKII and p38 MAPK pathways are strongly linked to metabolic and mitochondrial adaptation in skeletal muscle (Long *et al.*, 2004;Fluck, 2004;Chin, 2005;Jorgensen *et al.*, 2006). The metabolic milieu and the multiple extra- and intramuscular stimuli during contraction make it extremely difficult to isolate the exact contribution of each signalling pathway to measured changes in gene expression. Moreover, it has become increasingly apparent that these pathways, in broad terms, demonstrate some degree of dependence, cross-talk and redundancy in their regulation of metabolism (Hurley *et al.*, 2005;Wright *et al.*, 2007a). Conclusive data demonstrating continuity between activation these pathways to consequent alterations in gene expression is currently lacking in exercising human skeletal muscle. The mechanistic evidence is largely based on animal or cell-based experiments using pharmacological activation or inhibition of these kinases and/or knockout of signal pathway components and examining the downstream effects on gene expression.

Using divergent exercise intensities as a novel integrative approach to investigate the effect of altering metabolic stress (and consequently, signalling kinase activation), we hypothesised that altering the metabolic stress of an acute exercise bout would be reflected by corresponding alterations skeletal muscle gene expression. In experiment I, we have demonstrated an intensity-dependent activation of intracellular signalling kinases, and differential responses in mRNA expression of selected gene targets. In short, greater kinase activation of AMPK and CaMKII at the higher exercise intensity coincided with greater elevations in mRNA abundance of some (PGC-1α, FOXO1A, PPARδ) but not all gene targets (PDK4, RIP140) during recovery from high intensity exercise. However, the continuity between the activation of these kinases and the alterations in functional activity of transcriptional regulators regulating the expression of these genes was not established.

That the molecular signature of a single bout of exercise is sensitive and specific to the functional and metabolic demand imposed has been previously demonstrated in experiments that altered these demands by innovative means. Differential regulation of exercise-induced mRNA expression has been observed in studies that have reduced the muscle glycogen content (Keller *et al.*, 2001;Pilegaard *et al.*, 2002), fed carbohydrate during exercise (Civitarese *et al.*, 2005), varied carbohydrate supply during recovery (Cluberton *et al.*, 2005;Pilegaard *et al.*, 2005), suppressed circulating FFA prior to exercise (Watt *et al.*, 2004), or exercised the muscle under ischemia (Norrbom *et al.*, 2004). The divergent responses between metabolic genes and transcriptional coregulators to these interventions (Russell *et al.*, 2005;Civitarese *et al.*, 2005) suggests that the regulation of their expression on a gene-specific basis is sensitive to and dependent on specific signals. In some cases, the exercise-induced signal activation has been modulated under similar experimental conditions (Wadley *et al.*, 2006;Akerstrom *et al.*, 2006), which suggests that alterations in the activity of signal transduction pathways may underlie this transcriptional phenomenon. However, this has not been directly examined. Our results suggest that experimental manipulation of the exercise challenge modulates skeletal muscle gene expression, coincident with the differential activation of signal transduction pathways.

The intensity of exercise can be relatively easily modulated and quantified in a laboratory setting. Although we have only used two exercise intensities in our model, it would be possible to examine a variety of intensities between 30% and 90% VO_{2peak}, with a view to investigating the linearity of the signalling and transcriptional responses to acute exercise. This assumes each exercise bout is isocaloric, as the duration of an acute bout of exercise is known to modulate gene expression when exercise is performed at the same intensity (Hildebrandt *et al.*, 2003), presumably due to variation in energy expenditure.

This model would not be able to fully delineate the respective contributions of each signalling cascade. However, clearer relationships may be established between kinase activation and gene expression given the established intensity-dependent activation of these kinases (Wojtaszewski *et al.*, 2000;Widegren *et al.*, 2000;Rose *et al.*, 2006). For instance, during low intensity exercise, we observed minimal activation of AMPK and CaMKII but a robust elevation in mRNA abundance of PGC-1α was still observed during recovery.

7.1.2 Posttranslational modifications induced by exercise-induced signals

The lack on data demonstrating continuity between the contraction-induced signal activation and the regulation of gene expression reflects the fact that the majority of exercise-related studies on transcriptional regulation in humans have been descriptive rather than mechanistic. A network of nuclear receptors, transcription factors and coregulators are the key factors linking alterations in dietary, metabolic and endocrine pathways to the control of target gene expression (Feige & Auwerx, 2007). Their functional activity is regulated through spatial and temporal control of their expression and activity in response to metabolic cues. Accumulating evidence suggests that this regulation is controlled at the level of posttranslational modification of the existing transcriptional regulator protein. That these regulatory pathways are each modulated by exercise suggests that they could play some role in exercise-mediated metabolic regulation. For example, the regulation of PGC-1α functional activity can occur at the level of direct phosphorylation by p38 MAPK (Puigserver *et al.*, 2001) or AMPK (Jager *et al.*, 2007), or by acetylation status mediated by NAD^+/NADH-dependent control of Sirt1 and GCN5 activity (Rodgers *et al.*, 2005;Lerin *et al.*, 2006). Similarly, the regulation of FOXO1A activity can occur at the level of phosphorylation (Biggs III *et al.*, 1999) and acetylation (Nakae *et al.*, 2006). However, these mechanisms have not been investigated in an exercise context.

7.1.3 Untrained vs. trained muscle

The differences in exercise duration, energy expenditure and biopsy timing between experiments I and II make it impossible to directly compare the transcriptional response to exercise in the untrained and trained state. However, the general pattern of mRNA induction during recovery in either experiment is qualitatively similar. A rapid and robust increase in mRNA abundance of the PGC-1α, FOXO1A and PDK4 is consistent with a transcriptional response initiated to program a metabolic response in the recovery period after exercise that restores homeostasis through coordinated changes in substrate metabolism (Mahoney *et al.*, 2005) as discussed in earlier chapters.

Studies comparing the effect of training history or short-term training on the transcriptional response to acute exercise have generally concluded that the magnitude of the response is blunted for most genes (Nordsborg *et al.*, 2003;Fischer *et al.*, 2004;Lundby *et al.*, 2006;Schmutz *et al.*, 2006). Nordsborg et al. (2003) have suggested that possible that the mRNA changes during recovery from exercise would be the same in the trained and untrained state, if exercise had been carried out at the same relative, instead of the same absolute, workload. However, these effects in other studies were irrespective of whether the acute exercise bout after a period of training was conducted at the same relative or absolute exercise intensity. Conversely, our results suggest that irrespective of the training status of the muscle, it is likely that if the metabolic perturbation is sufficient, an appropriate transcriptional response is induced. In the case of glycogen-depleting exercise in experiment II, the appropriate transcriptional response in this case is likely to be similar, but not limited, to the one described above, i.e. sparing of glycogen precursors for glycogen resynthesis and increased lipid oxidation. Regarding the

extent of metabolic perturbation, it is worth remembering that the exercise challenge was more than three-fold higher in terms of calories expended during the respective bout in the trained compared to untrained experiments. Duration of exercise (and thus, total energy expenditure) has been previously shown to regulate the transcription of UCP3, PDK4 and HKII when exercise intensity is similar (Hildebrandt *et al*., 2003). These data are consistent with the notion that the muscle adapts appropriately and specifically to metabolic stress.

7.1.4 Continuity between effects of acute and chronic exercise on gene expression

Apart from the restoration of homeostasis, the transcriptional response to a single bout of exercise is hypothesised to increase mRNA abundance of factors that later play a role in adaptation to exercise (Mahoney *et al*., 2005). However, after an acute bout of exercise no measurable increase was observed in mRNA abundance of a number of genes known to regulate mitochondrial metabolism and regulate gene expression in skeletal muscle, i.e. ERRα, NRF-1, NRF-2. Despite this apparent lack of upregulation after acute exercise, we still observed an accumulation of ERRα transcript abundance during training, laterally reflected by an increase in ERRα protein content. Conversely, FOXO1A transcript abundance was rapidly and robustly increased after acute exercise, but neither mRNA nor protein content was altered during exercise training.

A generalised hypothesis regarding the role of transcriptional regulation in governing skeletal muscle plasticity and exercise training adaptation is widely proposed (Booth & Baldwin, 1996;Hood, 2001;Mahoney & Tarnopolsky, 2005;Fluck, 2006). Initial signalling events associated with acute exercise induce transient pulses of increased mRNA abundance for selected genes. These transcripts gradually accumulate with repeated stimulation due to the repetitive effect of exercise training. This accumulation of transcript abundance eventually leads to an increase in protein content and enzyme activity of the encoded targets. The functional consequences of these events are manifested as a training adaptation such as enhanced capacity to produce ATP from aerobic means during exercise.

The results reported herein add to our understanding of this framework. Rather than there being a linear relationship between mRNA expression during recovery from exercise with a gradual accumulation of transcript abundance during training, and a consequent increase in protein content, regulation must be considered on a gene-specific basis. There are several possible effects of acute and chronic exercise on any given gene that may be surmised from our data:

(i) robust and transient increase in mRNA abundance during recovery from a single bout of exercise consistent with role in homeostatic restoration; no change with exercise training and unlikely to have a role in the adaptation to training

(ii) robust and transient increase in mRNA abundance during recovery from a single bout of exercise, which results in gradual accumulation of transcript expression consistent with role in adaptation to exercise training

(iii) no change in mRNA abundance during recovery from a single bout of exercise, but gradual accumulation of transcript expression evident during training consistent with role in adaptation to exercise training

(iv) robust increase in mRNA abundance after one exercise session as part of a generalized stress response that is triggered in uninitiated skeletal muscle before a more specific response can be fine-tuned throughout the training period

7.2 Gene-specific regulation by exercise

7.2.1 PDK4 as a case study

PDK4-mediated inhibition of PDH via phosphorylation in muscle represents a mechanism for conserving carbohydrate substrates by gradually limiting the entry of glycolytic products into the mitochondria for oxidation and thereby conserving tricarboxylic acids for gluconeogenesis (Sugden & Holness, 2006). During recovery from exercise, skeletal muscle is characterized by a dramatic increase in insulin action and glycogen synthase activity, both of which promote the rapid resynthesis of muscle glycogen (Price *et al.*, 1994;Nielsen *et al.*, 2001). Partitioning of exogenous glucose towards glycogen resynthesis is of high metabolic priority during immediate post-exercise recovery, and is supported reduced PDH activity and increased fat oxidation (Kimber *et al.*, 2003).

Expression of PDK4 is highly sensitive to alterations in metabolic status such as acute exercise, fasting and high-fat/low-carbohydrate diets (Pilegaard *et al.*, 2000;Peters *et al.*, 2001;Pilegaard *et al.*, 2003;Tsintzas *et al.*, 2006). This is because skeletal muscle PDK4 expression increases in situations where carbohydrate availability and insulin levels are decreased and free fatty acids are increased (Sugden & Holness, 2006). In other words, during metabolic states in which there is a perceived deficit in whole body glucose availability. Irrespective of training status, we observed a marked increase in PDK4 mRNA abundance during recovery from a single bout of exercise (experiments I and II). An increase in PDK4 mRNA has been shown to correlate with an increased level of PDK4 protein, which increases PDK activity, reducing PDH activity (Peters *et al.*, 2001;Tsintzas *et al.*, 2006).

The induction pattern of PDK4 mRNA in response to metabolic perturbation illustrates the highly specific nature of the transcriptional response to a stimulus. Elevations in PDK4 mRNA are greater when the duration of exercise is extended at same exercise intensity (Hildebrandt *et al.*, 2003). Lowering muscle glycogen increases PDK4 mRNA at rest and augments the exercise-induced effects (Pilegaard *et al.*, 2002). Carbohydrate ingestion during exercise ablates the exercise-induced increase in PDK4 mRNA during recovery (Civatarese et al., 2005(Cluberton *et al.*, 2005). Similarly, high carbohydrate feeding during recovery from exercise blunts the post-exercise increase in PDK4 mRNA compared to low carbohydrate intake (Pilegaard *et al.*, 2005). Fasting (48 h) induces an upregulation in PDK4 mRNA expression, and additionally reduces PDH activity coincident with an increase in PDK4 protein expression in human skeletal muscle

(Tsintzas *et al.*, 2006). This response was reversed by refeeding with a CHO-rich diet. Without addressing factors regulating PDK4 gene expression (Pilegaard & Neufer, 2004;Sugden & Holness, 2006), taken together these results demonstrate that PDK4 mRNA is induced in response to acute or sustained perturbation to metabolism that require a shift in fuel partitioning, but is rapidly reversed when exogenous fuel is supplied.

A robust increase in PDK4 mRNA expression induced by a single bout exercise of exercise would be expected to be recapitulated by an accumulation in mRNA and latterly protein content during training. However, this is not the case. Timmons et al. (2005) reported steady-state levels of PDK4 mRNA robustly downregulated after six weeks of endurance training, whereas we found no change in PDK4 mRNA or protein content after fourteen days of training. After six weeks of high intensity training, acute exercise still induced an increase in PDK4 mRNA, despite an apparent downregulation of PDK4 mRNA abundance at rest (Nordsborg *et al.*, 2003). This suggests that PDK4 is one gene that is acutely responsive to exercise but does not participate in the adaptive response to exercise training. This type of transcriptional regulation has previously been proposed by Mahoney & Tarnopolsky (2005), and should be considered in any discussion of the relevance of an acute exercise-induced increase in expression of a given gene to a training-induced adaptation.

7.2.2 Transcription factors and transcriptional coregulators

A single bout of exercise increases the mRNA abundance of several transcription factors and transcriptional coregulators during recovery (Pilegaard *et al.*, 2003;Russell *et al.*, 2005;Cartoni *et al.*, 2005). The relevance of this phenomenon to the adaptive response to exercise training is not established. Intuitively, the induction of expression of regulators of mitochondrial gene expression and mitochondrial biogenesis by acute exercise may play a role in adaptation given the established effects of exercise training on mitochondrial adaptations (Holloszy & Coyle, 1984;Fluck, 2004).

We did not observe a change in transcript abundance of several transcription factors such as ERRα, NRF-1, NRF-2, MEF2 and CREB during recovery from acute exercise. Experimental design differences in factors such as timing of biopsies, exercise duration, or probe/primer design to name a few, cannot be ruled out as explanation for the discrepancy between studies. In the training study, the mRNA expression of several transcription factors was elevated 16 h after one session of exercise but returned to pre-training values thereafter for the remainder of the training period. Protein content for PGC-1α and FOXO1A was unaltered by training but ERRα protein increased during training. The increase in COXIV mRNA and protein content throughout the training period indicates that some adaptation at the level of mitochondrial metabolism/biogenesis had occurred.

Training-induced alterations in expression of transcription factors and coregulators are not well described. However, PPARα and PGC-1α mRNA and protein content have been previously shown to be elevated by six weeks of endurance training (Russell *et al.*, 2003;Burgomaster *et*

al., 2008). Similarly, ten weeks of exercise training resulted in a 50% increase in PGC-1α mRNA (Mortensen *et al*., 2007), whereas sixteen weeks of aerobic training increased the mRNA abundance of PGC-1α, NRF-1, and Tfam (Short *et al*., 2003).

At present, the generalised response of transcription factors to training and their precise role in exercise-mediated metabolic adaptation is unknown beyond a coincidental rise in mRNA and/or protein expression during training. What relevance this increase in expression has regarding functional activity is unknown. In some cases, it may be that the existing transcription factor or coregulator protein content is sufficient and exercise-induced adaptations are regulated by posttranslational modification of existing protein, thereby modifying its functional activity. This has been proposed in the case of exercise-induced PGC-1α-associated mitochondrial biogenesis (Wright *et al*., 2007b). One report currently in press (Mathai *et al*., 2008) has shown a ~20% increase in PGC-1α protein content in human skeletal muscle during 24 h of recovery from a single bout exhaustive exercise. PGC-1α knockout in skeletal muscle reduces the acute exercise-induced expression of mitochondrial gene targets (Leick *et al*., 2008). Together these results suggest PGC-1α expression is induced by exercise consistent with a role in acute exercise-induced transcriptome response, whereas PGC-1α is not mandatory for the exercise training-induced response of the same gene targets (Mathai *et al*., 2008).

7.3 Technical considerations

7.3.1 Timing of muscle biopsies

Given the rapid kinetics of contraction-induced signalling, posttranslational modifications, transcriptional activation, mRNA stability/half-life, and protein stability/half-life, studies that involve only single time point measurements may not be conclusive due to lack of time course data. Studies on skeletal muscle metabolism require the excision of muscle tissue, generally through means of a muscle biopsy. Although relatively painless, the invasive nature of the procedure reduces the number of volunteers willing to participate in experiments. This is one of the major difficulties in *in vivo* experiments in exercise metabolism. Our experimental designs have employed multiple muscle biopsies in an attempt to achieve adequate temporal resolution regarding the molecular responses to exercise.

However, it cannot be discounted that a lack of observed effects in the mRNA or protein expression of selected targets was simply due to the timing of biopsies. For example, the increase in PGC-1α mRNA reported during recovery from exercise can be seen 1 h after exercise (Russell *et al*., 2005) and remains elevated up to 8 h into recovery (Vissing *et al*., 2005). In contrast, increased mRNA abundance of CPT1 or COXIV may not be manifested until 24 h after exercise (Cartoni *et al*., 2005;Pilegaard *et al*., 2005). Therefore, in the acute exercise studies (experiments I and II), a lack of observed effects may be attributed to the timing of muscle biopsies.

In the training study, biopsies were taking in the fasted state, 16 h after the previous exercise bout. Again the kinetics of mRNA and protein stability cannot be discounted. However, we were interested in steady-state changes in gene expression and, as such, this design satisfied our aim. In addition, it is possible that accumulations in mRNA and protein content may have occurred later than the fourteen days of training measured here. Endurance training, employing an appropriate duration per day, frequency per week, and submaximal intensity per exercise bout, can produce an increase in mitochondrial content, usually ranging from 50 to 100% within six weeks, reaching a new, higher steady-state mitochondrial content (Holloszy & Coyle, 1984;Hood, 2001). Similarly, previous studies investigating PGC-1α protein content have shown changes in protein content after six weeks of training (Russell *et al.*, 2003;Burgomaster *et al.*, 2008). The nature of training adaptations is that individual components follow distinct time courses (Saltin *et al.*, 1977;Phillips *et al.*, 1996b), a fact that may be reflected by divergent responses of mRNA and protein content of specific targets.

7.3.2 mRNA and protein kinetics in skeletal muscle in response to contractile activity

The relationship between morphometric estimation of muscle mitochondrial volume density and biochemical changes in tissue oxidative capacity are highly correlated (Reichmann *et al.*, 1985). Therefore, mitochondrial content is generally estimated by the change in maximal activity, measured under optimal conditions *in vitro*, of a typical marker enzyme (e.g. citrate synthase, SDH) or by the change in the protein content of a single protein (e.g. cytochrome c, COX).

The approximate six week time period required to achieve a new steady-state mitochondrial content in response to endurance training clearly does not reflect the early molecular events that ultimately lead to the measurable morphological changes (Hood, 2001). A change in mitochondrial protein content is measurable at much earlier time points. Mitochondrial proteins turn over with a half-life of approximately one week after the onset of a new level of muscle contractile activity (Booth, 1977;Henriksson & Reitman, 1977;Terjung, 1979), whereas enzymatic activities are elevated after as little as two days of stimulation (Reichmann *et al.*, 1985).

A chronic increase in the contractile activity of skeletal muscle can stimulate translation of mitochondrial proteins from the existing pool of mRNAs, before the increase in mRNA content takes place (Freyssenet *et al.*, 1996). In chronically stimulated muscle, elevations in citrate synthase activity were observed before an increase in citrate synthase mRNA after eight days, but increases in citrate synthase mRNA were subsequently followed by a larger increase in citrate synthase activity up to fifty-two days (Seedorf *et al.*, 1986). However, the mRNA expression and enzymatic activity of LDH, a cytosolic enzyme, increased in an identical manner to one another in the same time frames.

7.3.3 Relationship of alterations in mRNA to functional consequences

Increments in mRNA abundance are often referred to as increases in gene expression. This is technically incorrect, because increased gene expression and its associated phenotypic/functional manifestations do not take place until there is an increase in the concentration of the protein encoded by the gene (Baar *et al*., 2002). Gene expression can be controlled at various points beyond transcription, so the extent to which a protein will increase in response to an adaptive stimulus cannot always be predicted from the increase in mRNA. In skeletal muscle, expression of mRNA does not always accurately reflect the abundance of proteins and can give no information regarding their posttranslational modifications (Hojlund *et al*., 2008).

For example, in order for PDK4 to alter fuel partitioning, it must phosphorylate PDC, thereby inactivating it. It is incorrect to say that an increase in PDK4 mRNA expression leads to sparing of glucose. There is no evidence that this mRNA change results in altered PDK4 protein content nor is there any evidence that its enzymatic activity, i.e. leading to phosphorylation of PDC, is increased. A more conservative statement is that a transcriptional response was induced that may form part of a metabolic response that contributes to glucose sparing and increased fat oxidation.

The observation that a specific mRNA was significantly increased following exercise indicates a regulatory effect of muscle contractions upon that mRNA level. However, the finding that subsequently the protein level does not change may demonstrate that acute exercise does not necessarily mediate a detectable increase in protein content at the translational level. Alternatively, the temporal resolution provided by muscle biopsies may be a confounding factor. Clearly, consideration and further investigation of the continuity between contractile activity-induced alterations in transcriptional regulation, protein synthesis and functional adaptation is required.

7.3.4 Measurement of PGC-1α protein content by immunoblot techniques

Measurement of protein content in these experiments is limited to those currently available from commercial sources. It must be acknowledged by this author that determination of PGC-1α protein content in human skeletal muscle has been an ongoing issue in this field for the past 12-18 months, something that has been widely acknowledged at conference proceedings. As such, there are currently doubts about the validity of certain commercially available antibodies, e.g. one commercially available PGC-1α antibody is known to identify an immunoreactive protein band in the tissue of PGC-1α-null mice (AV Chibalin, personal communication). The PGC-1α antibody used in the current experiments (PGC-1α H-300, sc-13067, Santa Cruz Biotechnology) was chosen on the basis of its use in previously published papers (Taylor *et al*., 2005;Akimoto *et al*., 2005;Hancock *et al*., 2008). The molecular weight of the immunoreactive protein band targeted by this antibody is approximately 75 kDa (Fig. 6.5A), as opposed to the predicted 92-105 kDa molecular weight of the full-length protein. Aside from assurances from the vendor as

to specificity of the antibody, and speculation on the cause of the discrepancy between the measured and expected molecular weight (posttranslational cleavage, splice variants, relative protein charge), there is little else under the control of the end user. However, it is carefully acknowledged that this issue must be resolved prior to publication of these results in a peer-reviewed publication.

7.4 Future directions

7.4.1 Establishing the mechanisms regulating exercise-induced gene transcription

The signalling and transcriptional responses to acute exercise have become increasingly well defined, but the direct link between these responses remains elusive *in vivo*. As discussed in other sections, transcription factors and their coregulators are responsible for integrating intracellular signals into functional consequences through their control of gene expression.

Only a few experiments have directly measured functional activity or associated changes of transcriptional regulators after exercise. In rodents, for example, the DNA binding activity of NRF-1 and NRF-2 to promoter regions of mitochondrial genes is increased by acute exercise (Baar *et al.*, 2002;Wright *et al.*, 2007b), an effect thought to be mediated by PGC-1α and indicative of stimulation of mitochondrial biogenesis by exercise. However, mechanistic data such as this remains unreported in humans. In humans, limited data does suggest acute exercise can alter the functional activity of transcription factors. A single bout of exercise has been shown to increase GEF (McGee *et al.*, 2006) and MEF2 (Yu *et al.*, 2001;McGee *et al.*, 2006) DNA binding activities, which may in part explain GLUT4 gene regulation by exercise.

Future studies in humans that aim to establish the mechanisms of exercise-induced mitochondrial biogenesis should focus on (i) establishing the effect of exercise on the functional activity of transcriptional factors and their coregulators, (ii) demonstrating continuity between activation of signal transduction pathways and the functional activity of these factors, and (iii) the regulation of translational mechanisms that translate these robust changes in mRNA expression to functional protein and enzymatic activity.

For example, p38 MAPK and AMPK phosphorylation sites modulating functional activity have been identified on the PGC-1α protein (Puigserver *et al.*, 2001;Jager *et al.*, 2007). If an exercise-induced increase in the activity of these kinases does in fact alter PGC-1α activity, one or more of these sites should be phosphorylated by exercise. The ability to examine these mechanisms is of course limited by the availability of methodologies such as, in this case, a primary antibody directed towards these sites.

7.4.2 Novel pathways/posttranslational modifications

We report the first data to demonstrate regulation of RIP140 and Sirt1 mRNA expression by acute exercise and exercise training, although a functional role in skeletal muscle metabolism or

adaptation to acute exercise or chronic training remains to be investigated. RIP140 has been proposed as a metabolic counterbalance to the effects of PGC-1α in regulation of metabolic homeostasis by acting on specific gene targets in common with PGC-1α (White *et al.*, 2008). The role of corepression in an exercise context has not been investigated. Sirt1 is a key regulator of skeletal muscle metabolism by regulating PGC-1α functional activity by its acetylation status (Lagouge *et al.*, 2006;Gerhart-Hines *et al.*, 2007). The growing body of evidence linking posttranslational modifications of factors, such as PGC-1 and FOXO1A among others, to metabolic regulation is worthy of investigation in the regulation of exercise-induced metabolic regulation.

As discussed in earlier chapters, the regulation of both Sirt1 and RIP140 activity is influenced by the cellular redox state via the NAD^+/NADH ratio (Rodgers *et al.*, 2008;White *et al.*, 2008). Fluctuations in this ratio are known to occur during exercise (Robergs *et al.*, 2004) and are potential contraction-induced metabolic signals (Hawley & Zierath, 2004). This suggests that the functional activity of these factors, rather than the expression, can also be modulated by contraction-induced mechanisms with possible downstream effects on gene expression. The role of cellular redox state in metabolic regulation at a transcriptional level requires further investigation in the context of exercise.

7.4.3 Identification of novel therapeutic targets

The identification of novel therapeutic targets if often cited as a meaningful outcome from studies on the molecular biology of exercise. However, the question should be asked “what does the identification of novel therapeutic targets really mean”?

Exercise as well as being a metabolic stressor and physiological stimulus to muscle plasticity also has the desirable outcome of ameliorating, short of ablating, metabolic dysfunction through favourable alterations in glycemic control, lipid profile and mitochondrial function. Alterations mediated by increases in exercise-mediated glucose disposal, possible clearing of lipid metabolism by-products/intermediaries, lipid oxidation, energy expenditure and mitochondrial biogenesis and oxidative phosphorylation gene expression. Exercise training alters substrate metabolism of working muscle but also the metabolic status of the resting muscle and improves metabolic flexibility. It seems logical to suggest that the induction of exercise-like responses in skeletal muscle through whatever means could be a panacea for the ills of metabolic dysfunction. Although targeting kinases activated by exercise may be one way to enhance the metabolic profile of a diseased muscle, the suitability of protein kinases as drug targets is debated (Coghlan & Smith, 2005). Currently, there are discrepancies between the exercise-induced alterations in gene expression and pharmacologically-induced effects (Jorgensen *et al.*, 2005).

Alternatively, the molecular genetic signature from acute exercise and training has been proposed as useful in predicting successful rehabilitation with a given disorder (Mahoney & Tarnopolsky, 2005). These authors suggest that transcriptome signatures could be used as a

standard by which to compare the exercise response from a variety of patient populations to identify which components of the response are altered by the disease in question. For example, insulin-resistant subjects have reduced responses of nuclear-encoded mitochondrial genes to exercise, and this could contribute to the origin and maintenance of mitochondrial dysfunction (De Filippis *et al*., 2008)

Genes that respond most robustly to exercise are regulatory genes (e.g. transcription factors, transcriptional coactivators), not effector genes (e.g. components of the electron transport chain, β-oxidation). According to Mahoney & Tarnopolsky (2005), therapeutically, it is advantageous to target genes that assert widespread effects on the multitude of cellular events that compose adaptation (e.g. mitochondrial biogenesis and elevated fat oxidation and glucose transport) as opposed to targeting individual genes within a given facet of adaptation.

7.4.4 Further investigations with the current tissue samples

There are several lines of enquiry that we intend to pursue with the remaining samples from this series of experiments, with a view to maximising the amount of knowledge garnered from human biopsy samples. Currently, there is both cDNA and whole muscle lysate available for most if not all experimental time points, which provides us with the means for further investigation to improve our current data set and investigate further as novel targets emerge.

Firstly as described above, methodological issues concerning the measurement of PGC-1α protein content must be addressed. Subject to confirmation of a commercially available and suitably validated PGC-1α antibody, or acquisition of an aliquot of antibody from the Spiegelman lab (Dana Farber Cancer Institute, Harvard Medical School, Boston MA), we will examine the protein content in samples from the training study. In addition, in light of a recent publication demonstrating rapid upregulation of PGC-1α protein during recovery from acute exercise (Mathai *et al*., 2008), we will return to the acute exercise samples from experiments I and II to investigate this observation in our samples.

Secondly, using the stored aliquots of whole muscle lysate, it will be po=ssible to investigate posttranslational modifications and protein-protein interactions under the experimental conditions described. We are currently developing a method to examine acetylation status by immunoprecipitating the protein of interest and probing for immunoreactive bands with an acetylated lysine antibody (#9441, Cell Signaling Technology). When optimised, this method will provide the first reported data on the effect of acute and regular exercise on the acetylation status of PGC-1α, RIP140 and FOXO1A. In addition, we intend to examine the colocalisation of PGC-1α and RIP140 in complex with transcriptional regulators such as NRF-1, NRF-2 and ERRα to investigate the role of transcription factor complex formation in the adaptive response to both acute exercise and exercise training.

Thirdly, given the role of AMPK in the regulation of skeletal muscle gene expression (section 2.6.2.2) and the robust activation by exercise, it is unsurprising that it has been implicated in the adaptive response to exercise training (Jorgensen *et al*., 2006). However, at present it is

unclear which AMPKα isoform is involved in the adaptation to training. Numerous studies have demonstrated that the α2 isoform is responsive to acute endurance-type exercise (Wojtaszewski *et al.*, 2000;Fujii *et al.*, 2000). However, the α1, but not α2 subunit, has been shown to be increased by exercise training (Frosig *et al.*, 2004). Given that the exercise training-induced response of several metabolic and mitochondrial genes is similar between wild-type and α2 AMPK knockout mice (Jorgensen *et al.*, 2007) but AMPK is required for mitochondrial biogenesis and the exercise-mimetic effects of β-GPA (Zong *et al.*, 2002), this suggests that AMPK isoform-specific regulation of training-induced responses remain to be investigated. With this in mind, we intend to measure the protein expression of the respective AMPK α-isoforms to explore this further.

Finally, allowing for the kinetic considerations and sample timing described in section 7.3 and further to the contention of Baar et al. (2002) (see section 7.3.3), we acknowledge that it would improve our results considerably to couple the measurement of mRNA abundance to alterations in protein content for each gene of interest. Currently, we are endeavouring to provide corresponding protein content measurements for each reported mRNA abundance, i.e. matching the transcript to the respective protein. For example, in experiment I, considering the mRNA abundance of PGC-1α, FOXO1A, PDK4 and RIP140 is robustly increased during the first 3 h of recovery from exercise, it will be interesting to examine the effect of the corresponding protein content during the same time period and up to 19 h after exercise.

7.5 Practical relevance

We have only measured a small number of genes in the context of a single bout of exercise but extrapolation of the intensity-dependent effect on the expression of selected genes after isocaloric exercise suggests that exercise training at a higher intensity for a shorter duration may promote greater magnitude of adaptations. This remains to be confirmed in an exercise training experiment. Previous research on rodents (Terada *et al.*, 2005) and humans (Gibala *et al.*, 2006;Burgomaster *et al.*, 2008) suggest that shorter duration, but higher intensity, training is at least as effective as more traditional endurance-type training in terms of the degree of adaptation. In a human study (Gibala *et al.*, 2006), during two weeks of training (six sessions) the total training volume for the high intensity group was only 10% that of the endurance training group (i.e. 150 vs. 1500 kcal). In terms of total training time, commitment over two weeks was 2.5 h for the high intensity group, whereas the endurance group performed continuous exercise each training day for a total of 10.5 h. Training adaptations in terms of maximal oxygen uptake and muscle oxidative capacity were not different between groups. Therefore, intense interval training is a time-efficient strategy to induce rapid muscle and performance adaptations comparable to traditional endurance training.

Our acute exercise results support the contention that reduced duration, high intensity exercise is as effective as traditional training (Coyle, 2005b;Baar, 2006). The stimulus for adaptation, particularly in terms of substrate metabolism and mitochondrial biogenesis, may be greater

when the intensity of the exercise training is higher. This study may provide a molecular insight into findings that first established this principle over a quarter of a century ago (Dudley *et al.*, 1982): “for the same adaptive response, the length of daily exercise necessary to bring about the change becomes less as the intensity of training increases”.

The most commonly cited reason for not exercising is a “lack of time” (Godin *et al.*, 1994). Training at higher intensities provides an alternative to current exercise prescriptions for time pressed individuals (Gibala & McGee, 2008). The price for the effectiveness and time-efficiency of training at a higher intensity is that it is very hard to get people to exercise at a high intensity for any period of time (Baar, 2006). It remains to be determined which populations, depending on age, health status, and psychology, are mostly likely to adhere and benefit from high intensity training.

Finally, we chose to limit the caloric expenditure of the exercise bout within a physiologically relevant range consistent with industry exercise guidelines (ACSM, 1998). High intensity sprint training does not utilize as many calories as longer duration endurance exercise (Gibala & McGee, 2008). This means that the efficacy of this type of training is reduced if the goal of training weight loss. However, high intensity endurance exercise may expend sufficient calories, require less time commitment and result in more pronounced cellular adaptations than low intensity recreational exercise.

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