The regulation of metabolic gene expression in human skeletal muscle by exercise: the influence of exercise intensity and contraction frequency

A thesis submitted to Dublin City University for the degree of Doctor of Philosophy in the Faculty of Science and Health
2010

By
Brian Carson BSc
School of Health and Human Performance

Supervisor
Dr. Donal J. O'Gorman
School of Health and Human Performance
Dublin City University

Submitted to Dublin City University September 22nd 2010

Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of PhD is entirely my own work, that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: _____ (Candidate)
Brian Carson

ID No.: 514722701

Date: _____

Acknowledgements

The author would like to acknowledge and thank the following for their invaluable contribution to this research.

Dr. Donal O'Gorman for his supervision, advice, dedication and understanding.

Our collaborators Prof. Juleen Zierath, Dr. Alexander Chibalin, Dr. Pablo Garcia-Roves, and Dr. David Kramer from the Section of Integrative Physiology at the Departments of Molecular Medicine and Surgery, and Physiology and Pharmacology at the Karolinska Institute, Stockholm, Sweden for spending the time and effort to pass on their expertise and their continued support.

Dr. Niall Barron at the National Institute for Cellular Biotechnology (NICB) at DCU for assistance with and facilitating the qPCR analysis

Dr. Ronan Murphy, Dr. Phil Cummins and the rest of the research team in the Vascular Health Research Centre at DCU for their assistance and support throughout this process.

Staff at the School of Health and Human Performance at DCU for their guidance, assistance and support with a special mention for Prof. Niall Moyna, Aisling Scally and Christine Stears.

Dr. Noel McCaffrey, School of Health and Human Performance at DCU, Dr. Gavin McHugh and Dr. Ray Walls, Cappagh National Orthopaedic Hospital, Dublin 11, for performing the muscle biopsies, and at all times being reliable, professional and most importantly, available.

Members of the Metabolic Physiology Research Unit at DCU, Brendan Egan, David Ashley, Diane Cooper and Adam McDonnell, technicians Paul O'Connor and Javier Monedero, and all the cardiovascular research group for providing excellent research support as well as their friendship.

All sixteen participants, without whom, this would not be possible, for having the interest to volunteer and the commitment to see the study out.

The Irish Research Council for Science, Engineering and Technology (IRCSET) for their funding which enabled me to carry out the research.

Finally, all my friends and particularly my family, who throughout this process offered me comfort, understanding, their love and full support. Mam, Dad, Robert and Amy, it is really appreciated and will not be forgotten.

Publications

Harrison M., O'Gorman D.J., McCaffrey N., Hamilton M.T., Zderic T.W., Carson B.P., Moyna NM (2009). *Influence of acute exercise with and without carbohydrate replacement on postprandial lipid metabolism.* Mar;106(3):943-9. J Appl Physiol.

Brady, S., Carson, B.P., O'Gorman, D.J., Moyna, N.M. and Diamond, D. (2006). *Combining Wireless with Wearable Technology for the Development of On-Body Networks.* Journal of Communications. 2, pp1-6.

Submitted:

Egan, B., Carson, B.P., Garcia-Roves, P.M., Sarsfield, F.M., Chibalin, A.V., Monedero, J., O'Connor, P., Moyna, N.M., McCaffrey, N., Zierath, J.R., O'Gorman, D.J. (2009). *Acute exercise increases expression of the transcription factor co-regulators PGC-1α and RIP140: implications for the regulation of gene expression regulation.* Manuscript under review at the American Journal of Physiology.

In preparation:

Carson, B.P., Egan, B., Garcia-Roves, P.M., Sarsfield, F.M., Chibalin, A.V., Moyna, N.M., Zierath, J.R., O'Gorman, D.J. *The influence of contraction frequency on PGC-1α and metabolic gene expression in human skeletal muscle.*

Egan, B., Carson, B.P., Garcia-Roves, P.M., Sarsfield, F.M., Chibalin, A.V., Monedero, J., O'Connor, P., Moyna, N.M., McCaffrey, N., Zierath, J.R., O'Gorman, D.J. *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.*

Abstract

The regulation of metabolic gene expression in human skeletal muscle by exercise: the influence of exercise intensity and contraction frequency

Skeletal muscle contraction increases energy expenditure and improves metabolic flexibility, but is also a key regulator of metabolic gene expression. Acute exercise stimulates a unique set of intracellular signalling cascades resulting in the activation of kinases which can subsequently control muscle metabolism. The signalling pathways involved are activated by alterations in ATP turnover, calcium flux, cellular stress and the redox state in the muscle cell. The aim of this thesis was to impact the main variables influencing muscle contraction, the contraction force and frequency, during an acute bout of exercise and to investigate the subsequent outcome of altering these variables on intracellular signalling and metabolic gene expression in human skeletal muscle.

High intensity isocaloric exercise (400 kcal, 80%VO_{2peak}) resulted in greater activation of the signalling kinases AMPK and CaMKII than low intensity exercise (40% VO_{2peak}), whereas the frequency of contraction (50 vs 80 RPM) had no effect on AMPK and CaMKII phosphorylation. PGC-1α mRNA was upregulated after exercise with a greater increase observed after high compared to low intensity. PGC-1α mRNA was also regulated by the frequency of contraction with a greater increase observed after exercising at a higher contraction frequency. Exercise induced a response in a number of metabolic genes associated with the regulation of substrate utilisation including FOXO1A and PDK4. This may form part of a transcriptional response to exercise that promotes fat oxidation and glucose sparing in the recovery from exercise.

These results suggest that an acute bout of exercise induces a transient response in intracellular signaling and metabolic gene expression in human skeletal muscle specific to the demands placed upon the tissue.

Abbreviations

ACC	acetyl CoA carboxylase
ADP	Adenosine diphosphate
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
AMP	Adenosine monohosphate
AMPK	adenosine monophosphate-activated protein kinase
AS160	Akt substrate at 160 kDa
ATF2	activating transcription factor 2
ATP	Adnosine triphosphate
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
COX	cytochrome c oxidase
CPT1	carnitine palmitoyltransferase 1
CRE	cyclic AMP response element
CREB	CRE binding protein
CtBP	carboxyl-terminal binding protein
DRIP	vitamin D receptor-interacting protein
EPOC	Excess post-exercise oxygen consumption
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
FFA	Free fatty acid
FOXO1A	forkhead transcription factor, O-box subfamily, 1A (a.k.a. FKHR)
G-6-P	Glucose-6-phosphate
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
HSL	Hormone sensitive lipase
IDH	Isocitrate dehydrogenase
IMTG	Intramuscular triglyceride
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
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
PDH	pyruvate dehydrogenase
PDK4	pyruvate dehydrogenase kinase 4
PFK	phosphofructokinase
PIC	pre-initiation complex
PK	pyruvate kinase
PKC	Protein kinase C
PKD	Protein kinase D
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
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
TG	Triglyceride
TRAP	thyroid hormone receptor-associated protein
UCP3	uncoupling protein 3
VO_{2peak}	maximal oxygen uptake
W_{max}	maximal workrate

Table of Contents

List of figures

List of Tables

Chapter I Introduction

1.1 General Introduction

The overall genetic make-up of *Homo Sapiens* has changed little in the last 10,000 years and I remain genetically predisposed to a pre-agricultural hunter-gatherer lifestyle (Eaton *et al.*, 1988); (Fernandez-Real & Ricart, 1999). Pre-agricultural hunter-gatherer societies were forced to undertake physical activity on a daily basis in order to provide necessities for survival such as food, water, shelter and clothing. Therefore, it is likely that we developed metabolic features capable of supporting a physically active lifestyle (Fernandez-Real & Ricart, 1999); (Wendorf & Goldfine, 1991). Current daily levels of physical activity are likely to be below the levels our genetically-determined physiology has evolved to carry out (Cordain *et al.*, 1998). Thus, a sedentary lifestyle is a disruption to the homeostatic mechanisms programmed for maintaining metabolic balance and subsequently the health of the population. There is evidence that physical inactivity increases the incidence of metabolic diseases, cardiovascular diseases, cancer, pulmonary diseases, immune dysfunction, musculoskeletal and neurological disorders (Booth *et al.*, 2002a). For the purpose of this thesis, the focus will be on the effects of exercise on metabolism.

1.1.1 Metabolic Dysfunction

Metabolic homeostasis requires the coordinated regulation of energy intake, storage and expenditure. With people leading increasingly sedentary lives, their lifestyles are giving rise to an imbalance in metabolic homeostasis resulting in the increased prevalence of Type 2 Diabetes (T2D), obesity and the metabolic syndrome. The onset of these disease states are preceded by resistance to the action of insulin, termed insulin resistance, which is present in almost all patients with T2D (DeFronzo *et al.*, 1982). Insulin resistance is associated with an impaired ability to utilise fats as a fuel source. Patients with T2D and obesity display reduced oxidative enzyme capacity, decreased fatty acid (FA) oxidation and inflexibility in regulating fat oxidation when compared with their lean, healthy counterparts (He *et al.*, 2001), (Simoneau & Kelley, 1997); (Kelley *et al.*, 1999). This impaired metabolism leads to increased accumulation of fat stores in the muscle (Intramuscular triglycerides, (IMTG)) (Goodpaster *et al.*, 2000).

1.1.2 Exercise as a therapeutic intervention

Physical activity and exercise represents a therapeutic strategy for the prevention and treatment of obesity and T2D. Exercise achieves this by providing a metabolic stress that acutely increases energy production and expenditure which immediately improves the balance of metabolic homeostasis. Furthermore, physical activity and exercise result in a number of beneficial metabolic adaptations including a decrease in insulin resistance and improved glucose tolerance in obese patients, increased mitochondrial size and number, and increased activity of Kreb's cycle, β-oxidation and the electron transport chain (Kelley *et al.*, 1999); (Holloszy & Coyle, 1984). Fat and carbohydrates are both utilised during contraction in skeletal muscle (Hargreaves, 2000). Exercise training also results in an increase in the relative contribution of fat to total oxidation during exercise (Goodpaster *et al.*, 2003). In addition, during the recovery from exercise, muscle metabolism primarily depends upon the oxidation of fat as a fuel source (van Loon *et al.*, 2003). Regular physical activity is therefore a critical tool in the treatment and primary prevention of these chronic diseases (Booth *et al.*, 2002b;Hawley, 2004).

1.1.3 Regulation of metabolic gene expression

Skeletal muscle contraction increases energy expenditure and improves metabolic flexibility, but is also a key regulator of metabolic gene expression. Acute exercise stimulates a unique set of intracellular signalling cascades resulting in the activation of kinases which can subsequently control muscle metabolism. The signalling pathways involved are activated by alterations in ATP turnover, calcium flux, cellular stress and the redox state in the muscle cell. These metabolic pathways act as a coordinated network to generate either an acute change in substrate utilisation or a transcriptional metabolic adaptation in response to exercise. Transcriptional control of skeletal muscle metabolic pathways by intracellular signalling cascades is achieved by the action of numerous transcription factors and transcriptional co-regulators by translating these signals to a specific subset of genes in response to exercise.

An acute bout of exercise can result in transient increases in the mRNA of genes regulating these metabolic processes. However, the molecular mechanisms through which exercise induces these proteins are not fully elucidated as of yet. This information has practical relevance for maintaining metabolic health. Knowledge of the molecular and cellular events that regulate skeletal muscle plasticity can define the potential for

adaptation in metabolism and may lead to the discovery of novel pathways in common clinical disease states (Hawley & Holloszy, 2009). Much of this work has previously been carried out in animal and cell models, but there is a lack of analysis of these processes in humans. The aim of this thesis is to further the understanding of the coordinated regulation of genes controlling metabolism and, in particular, substrate utilisation in response to an acute bout of exercise. This thesis aims to further our knowledge of the transcriptional response to acute exercise by investigating the modulation of gene expression in human skeletal muscle under varying physiological conditions, including alternate contraction frequencies and divergent exercise intensities. This is important because understanding the biochemical, molecular and cellular mechanisms of physical activity in the prevention of metabolic disease will provide the scientific foundation for appropriate individual prescription of physical activity for health (Booth *et al.*, 2000).

1.2 Thesis overview: aims, objectives and hypotheses

This thesis will examine the regulation of metabolic gene expression in human skeletal muscle following an acute bout of exercise. The main variables influencing muscle contraction are the force and the frequency of contraction. This research investigated the outcome of altering these variables during an acute bout of exercise on metabolic gene expression. Firstly, the effect of divergent exercise intensities on contraction-activated signalling cascades and subsequent gene expression of metabolic genes in untrained males will be described. Finally, the effect of altering the calcium transients of the muscle by varying the rate of contraction frequency during cycling exercise on contraction-induced signalling cascades and subsequent metabolic gene expression in untrained males will be investigated.

1.2.1 Experiment I

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

1.2.1.1 Overview

Eight healthy, sedentary males performed two isocaloric (400 kcal) cycle exercise trials at 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.

1.2.1.2 Specific aims

(i) To examine the potential for differential activation of contraction-induced signal transduction pathways in response to divergent exercise intensities in human skeletal muscle.
(ii) To investigate the impact of exercise intensity on the expression of genes involved in mitochondrial and substrate metabolism in skeletal muscle.

1.2.1.3 Hypothesis

(i) There will be greater activation of contraction-induced signalling cascades (AMPK, CaMKII) and subsequent expression of genes involved in metabolism and substrate selection (PGC-1α, FOXO1A, PDK4) after a single bout of high-intensity compared to low-intensity isocaloric exercise.

1.3.1 Experiment II

The impact of contraction frequency during an acute bout of exercise on the expression of genes involved in metabolism and substrate selection in human skeletal muscle.

1.3.1.2 Overview

Eight healthy, sedentary males performed two isocaloric (400 kcal) cycle exercise trials at identical power outputs, eliciting approximately 50% VO_{2peak}, pedalling at a cadence of either 50 RPM or 80 RPM. Skeletal muscle biopsies from the *m. vastus lateralis* were taken at rest and at +0 h, +3 h and +19 h after exercise.

1.3.1.3 Specific aims

(i) To investigate the effect of altering the calcium transient, by varying the frequency of contraction, on the activation of the contraction-induced signalling cascades in human skeletal muscle in response to an acute bout of exercise.
(ii) To examine the effect of the frequency of contraction during an acute bout of exercise on the expression of genes involved in metabolism and substrate selection in human skeletal muscle.

1.3.1.4 Hypothesis

(i) There will be greater activation of the calcium-induced signalling cascades (CaMKII in particular) in response to cycling exercise with a greater frequency of contraction.

(ii) Cycling exercise at a higher contraction frequency will result in greater induction of the metabolic genes regulated by the calcium-activated signalling pathways compared with low-contraction frequency.

1.4 Limitations

The experiments described within this thesis have a number of limitations. Firstly, carrying out human research carries with it a lot of difficulties. For example, the number of biopsies that could be performed as well as the amount of tissue that could be extracted were limited due to ethical considerations. This limited the analysis in terms of the number and type of laboratory techniques that were carried out. The invasive nature of the study and the time commitment involved made subject recruitment a difficult and lengthy process. This, in turn, made the task of scheduling subjects and the research team (including Doctor qualified to perform biopsies) difficult.

Controlling for variability in a human population provided a tough task. It is important to control for factors such as diet and activity and several measures were taken to ensure this (see Methodologies), however, it is impossible to say conclusively if subjects followed all directions accurately or reported behaviour outside the laboratory honestly.

There were some issues with the analysis. A considerable period of time was dedicated to developing a technique which had not previously been reported in human muscle samples. Eventually it became apparent that the antibody being used may not actually bind the correct target which could explain, in part at least, the difficulties encountered with setting up the technique. This is discussed in further detail in the methodologies section.

Chapter II Review of Literature

2.1 Muscle metabolism and ATP production

2.1.1 General Introduction

To understand the relationship between exercise and metabolic gene expression in muscle one must first have a background knowledge in the cellular processes involved in energy storage, production and use and how exercise affects these processes. In this chapter the systems capable of producing energy for muscle contraction as well as the exercise-related factors affecting these systems will be reviewed. A review of the intracellular signalling mechanisms involved in the control of energy production and consumption and the effect of exercise on which fuels are utilised under varying conditions will also be included. The biochemical processes that facilitate the adaptation of the muscle and the main gene players that regulate muscle metabolism will also be reviewed in detail. Finally, the impact of exercise on intracellular signalling and gene targets, and ultimately the adaptation of metabolism in human skeletal muscle will be reviewed in detail.

2.1.2 Cellular Bioenergetics

Energy is defined as the ability to perform work and is required for all physiological processes. Human movement occurs following the conversion of chemical energy, derived from nutrients, to mechanical energy for skeletal muscle contraction. Each cross-bridge cycle in skeletal muscle requires hydrolysis of adenosine triphosphate (ATP) by myosin ATPase in order for muscle contraction to occur. The muscle cells require a large and continuous supply of ATP for exercise. Intramuscular stores of ATP are very limited (~5mmol/kg wet weight) and is thought to be owed to the fact that many metabolic processes are highly sensitive to ATP, ADP and AMP levels. This sensitivity enables the muscle to produce a rapid response to replenish ATP stores. There is only enough ATP stored in the muscle to sustain maximal exercise for a couple of seconds; therefore, prolonged exercise requires rapid resynthesis of ATP from ADP.

In addition to a limited store of ATP in the muscle, there is also a limited store of phosphocreatine. Skeletal muscle contains about 3-4 times more phosphocreatine than ATP (20mmol/kg ww). The fasted method to resynthesise ATP involves the donation of a phosphate group from Phosphocreatine (PC) to ADP, catalysed by the enzyme creatine kinase. When ATP is split into ADP + Pi to provide energy for muscle contraction, the increased concentration of ADP in the cell stimulates creatine kinase to break down PC and resynthesise ATP. This sensing of ADP levels acts as a crucial feedback mechanism for rapidly forming ATP when stores are depleted. However, there are only small amounts of PC stored in a muscle cell so the total amount of ATP that can be produced by this reaction is limited (less than 5 seconds). This process, referred to as the Creatine Phosphate System, functions to maintain ATP homeostasis during contraction at the expense of phosphocreatine. This system is generally utilised at the onset of exercise or during high-intensity exercise of a short duration, and crucially, does not require oxygen to proceed (Bessman & Carpenter, 1985).

Figure 2.1 Representative diagram of ATP-PCr reaction.

For exercise to continue following the depletion of the limited ATP and phosphocreatine stores, ATP must be resynthesised. As energy is not readily available in the cell, the breakdown of energy from substrate stores is required.

2.1.2.1 Sources of Substrate for ATP production

Fat and carbohydrates are the primary metabolic substrates utilised during contraction in skeletal muscle (Hargreaves, 2000). While there are circulating FFAs, fat is mainly stored as a fuel reserve in the form of triglycerides (TGs) in adipocytes and in muscle as IMTG. Carbohydrate is stored as glycogen in the muscle and liver, and circulates as plasma glucose (Watt *et al.*, 2003a). Muscle glycogen provides a direct source of carbohydrate for muscle energy metabolism and is the most readily available and preferentially used source of carbohydrate. Muscle glycogen undergoes glycogenolysis which is the breakdown of glycogen to Glucose-6-Phosphate (G-6-P), a process controlled by the activation of the rate-limiting enzyme glycogen phosphorylase. Following breakdown from muscle glycogen, G-6-P enters glycolysis for production of ATP.

Fat is mainly stored as a fuel reserve in the form of TGs in adipocytes and in muscle as IMTG. There is also a certain concentration of circulating fre fatty acids (FFAs) in the blood. Lipoprotein lipase (LPL) hydrolyses TGs to release FFAs into the plasma or sarcoplasm for transport to the mitochondria for oxidation (Hargreaves, 2000). IMTGs are usually located adjacent to the muscle mitochondria suggesting they act as a source of stored FFA for muscle contraction (Hoppeler *et al.*, 1985). Following release of FFAs into circulation, their uptake into the cell occurs at the plasma membrane for subsequent oxidation in the mitochondria via β-oxidation as described earlier (Rasmussen & Wolfe, 1999). Glycogenolysis in the liver leads to an increase in circulating plasma glucose; whereas in the muscle following breakdown from glycogen, G-6-P enters glycolysis for production of ATP as described earlier. Circulating glucose uptake into the muscle cell is regulated by insulin at rest, as insulin binds to its receptor to activate the PI 3-Kinase pathway which stimulates glucose transporter 4 (GLUT4) translocation to the plasma membrane for subsequent glucose uptake into the muscle (Ryder *et al.*, 2001).

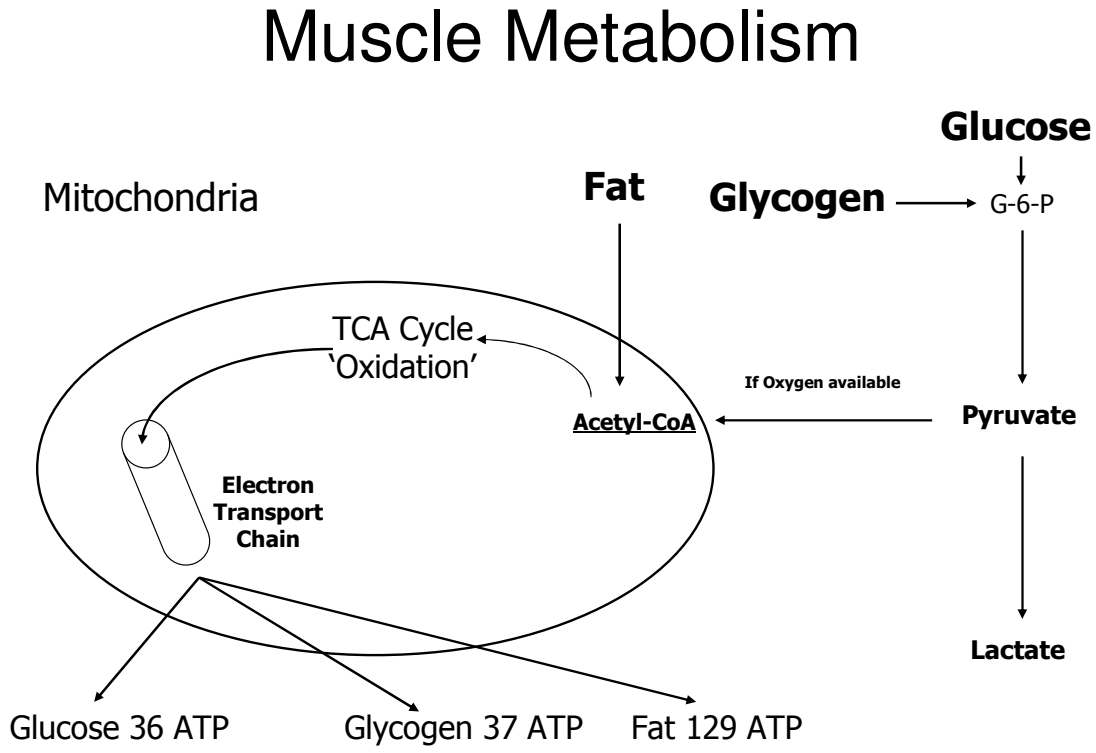

Figure 2.2 Overview of metabolic processes in muscle

2.1.2.2 Anaerobic metabolism

2.1.2.2.1 Glycolysis

Energy can be derived in both the presence and absence of oxygen by aerobic or anaerobic metabolism. Glycolysis is an anaerobic pathway to break down glucose and glycogen to form two molecules of pyruvate or lactate. This process produces a net gain of two molecules of ATP and two molecules of pyruvate per molecule of glucose. If muscle glycogen is the starting point for glycolysis, a single glucose molecule, glucose 1-phosphate, must be split from the large glycogen molecule and this reaction is catalysed by glycogen phosphorylase. Glycogen phosphorylase adds a phosphate group to the glucose molecule, effectively trapping it in the cell. The activity of glycogen phosphorylase is regulated by the release of calcium during contraction. In this way, breakdown of glycogen is only activated when the energy demand is high, but is rapid in response to contraction. Glucose 1-phosphate is quickly converted to glucose 6-phosphate. Alternatively, if the starting point of glycolysis is blood glucose, the glucose transporter (GLUT 4) takes glucose into the muscle where a phosphate group is added to glucose at the expense of ATP by hexokinase to form glucose 6-phosphate. A series of biochemical reactions occur, as outlined in Fig. 2.4, with each molecule of 1,3-diphosphoglycerate producing 2 ATP and 1 pyruvate, for a total of 4 ATP and 2 pyruvate molecules. This is a net gain of 2 or 3 ATP depending on whether glucose or glycogen was the starting point. Under anaerobic conditions, pyruvate accepts the hydrogens from NADH to form lactic acid. However, if sufficient O_2 is available, NADH is shuttled to the mitochondria for later production of ATP. When O_2 is available, pyruvate dehydrogenase (PDH) catalyses the decarboxylation of pyruvate to Acetyl CoA, which enters the Tricarboxylix Acid Cycle (TCA) cycle. In this case, glycolysis is no longer an anaerobic process but is the first step in the aerobic breakdown of carbohydrates.

2.1.2.2.2 Aerobic Metabolism

The energy systems we have described so far represent ATP production in the absence of oxygen, also known as anaerobic metabolism. However, anaerobic metabolism cannot continue for a sustained period of time as stores of ATP and phosphocreatine are limited. Anaerobic metabolism of glucose will result in the accumulation of lactate, reducing the ability to perform exercise. Prolonged exercise requires constant

resynthesis of ATP at a rate matching its consumption. For this to occur, the availability of oxygen is essential. We will now describe the processes involved in aerobic metabolism.

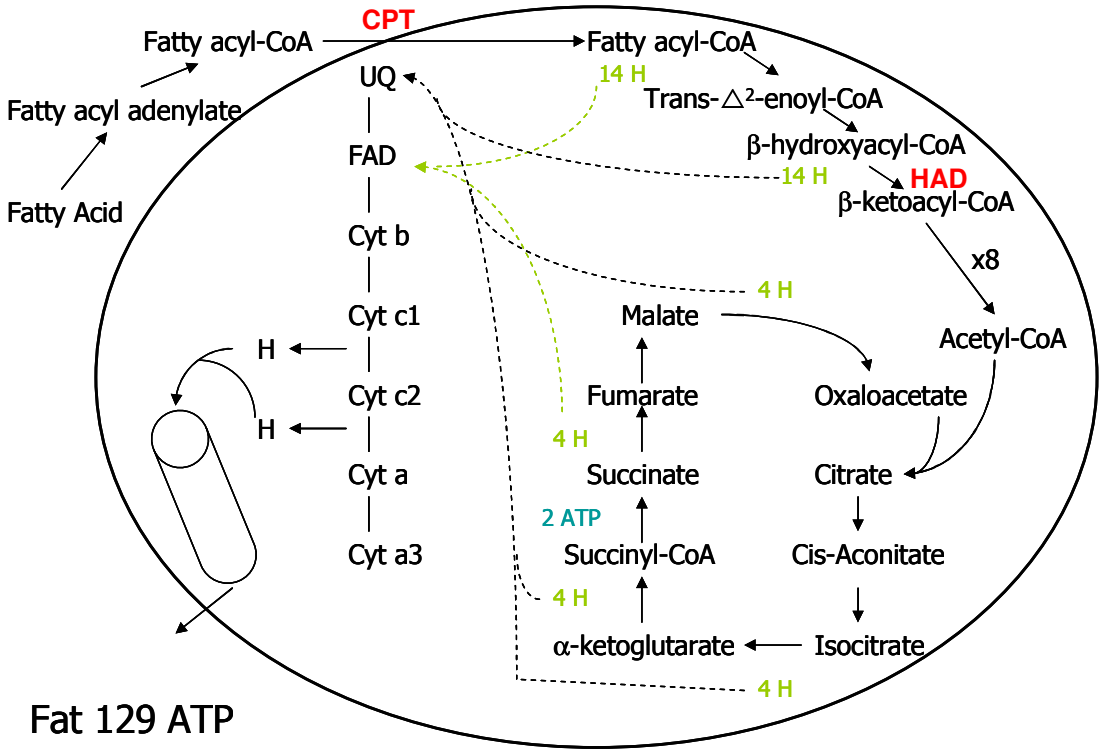

Figure 2.3 Diagram of β-oxidation and ATP production from fat sources

***2.1.2.2.3 β-oxidation*:** FFAs are the primary source of lipids used for oxidation during exercise. Lipolysis of adipose tissue triglycerides must occur to mobilise FFAs, allowing their subsequent uptake into the muscle for oxidation to provide ATP for contracting muscle cells. The rate-limiting enzyme in adipose tissue lipolysis is Hormone Sensitive Lipase (HSL) (Rasmussen & Wolfe, 1999). HSL is regulated by the hormones epinephrine and insulin. Increased concentrations of epinephrine stimulate lipolysis through phosphorylation of HSL whereas an increase in insulin is inhibitory. Upon activation, HSL hydrolyses two FAs from the glycerol backbone of TGs with the third FA being hydrolysed by monoglycerol lipase. The three FFAs leave the adipose tissue and enter circulation where they bind to albumin. These FFAs are then taken up by the muscle at the plasma membrane by the FA binding protein FAT/CD36. FFA uptake into muscle only occurs if the concentration of intacellular FFA is less than the extracellular concentration. Once FFAs have entered the cell they are bound by the FA-binding protein (FABP). Acyl-CoA synthetase then esterifies the FFAs to fatty acyl-

CoA with the hydrolysis of one molecule of ATP. Following esterification, carnitine and the enzyme carnitine palmitoyltransferase I (CPT-I) mediate fatty acyl-CoA transport through the inner membrane of the mitochondrion. Malonyl-CoA is a potent inhibitor of CPT-I, the rate limiting enzyme in mitochondrial FA uptake. Acetyl-CoA carboxylase (ACC), activated by citrate, increases malonyl-CoA and inhibits CPT-1 when ATP levels are high (Winder & Hardie, 1996). However, ACC is phosphorylated and deactivated by an increase in the AMP:ATP ratio, allowing acyl-CoA enter the mitochondria. Once inside the mitochondrial matrix the enzymes of the β-oxidative pathway act on fatty acyl-CoA resulting in the production of Acetyl CoA and 2 pairs of hydrogen atoms. The FA can re-enter the β-oxidative pathway creating 1 Acetyl CoA and 2 pairs of hydrogen atoms after each cycle. Palmitate, a long chain FA, undergoes a total of 7 cycles to yield a total of 8 molecules of Acetyl-CoA and 14 pairs of hydrogen atoms. The 14 pairs of hydrogen atoms enter the electron transport chain as FADH2 and NADH. If sufficient oxygen is available, Acetyl-CoA then enters the TCA cycle for the production of ATP via oxidative phosphorylation (Rasmussen & Wolfe, 1999).

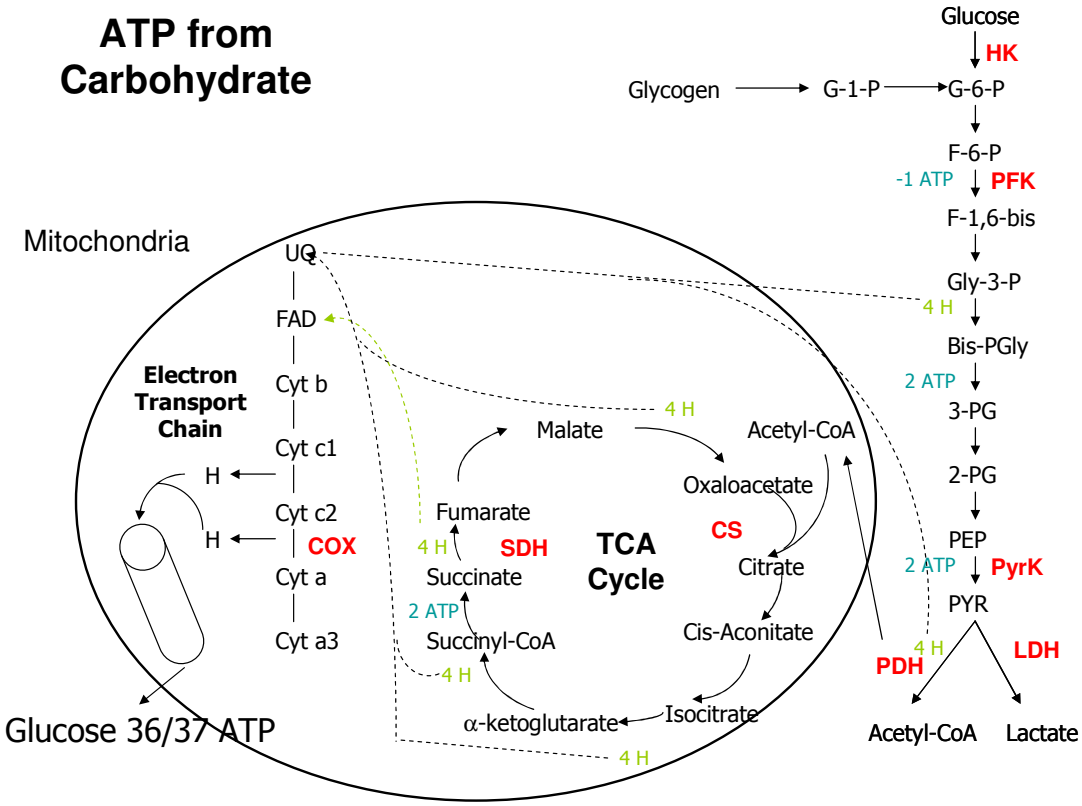

Figure 2.4 Diagram of Glycolysis and ATP production from carbohydrate sources

2.1.2.2.4 ***TCA Cycle***: The tricarboxylic cycle (TCA cycle) takes place in the inner mitochondrial matrix of the mitochondria. This involves the oxidation of Acetyl-CoA, the common product of carbohydrate and fatty acid breakdown, to CO_2 with the production of hydrogen ions for their subsequent passage to the electron transport chain. Acetyl CoA is combined with oxaloacetatic acid (OAA) and catalysed by the enzyme citrate synthetase to produce citric acid. For each Acetyl-CoA that enters the cycle, 3 molecules of NADH, 1 molecule of FADH and 1 ATP are formed. NADH and $FADH_2$ are transported to the electron transport chain. Isocitrate dehydrogenase (IDH), which catalyses the conversion of isocitrate to α-ketoglutarate with the removal of a hydrogen ion, is the rate-limiting step of the TCA cycle. IDH is allosterically stimulated by ADP and inhibited by ATP (Stanley & Connett, 1991). This shows that the energy producing systems are under tight regulatory control in the production of ATP.

***2.1.2.2.5 Electron Transport Chain*:** The ETC is located on the inner mitochondrial membrane and consists of a series of cytochromes, iron-protein electron carriers. These cytochromes each pass the electrons carried by the hydrogen atom carriers NADH and $FADH_2$ along the inner membrane of the mitochondria. The oxidised iron portion of each cytochrome is reduced upon accepting an electron. This reduced iron donates its electron to the next cytochrome and so on down the membrane. Electrons are carried from Complex I to Complex III by coenzyme Q and from complex III to complex IV by cytochrome c. By shuttling between the oxidised and reduced state, the cytochromes transfer electrons to ATP-synthase (Complex V) where they reduce oxygen to form H_2O as a by-product. For each pair of hydrogen atoms, 2 electrons flow down the chain and reduce one molecule of oxygen to water. As the electrons are passed down to Complex V, energy is released and is coupled to form ATP from ADP and Pi. For each NADH entering the mitochondria 3 pairs of H^+ are pumped out and for each FADH entering 2 pairs of H+ are pumped out. The movement of hydrogens from the inner mitochondrial space to the matrix through an inner mitochondrial channel causes a region of decreased pH and positive charge to be created outside the mitochondria. This activates ATP synthase which phosphorylates ADP to ATP. NAD^+ and FAD are now available to accept hydrogen ions during glycolysis and the TCA cycle again. The complete aerobic oxidation of one molecule of glucose and glycogen is 36 and 37 ATP respectively; whereas the complete oxidation of palmitate yields 129 ATP. If an adequate supply of oxygen is not available there will be a decrease in the supply of

reduced NAD^+ and FAD and the ETC will not be able to generate ATP by cytochrome oxidase.

2.1.3 Exercise Metabolism

The immediate source of energy for contraction in the muscle cell is the high-energy phosphate compound ATP (Tullson & Terjung, 1991). As discussed earlier, muscle cells store limited amounts of ATP so a number of metabolic pathways exist to produce ATP when required for muscle contraction. At the onset of exercise, skeletal muscle requires an immediate increase in ATP production for muscular contraction. Oxygen consumption increases rapidly to resynthesise ATP aerobically, but there is a lag in oxygen uptake at the beginning of exercise, termed oxygen deficit, where ATP production is anaerobic (Medbo *et al.*, 1988). When oxygen consumption reaches steady state, ATP is produced aerobically by oxidative phosphorylation (Hultman, 1973) and the rate of ATP production can be maintained for a prolonged period of time, depending on substrate and oxygen availability. However, if the intensity of exercise is greater than ~75% VO_2max, there is a slow rise in oxygen consumption over time owing to increased body temperature and increasing levels of epinephrine and norepinephrine (Brooks *et al.*, 1971); (Gladden *et al.*, 1982); (Harris *et al.*, 1976).

As discussed above, there are several energy systems utilised during exercise. This review will focus on aerobic metabolism. During exercise, fat and carbohydrate are the major substrates used for energy supply as proteins contribute less than 2% of the substrate used during exercise of less than 1 h in duration (Hood & Terjung, 1990). Whether fat or carbohydrate is the primary source of fuel in working muscle depends on several factors including diet and the intensity and duration of exercise.

2.1.3.1 Exercise Intensity and Fuel Selection

As the intensity of exercise increases there is a progressive shift from mainly fat to predominately carbohydrate metabolism (Brooks & Mercier, 1994). Plasma FFAs have been shown to be the major substrate during low (25% and 28 VO_2max) and moderate intensity exercise (65% VO_2max) (Romijn *et al.*, 1993); (Klein *et al.*, 1994). A similar study compared different intensities and found that during exercise at 40% of maximal workload (Wmax) fat was the primary fuel source, but there was an intensity-dependent shift to carbohydrate at 55% and 75% Wmax (van Loon *et al.*, 2001). This shift results

in a decrease in both the relative and absolute contribution of fat oxidation. Fat oxidation peaked at 55% Wmax, but a decrease in the oxidation rates of plasma FFA and TG stores resulted in a decline in lipid metabolism at 75% Wmax (van Loon *et al.*, 2001). This evidence of lower fat oxidation with increasing exercise intensity has been supported by others, including a study comparing cycling at 40% and 80% VO_2peak (Sidossis *et al.*, 1997). Much work has been done to elucidate the exercise intensity that elicits the maximal rate of fat oxidation. In cycling, the maximal rate of fat oxidation was found to occur at ~64% VO_2peak, with maximum rates of 0.60 $g \cdot min^{-1}$ (Achten *et al.*, 2002). The respiratory exchange ratio, which indicates the substrate source, has been shown to increase linearly with increments in power output, signifying greater reliance on carbohydrate metabolism at higher power outputs (Howlett *et al.*, 1998); (Odland *et al.*, 1998). At 90% VO_2peak, the respiratory exchange ratio (RER) was greater than 1, which suggests ATP production was solely dependent on carbohydrate oxidation at this intensity. In agreement with this, the same studies showed PDH activation increased as a function of power output, indicating greater carbohydrate breakdown as PDH catalyses the conversion of pyruvate to acetyl-CoA (Howlett *et al.*, 1998); (Odland *et al.*, 1998).

This apparent shift from fat to carbohydrate metabolism at higher exercise intensities may be owed to an increase in glycogenolysis. At these intensities, muscle glycogen becomes the primary source of substrate. Romijn et al. (1993) found that whole-body fat and plasma FFA oxidation rates declined during high-intensity exercise (85% VO_2max) when muscle glycogen became the primary source of fuel and carbohydrate oxidation increased in line with exercise intensity. Similar results were seen by van Loon et al. (2001) where muscle glycogen and plasma glucose oxidation rates increased as the exercise intensity increased from 40% to 55% and 75% Wmax. Muscle glycogen becomes the primary source of substrate for ATP production above 50% of Wmax (van Loon *et al.*, 2001); (Romijn *et al.*, 1993). Muscle glycogen utilisation is greater during 80% VO_2peak compared with cycling at 40% VO_2peak even though energy expenditure was no different between trials (Kraniou *et al.*, 2006). These results are supported by Vollestad and Blom who observed greater depletion of muscle glycogen stores with increments in intensity (Vollestad & Blom, 1985).

In addition to the switch in substrate utilisation from fat to carbohydrate with increased exercise intensity, there is also a change in the lipid source. Romijn et al. (1993) showed

that peripheral lipolysis was high at 25% VO_2max and did not change with increasing intensity, unlike the lipolysis of IMTG which increased with the rise in intensity to peak at 65% VO_2max. This coincided with a decrease in FFA oxidation and an overall decline in fat metabolism (Romijn *et al.*, 1993). In support of these findings, moderate intensity exercise (60-70% VO_2max) decreased IMTG by 10-36% in both the soleus and tibialis anterior of runners; however, running at 83-85% VO_2max for approximately the same time saw no change in IMTG stores in either muscle (Brechtel *et al.*, 2001).

As the intensity of exercise rises, there is a greater demand for the delivery of ATP to the working muscles. The ability to take up oxygen into the muscle is limited. VO_2max varies between individuals and is dependent on a number of factors such as training status, body composition and genetics (McArdle *et al.*, 2006). VO_2max indicates an individual's capacity for aerobically synthesising ATP. Since the availability of oxygen is limited, the muscle requires the most efficient mechanism of ATP production. Oxidative metabolism of carbohydrate, muscle glycogen in particular, provides the most efficient supply of ATP to the muscle as more energy is released per litre of oxygen used (Jeukendrup & Wallis, 2005). Glycogen provides 5.02 kcal per litre of oxygen compared with an average fatty acid which only provides 4.85 kcal (Jeukendrup & Wallis, 2005). It is for this reason, the increased requirement of rapid delivery of ATP, that there is a progressive switch to carbohydrate metabolism at increasing exercise intensities.

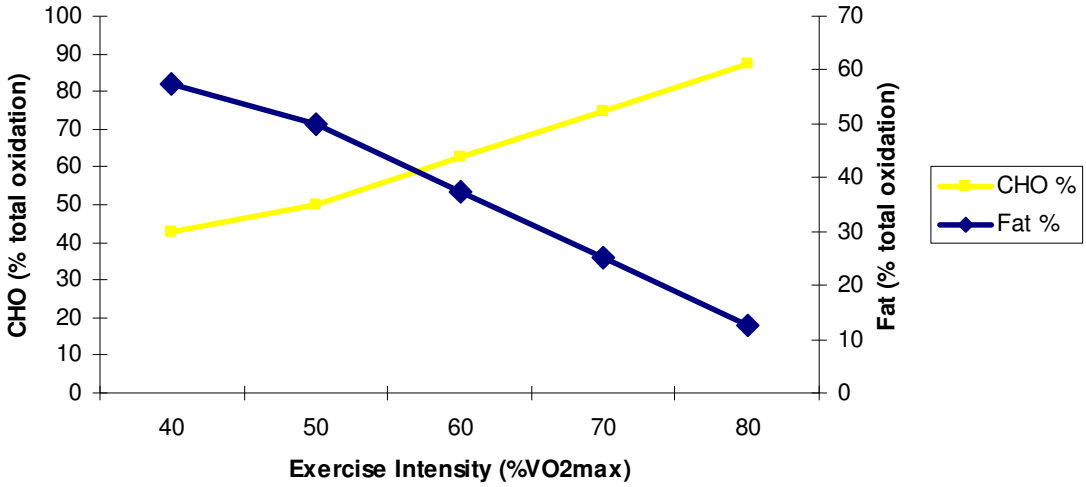

Figure 2.5 Relative contribution of carbohydrate and fat energy sources during exercise of increasing intensity. Adapted from Holloszy et al. (1998).

2.1.3.2 Exercise Duration and Fuel Selection

The duration of exercise can also affect the substrate source utilised by the muscle. During moderate-intensity exercise (65% VO_2max) over 2 hours there is a progressive decline in the relative proportion of energy produced from muscle glycogen and IMTG to an increase in plasma FFA oxidation (Romijn *et al.*, 1993). In support of this, cycling at 60% of VO_2peak for 2 hours resulted in an increase in FFA oxidation rates with a concurrent decline in oxidation of muscle glycogen and 'other fat sources' (thought to be IMTG in TypeI fibers) (van Loon *et al.*, 2003). In this study there was also a gradual increase in plasma glucose utilisation suggesting a duration depedent shift towards the use of energy from extramuscular sources. In support of this, muscle glucose uptake during exercise increases with the duration of exercise (Katz *et al*., 1991). Krssak *et al*. (2000) found similar results during exercise at 65-70% VO_2peak over 2 hours duration (Krssak *et al.*, 2000). Glycogen and IMTG depleted over the course of the trial with a decrease in the rate of glycogenolysis towards the end of exercise while simultaneously FFA oxidation increased throughout exercise. In addition, a study in trained males who cycled for 240 minutes at 57% VO_2peak, reported a significant increase in plasma FFA concentration after 90 minutes compared with rest and this continued to rise until exercise cessation (Watt *et al.*, 2002). Furthermore, IMTG levels were reduced after 120 minutes but there was no further reduction after 240 minutes. Glycogen stores declined at 120 and 240 minutes. Interestingly, fat accounted for almost 40% of the energy expended during the first half of exercise, whereas fat supplied just less than 60% in the latter half (Watt *et al.*, 2002). As the duration of exercise increases, muscle and hepatic glycogen stores become depleted resulting in greater reliance on FA oxidation. As fatty acid oxidation requires approximately 2.5 times the amount of oxygen, there is an upward drift in oxygen consumption to maintain the rate of ATP production (Jeukendrup & Wallis, 2005). This often results in fatigue as there is a limit to oxygen consumption rates (Maughan & Gleeson, 2004) .

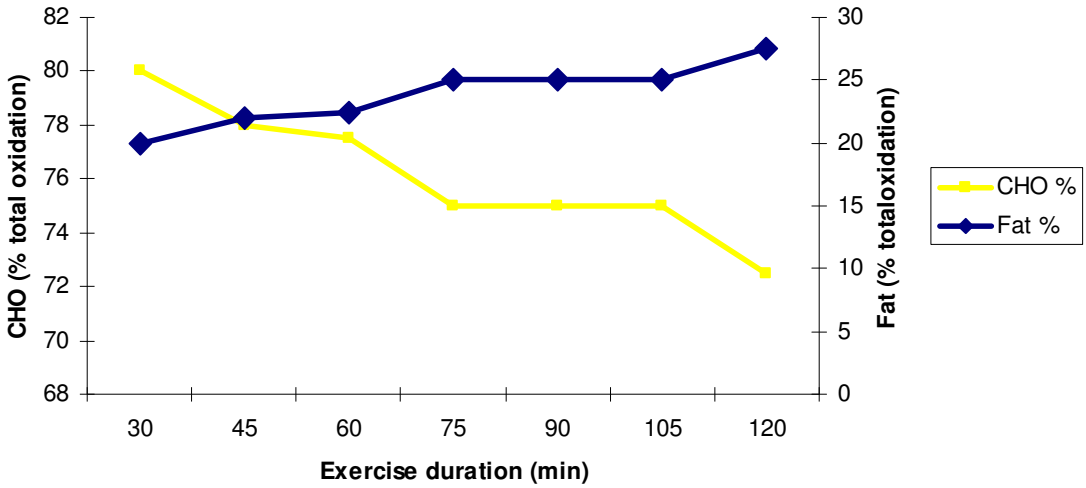

Figure 2.6 Relative contribution of carbohydrate and fat energy sources during exercise. Adapted from Van Loon et al (2003).

2.1.3.3 Fuel selection during recovery from exercise

For a period of time after exercise, oxygen consumption remains above resting levels, termed "Excess Post-Exercise Oxygen Consumption" (EPOC) (Gaesser & Brooks, 1984). This sustained increase in metabolism contributes to the energy cost of exercise. The degree of EPOC is dependent upon the intensity and duration of the preceding exercise. EPOC has been shown to increase with a rise in intensity when the total work is equated between trials (Dawson *et al.*, 1996); (Phelain *et al.*, 1997); (LaForgia *et al.*, 1997). In a treadmill study where subjects ran for 30 minutes at 70% VO_2max or 20 X 1 minutes at 105% VO_2max, the higher intensity exercise resulted in a two-fold increase in EPOC over the subsequent 9 hours post exercise (LaForgia *et al.*, 1997). EPOC increases linearly with exercise duration (Bahr *et al.*, 1987), (Imamura *et al.*, 2004); (Chad & Wenger, 1988); (Knuttgen, 1970). Sixty minutes of cycling at the same intensity (60% VO_2peak) resulted in a two-fold increase in EPOC compared with the thirty-minute exercise bout (Imamura *et al.*, 2004). This increase in metabolism, post exercise, allows the body to respond to the metabolic perturbation of exercise and a return to energy homeostasis. EPOC supplies energy for phosphagen restoration, lactate metabolism and TG/FA cycling in the muscle following exercise (Bahr, 1992); (Bahr *et al.*, 1990); (Trost *et al.*, 1997).

During recovery from exercise, muscle metabolism primarily depends upon the oxidation of fat as a fuel source. In the 2 h following exercise fat sources provided 75% of the total energy expended (van Loon *et al.*, 2003). FFA availability and oxidation rates increase above resting levels, peaking 15 minutes after exercise and may be responsible for the switch to fat as the major substrate (van Loon *et al.*, 2003). Similarly, cycling for 3 hours at 44% VO_2peak resulted in a decrease in RER suggesting a switch to greater fat metabolism and this was supported by an increase in FFA uptake (Mourtzakis *et al.*, 2006). Kimber *et al*. (2003) reported a similar decrease in RER and a corresponding increase in plasma FA concentration following exercise to exhaustion (Kimber *et al.*, 2003). Furthermore, Kiens & Richter (1998) witnessed a similar RER and an increase in FFA concentration which was maintained for 2 h post exercise. However, it was also reported that there was a significant decrease in IMTG in the post-exercise period suggesting an intramuscular contribution to the increase in fat metabolism (Kiens & Richter, 1998). This is in contrast with the findings of Kimber *et al*. (2003) who did not find a decrease in IMTG. In fact, IMTG levels have been shown to be resynthesised rather than oxidised in the post-exercise period (Krssak *et al.*, 2000). This may result from the increased lipolytic rate, FFA mobilisation and plasma TG in the post-exercise period (Mulla *et al.*, 2000); (van Loon *et al.*, 2003).

The increase in fat metabolism after exercise coincides with the resynthesis of glycogen. Muscle glycogen is replenished from its nadir post exercise back to resting levels in 30 hours post exercise (Kiens & Richter, 1998). Kimber *et al*. (2003) also reported similar glycogen restoration rates following exercise to exhaustion. The switch from carbohydrate to fat metabolism may drive the resynthesis of glycogen in an effort to return to metabolic homeostasis.

The regulation of substrate utilisation during and in response to exercise has been studied for over a century. However, it is only in more recent years that we have been able to investigate the regulatory mechanisms in skeletal muscle. One of the important advances made has been the identification and characterisation of intracellular signalling cascades that are activated by changes in the bioenergetic status of the cell or by muscle contraction. The next section of this review will focus on the action and regulation of these signalling cascades.

2.2 Intracellular signalling and the regulation of metabolism

Muscle contraction activates a unique set of intracellular signalling cascades that regulate muscle metabolism. The major signalling pathways involved are activated by (i) the rate of ATP turnover, (ii) calcium flux, (iii) cellular stress, and (iv) the redox state in the muscle cell. These metabolic pathways do not act independently and respond to exercise in a coordinated manner to regulate substrate utilisation or transcriptional activity. For the purpose of this review each of the signalling pathways will be defined in terms of their structure, function and activation by exercise independently. The consequences of the activation of these signalling cascades on metabolic processes and transcriptional regulation will be described collectively.

2.2.1 AMPK Signalling cascade

2.2.1.1 ATP Turnover

The important role for ATP in energy expenditure has been previously described. Almost all energy-requiring processes in the cell are driven, either directly or indirectly, by the hydrolysis of an acid anhydride bond leading to the formation of ADP or AMP and free energy. In eukaryotic cells the enzyme adenylate kinase interconverts ATP, ADP and AMP, and maintains this reaction close to equilibrium (Hardie & Hawley, 2001). During aerobic exercise the demand for ATP increases and must be matched by the rate of mitochondrial resynthesis. If demand exceeds supply there will be an increase in lactate production and a further increase in the free ADP/ATP ratio. This results in an amplification of the AMP/ATP ratio via the adenylate kinase reaction as the AMP/ATP ratio is the square of the ADP/ATP ratio (Hardie & Hawley, 2001); (Freyssenet, 2007). AMP-activated protein kinase (AMPK) is an AMP-responsive enzyme that is allosterically activated by 5'-AMP in response to stresses that increase the cellular concentration of AMP relative to ATP owing to either limited ATP production or increased energy expenditure (Richter & Ruderman, 2009). Therefore,

AMPK functions as a 'fuel guage', sensing the energy status of the cell (Jessen & Goodyear, 2005).

2.2.1.2 AMPK Structure and Function

AMPK is an αβγ heterotrimer consisting of an α catalytic subunit and β and γ regulatory subunits, with corresponding homologues in all eukaryotes. Multiple isoforms exist for each subunit in mammals (α1, α2, β1, β2, γ1, γ2 and γ3), enabling the formation of 12 heterotrimer combinations that are thought to exhibit differences in subcellular localization and signalling functions (Hardie, 2007). 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 as essential for full enzymatic activity and cellular localisation of AMPK (Kahn *et al.*, 2005;Kahn *et al.*, 2005). In addition, β subunits contain an evolutionally conserved glycogen-binding domain that allows AMPK to interact with glycogen particles (Bright *et al.*, 2009). AMP binds to AMPK at the γ subunit resulting in a conformational change that allows phosphorylation (and activation) by the upstream AMPK kinase, LKB1 (Hawley *et al.*, 2003). Calcium-activated kinases have also been identified as AMPK kinases capable of inducing phosphorylation at Thr^{172} and thus activating AMPK (Jensen *et al.*, 2007;Hawley *et al.*, 2005;Hurley *et al.*, 2005;Hurley *et al.*, 2005). In fact, AMPK activation occurs directly as a result of calcium signalling in myotubes (Freyssenet, 2007;Freyssenet *et al.*, 2004;Freyssenet *et al.*, 2004). Calcium-activated signalling cascades will be described later but these findings demonstrate that the signalling cascades do not act independently of each other.

2.2.1.3 Regulation of AMPK activity in skeletal muscle

Physiological activation of AMPK occurs in skeletal muscle during exercise in response to an increase in the AMP/ATP ratio, whereby there is an increase in binding of AMP and decreased binding of ATP to the γ–subunit (Richter & Ruderman, 2009). The elevated AMP/ATP ratio during exercise activates AMPK by increasing the binding of AMP and decreasing the binding of ATP to the γ–subunit (Richter & Ruderman, 2009).

Acute exercise at intensities above 50–60% VO_2peak and varying duration have reported an increase in AMPK phosphorylation and its enzymatic activity (Wojtaszewski *et al.*, 2000); (Chen *et al.*, 2003); (Park *et al.*, 2002); (Musi *et al.*, 2001); (Stephens *et al.*, 2002); (Wojtaszewski *et al.*, 2002), (Fujii *et al.*, 2000); (Wojtaszewski *et al.*, 2003b). Exercise in rodents and electrical stimulation of muscle increases AMPK activation, supporting the findings in humans (Rasmussen & Winder, 1997); (Winder & Hardie, 1996); (Jorgensen *et al.*, 2005); (Jorgensen *et al.*, 2007); (Vavvas *et al.*, 1997). Similarly, phosphorylation in the animal model is also intensity dependent (Rasmussen & Winder, 1997). However, exercise and electrical stimulation in rodent muscle, phosphorylates both the α_1 and α_2 isoforms of AMPK (Jorgensen *et al.*, 2005); (Toyoda *et al.*, 2006;Jorgensen *et al.*, 2007); (Klein *et al.*, 2007), whereas the α_1 isoform is not activated in humans (Wojtaszewski *et al.*, 2002); (Fujii *et al.*, 2000); (Musi *et al.*, 2001).

AMPK can also be activated during low-intensity exercise as long as the duration of exercise is of a sufficient duration to alter energy metabolism (Wojtaszewski *et al.*, 2002). The increased AMPK activity in this study was tightly associated with the degree of Thr^{172} phosphorylation. As with previous studies in humans there was no activation of the α_1 isoform, and α_2 AMPK activity had returned to basal levels 1 hour post exercise. At this low intensity, it is likely that decreases in blood glucose and muscle glycogen are more important regulators of AMPK activity given the fact that the AMP/ATP ratio was unchanged throughout exercise and that AMPK activity increased as fuel stores decreased. This suggests that AMPK activity is also regulated by the muscle glycogen content. In support of this, the AMPK β-subunit was found to have a glycogen binding domain (Hudson *et al.*, 2003); (Polekhina *et al.*, 2003). When muscle glycogen is low, AMPK activity is elevated at rest and it increases significantly more during exercise than when glycogen is high (Wojtaszewski *et al.*, 2003b); (Derave *et al.*, 2000); (Steinberg *et al.*, 2006); (Roepstorff *et al.*, 2004a). 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).

In human muscle, only three heterotrimeric AMPK complexes are expressed, $\alpha 1\beta 2\gamma 1$, $\alpha 2\beta 2\gamma 1$ and $\alpha 2\beta 2\gamma 3$, and, during intense exercise of up to 20-minutes duration, only the $\alpha 2\beta 2\gamma 3$ complex is activated. The other complexes, which comprise as much as 80% of the total AMPK pool, are unchanged or even decreased during contraction (Birk & Wojtaszewski, 2006). Only after moderate intensity exercise of 60 minutes or more

does the activity of the $\alpha 2\beta 2\gamma 1$ complex increase (Treebak *et al.*, 2007). This evidence suggests that AMPK is activated in both an intensity-dependent and isoform-specific manner in human skeletal muscle while the duration of exercise and glycogen content also play a role in the exercise response.

AMPK has been shown to be activated pharmacologically by 5-amino-4-imidazolecarboxamide ribonucleoside (AICAR) in a time and dose-dependent manner (Sullivan *et al.*, 1994). AMPK activation occurs directly as a result of calcium signalling following incubation with the calcium ionophore A-23187 in myotubes (Freyssenet, 2007;Freyssenet *et al.*, 2004;Freyssenet *et al.*, 2004). Calcium-activated kinases have also been identified as upstream AMPK kinases capable of inducing phosphorylation at Thr^{172} and thus activating AMPK (Jensen *et al.*, 2007;Hawley *et al.*, 2005;Hurley *et al.*, 2005;Hurley *et al.*, 2005).

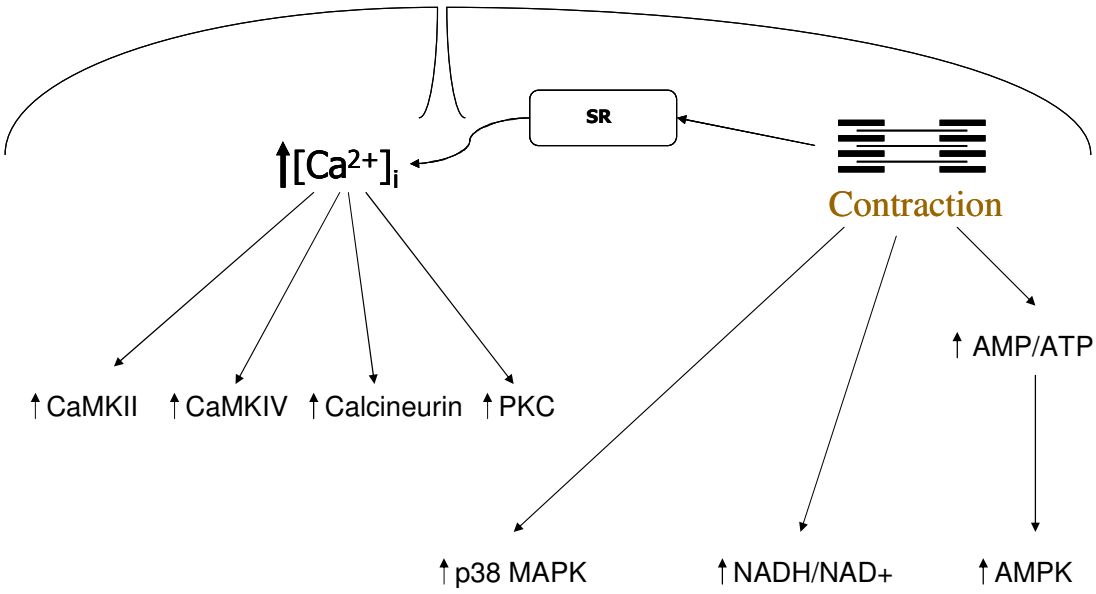

Figure 2.7 Diagram of signalling cascades regulated by exercise

2.2.2 Calcium Flux

Calcium release from the sarcoplasmic reticulum following membrane depolarisation is an essential regulator of the actin morphology and the subsequent formation of actin-myosin cross-bridges. The somatic nervous system releases acetylcholine into the

synaptic cleft and causes depolarisation of the muscle cell. 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 the adaptive response to exercise (Chin, 2004;Chin, 2004;Ojuka, 2004;Ojuka, 2004). It is thought that the amplitude and duration of these Ca^{2+} transients in response to activity can determine the set of genes expressed, coupling the extent of muscle excitation to transcription and allowing muscles to adapt to the demands placed on them (Chin, 2005). The transient release of intracellular Ca^{2+} ($[Ca^{2+}]_I$) activates a number of signaling pathways that translate this signal into a prolonged metabolic response. The kinases involved in the response include Ca^{2+}-dependent phosphatase calcineurin (CnA) (Chin *et al.*, 1998); (Ojuka *et al.*, 2002), Ca^{2+}/calmodulin-dependent kinase II (CaMKII) (Fluck *et al.*, 2000), Ca^{2+}/calmodulin-dependent kinase IV (CaMKIV) (Zhang *et al.*, 2002a), and Ca^{2+}-dependent protein kinase C (PKC) (Freyssenet *et al.*, 1999).

2.2.2.1 CaMKs

The increase in cytosolic calcium following depolarisation is decoded by the intermediate binding protein calmodulin (CaM). CaM is a multifunctional signal transducer that acts as an intermediate in the activation of downstream signalling pathways. By binding Ca^{2+}, CaM undergoes conformational and subcellular localisation changes before activating other CaM binding proteins such as the CaM-kinases (Chin, 2005). The specificity of CaM signalling is determined by the CaMK isoforms activated, its localisation and the duration, amplitude and frequency of the Ca^{2+} signal (Chin, 2005). CaMKII is a multimeric enzyme composed of twelve subunits arranged in two sets of six subunits in a spoke and wheel pattern (Soderling *et al.*, 2001). The central hub contains the carboxy terminus association domain and the spoke portions contain the amino terminus catalytic domain and the intervening regulatory domains. The calcium-activated CaM binds to the CaM-binding domain of CaMKII, activating intramolecular autophosphorylation on Thr^{286} which results in Ca^{2+}-independent activity (Hook & Means, 2001); (Hudmon & Schulman, 2002). Thus, when the $[Ca^{2+}]_I$ returns to basal levels there is still an increase in CaMKII activity. When pulses are more frequent and of a longer duration this leads to greater activation allowing CaMKII to decode the Ca^{2+} signal into discrete levels of kinase activity (Chin, 2005).

There are numerous CaMK isoforms, however, in human skeletal muscle. CaMKII has been shown to be the dominant isoform expressed. In animal studies CaMKII and CaMKIV have been implicated in the regulation of skeletal muscle plasticity (Fluck *et al.*, 2000;Wu *et al.*, 2002). For these reasons, this review will focus on the role of CaMKII and CaMKIV in skeletal muscle metabolism in response to exercise/contraction.

2.2.2.2 CaMKII

Acute exercise increases CaMKII phosphorylation in an intensity- but not time-dependent manner in human skeletal muscle (Rose & Hargreaves, 2003;Rose *et al.*, 2006). The intensity-dependent increase in CaMKII activity may be related to (i) the recruitment of individual skeletal muscle fibres (Sale, 1987) (ii) the recruitment of different fibre types (Baylor & Hollingworth, 2003); or (iii) greater Ca^{2+}–CaM signalling in recruited fibres. Phosphorylation of CaMKII at Thr^{287} is highly correlated with autonomous enzymatic activity (Rose *et al.*, 2006;Rose *et al.*, 2007). Autophosphorylation of CaMKII allows it to sustain autonomous enzymatic activity between calcium transients, allowing for sustained activity between calcium transients and a prolonged activation of downstream ligands (Hudmon & Schulman, 2002). Electrical stimulation of isolated rat skeletal muscle and caffeine-stimulated release of intracellular calcium have been shown to increase CaMKII phsophorylation (Wright *et al*., 2004).

2.2.2.3 CaMKIV

CaMKIV, a multi-functional kinase considered to have multiple isoforms and multiple downstream targets, plays an important role in muscle plasticity. It is not widely expressed and is believed to be found mostly in neural tissue. CaMKIV autoinhibition is relieved by the binding of Ca^{2+}/CaM, thus allowing phosphorylation by one of the upstream CaMKKs (Hook & Means, 2001). CaMKIV is phosphorylated within its activation loop on Thr^{196} in the rat enzyme and Thr^{200} in the human enzyme by both CaMKKα and CaMKKβ (Edelman *et al.*, 1996;Selbert *et al.*, 1995). Activation of CaMKIV by CaMKKs has been shown to increase CREB-mediated transcription *in vitro* (Enslen *et al.*, 1994).

2.2.2.4 Calcineurin

Calcineurin is a heterodimeric protein phophatase also proposed to act as a Ca^{2+} sensor, (Bassel-Duby & Olson, 2006); (Chin *et al.*, 1998). Calcineurin is a cyclosporin-sensitive, calcium-regulated Ser/Thr phosphatase (Chin *et al.*, 1998); (Derave *et al.*, 2000); (Wu *et al.*, 2001). Binding of calcium to a calmodulin-calcineurin complex stimulates serine/threonine phosphatase activity of calcineurin (Chin *et al.*, 1998); (Derave *et al.*, 2000); (Wu *et al.*, 2001).

2.2.2.5 Protein Kinase C

Members of the protein kinase C (PKC) family are single polypeptide chains, comprised of an N-terminal regulatory region and a C-terminal catalytic region (Newton, 1995). The PKC superfamily is divided into three subfamilies, conventional (c) (α, βI, βII, and γ isoforms), novel (n) (δ, ε, θ, and η isoforms), and atypical PKCs (ζ and λ/ι isoforms), based on differences in structure and responsiveness to the second messengers, Ca^{2+} and diacylglycerol (DAG) (Newton, 2003). The conventional PKC isoforms are activated by Ca^{2+} as they have a putative Ca^{2+} binding site. Skeletal muscle contraction increases intracellular Ca^{2+} and has been demonstrated to increase DAG following *in situ* contraction in rat muscle (Cleland *et al.*, 1989). This induces a rapid translocation of cPKC isoforms, synergistically activated by Ca^{2+} and DAG (Newton, 2001) from a cytosolic to a particulate fraction (Richter *et al.*, 1987), suggesting their activation. Treadmill running in mice (Chen *et al.*, 2002) and bicycle exercise in human subjects (Beeson *et al.*, 2003); (Nielsen *et al.*, 2003) increases the activity of aPKC and abundance and phosphorylation of PKCζ in the membrane fraction (Perrini *et al.*, 2004) in skeletal muscle. Electrical stimulation of muscle cells leads to an increase in PKC activity in the nucleus (Huang *et al.*, 1992).

2.2.3 MAPK signalling

The MAPK family of proteins is composed of three distinct signaling cascades capable of altering metabolism in skeletal muscle: *1*) extracellular signal regulated kinases (ERK) 1 and 2 (ERK1/2); *2*) p38 MAPK; and *3*) c-Jun NH_2-terminal kinases (JNK) (Kramer & Goodyear, 2007). The MAPK family can be activated by a variety of stimuli including cytokines, growth factors, and cellular stress (Long *et al.*, 2004). Exercise, a

form of cellular stress, has been shown to act on each of these signalling pathways in rat skeletal muscle (Goodyear *et al.*, 1996). MAPKs phosphorylate diverse substrates, including transcription factors and coactivators localized in the cytoplasm or nucleus, thereby form a basis for the regulation of transcriptional events (Long *et al.*, 2004). The individual signalling cascades will be discussed briefly.

2.2.3.1 p38 MAPK

p38 MAPK consists of four isoforms (p38α, p38β, p38δ, and p38γ) and there is evidence to suggest tissue-specific expression and regulation of the p38 MAPK family with exercise. Whereas the p38α and p38β MAPK isoforms are ubiquitously expressed, p38δ mRNA is detected mainly in the lung and kidney (Ji *et al.*, 2006), and the p38γ isoform is almost exclusively expressed in skeletal muscle (Li *et al.*, 1996). Exercise phosphorylates and activates p38 MAPK in rodent skeletal muscle and during cycling and marathon running in humans (Widegren *et al.*, 1998); (Yu *et al.*, 2001); (Boppart *et al.*, 2000). Interestingly, only p38γ (expressed exclusively in skeletal muscle) is phosphorylated in response to marathon running (Boppart *et al.*, 2000). The activation of p38 is influenced by training status as p38 phosphorylation is greater in untrained males following high intensity cycling (Yu *et al.*, 2003). 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, suggesting the muscle is adapting to the new demands being placed on it (Coffey *et al.*, 2006). The intensty of exercise is also an important regulatory factor with p38 phosphorylation primarily occurring during high-intensity muscle contractions in isolated rat skeletal muscle (Russ & Lovering, 2006); (Wretman *et al.*, 2001). These results provide evidence that p38 MAPK is activated in response to exercise and may have a role to play in the adaptive response to training.

2.2.3.2 ERK1/2

Activation of ERK1/2 by phosphporylation and activation has been observed in response to exercise in human (Widegren *et al.*, 1998); (Widegren *et al.*, 2000); (Yu *et al.*, 2003); (Yu *et al.*, 2001) and rodent skeletal muscle (Nader & Esser, 2001); (Goodyear *et al.*, 1996); (Dufresne *et al.*, 2001). The phosphorylation and activation of ERK1/2 is dependent on the exercise intensity (Widegren *et al.*, 2000). In this study

subjects completed 30 minutes of one-leg exercise at low (40% VO_2max) and high (75% VO_2max) intensity. Activation of both ERK1/2 and its upstream kinase MEK1/2 increased with high but not low intensity exercise. Resistance exercise has also been shown to upregulate ERK1/2 activation in humans (Creer *et al.*, 2005); (Karlsson *et al.*, 2004). Activation of ERK1/2 in human skeletal muscle by exercise appears to be rapid as it is increased after only 10 minutes of cycling but this increase is not sustained as it diminishes shortly after exercise. This exercise-induced activation of ERK1/2 is also thought to be local as it is only observed in the exercised leg (Widegren *et al.*, 1998).

2.2.3.3 JNK

The JNK pathway responds to intense exercise and those inducing muscular damage in human skeletal muscle (Boppart *et al.*, 2000;Boppart *et al.*, 1999;Aronson *et al.*, 1998). JNK activation, as well as its upstream kinase MAPKK4, was increased following both concentric and eccentric resistance exercise in humans. However, JNK phosphorylation was significantly greater following eccentric exercise, suggesting JNK responds to muscular damage/injury (Boppart *et al.*, 1999). *In vivo* stimulation of rat skeletal muscle increased JNK phosphorylation after 15 minutes and this remained elevated at 60 minutes, with a similar pattern being observed in MAPKK4 (Aronson *et al.*, 1997). In isolated rat skeletal muscle, electrical stimulation increased JNK phosphorylation; however, a more profound activation was observed with static stretch (similar to the stretch component of eccentric contractions) of the isolated muscles (Boppart *et al.*, 2001). JNK phosphorylation has been shown to increase linearly with escalating levels of muscular contraction force (Martineau & Gardiner, 2001).

2.2.4 Cellular Redox State NAD:NADH

The oxidation of glucose and fatty acids produce 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 in the electron transport chain. During exercise or muscle contraction, the NADH/NAD^+ ratio increases as a result of increased cellular metabolic processes. Previous studies have demonstarted this change in the redox state of the muscle cell where exercise of moderate to high intensity increases NADH concentration (Odland *et al.*, 2000;Sahlin *et al.*, 1987) or decreases NAD^+ concentration (Graham *et al.*, 1978). (Green *et al.*, 1992)

have also shown a progressive increase in the NADH/NAD^+ ratio during 60 minutes of exercise at 67-76% VO_{2peak}. Lactate also affects the redox state in an intensity-dependent manner, as NAD^+ production is a bi-product of the lactate dehydrogenase reaction (Denis *et al.*, 1991). Fluctuations in NADH/NAD^+ ratio and the production of lactate are potential intracellular signals affecting skeletal muscle gene expression (Hawley & Zierath, 2004). An example of this is the NAD^+-dependent changes in the protein and enzymatic activity of the deacetylase SIRT1 (Rodgers *et al.*, 2005); (Lagouge *et al.*, 2006).

2.2.5 The regulation of metabolic processes by intracellular cascade activation

Activation of the aforementioned signalling cascades enables the muscle to respond to any external stimuli such as exercise to regulate muscle metabolism accordingly. At any given time point these kinases are modulating the processes involved in muscle metabolism such as substrate utilisation to elicit the desired response.

2.2.5.1 Glucose Transport

During exercise AMPK is thought to help regulate cellular energy homeostasis by limiting anabolic processes and switching on alternative pathways for ATP regeneration. AMPK phosphorylates glycogen synthase on Ser^7, reducing its enzymatic activity (Carling & Hardie, 1989;Jorgensen *et al.*, 2004), and thereby attenuating glycogen synthesis. AMPK has also been proposed as a key player in contraction-stimulated glucose uptake (Merrill *et al.*, 1997). AMPK activity regulates glucose uptake in resting muscle and is at least partly responsible for contraction-mediated glucose uptake (Jorgensen *et al.*, 2004). There are strong correlations between glucose uptake and AMPK activity in muscle biopsies following exercise (Wojtaszewski *et al.*, 2003a). AMPK-stimulated increases in glucose transport are mediated by the translocation of existing GLUT4 containing vesicles to the plasma membrane (Koistinen *et al.*, 2003). However, it is unclear if there are other signalling cascades such as CaMK which may also be mediating this increase in glucose uptake (Rose & Richter, 2005). Evidence for a stimulatory role of intracellular Ca^{2+} or CaMK on muscle glucose transport comes from studies showing that a pharmacologically-induced increase in myoplasmic Ca^{2+} concentration increases glucose transport in non-contracting muscle (Holloszy &

Narahara, 1967); (Youn *et al.*, 1991). Similarly, elevating calcium concentrations in myotubes leads to an acute increase in glucose uptake (Wright *et al.*, 2004). Furthermore, incubating epitrochlearis muscle in concentrations of caffeine that increase cytosolic Ca^{2+} levels, but do not induce contraction resulted in increased glucose uptake, an action inhibited by the CaMK inhibitors KN62 and KN63. In the same study, rat epitrochlearis muscle was incubated with both AICAR and caffeine and the effects of both AMPK and CaMKII on glucose transport were additive. When these muscles were contracted, both AMPK and CaMKII were phosphorylated with a subsequent increase in glucose uptake observed. Incubation with the CaMK inhibitor, KN62, was shown to decrease this contraction-induced glucose transport by about 50% in the same study by inhibiting CaMKII phosphorylation, while AMPK remained unaffected (Wright *et al.*, 2004). Caffeine has also been shown to increase the phospohorylation of p38 MAPK and its downstream target, ATF-2, an effect that is blocked when CaMKII is inhibited. This suggests CaMKII is involved in the activation of p38 MAPK and ATF-2 (Wright *et al.*, 2007a). Electrically-induced contraction of isolated EDL muscle increased the activity of p38α and p38β MAPK approximately 2-fold causing an increase in 2-deoxyglucose uptake by the muscle (Somwar *et al.*, 2000). SB203580, an inhibitor of p38 MAPK, caused a significant reduction in p38 MAPK phosphorylation and subsequent contraction stimulated 2-deoxyglucose uptake (Somwar *et al.*, 2000). This suggests that p38 MAPK is involved in contraction-mediated glucose uptake possibly through a CaMKII-mediated pathway. These studies demonstrate an integrated approach to contraction-mediated glucose uptake involving AMPK, CaMKII and p38 MAPK.

The stimulation frequency of the muscle cell may be another way in which calcium regulates glucose uptake. In rat skeletal muscle, glucose transport increases in parallel with the stimulation frequency despite a constant mechanical output of the muscle (Ihlemann *et al.*, 2000). As CaMKII is known to be sensitive to the stimulation frequency, it is possible that it could decode this signal to influence glucose uptake (De Koninck & Schulman, 1998). These data indicate that CaMKII may be involved in the Ca^{2+}/contraction-stimulated increases in glucose transport and suggest that CaMKII signalling may coordinate muscle energy supply with energy demand. PKC has been implicated in the regulation of contraction-stimulated muscle glucose transport and pharmacological inhibition of cPKCs and nPKCs blunts contraction-stimulated skeletal

muscle glucose uptake in a fibre-type specific manner (Cleland *et al.*, 1989); (Wojtaszewski *et al.*, 1998).

Although p38 MAPK is implicated in regulation of glucose transport (as described), the other kinases of the MAPK cascade do not appear to be involved. Acute exercise effects on glucose transport and glycogen synthesis are unlikely to be mediated by ERK1/2, as inhibitors of MAPKK and ERKK abolish contraction-stimulated ERK1/2 phosphorylation without affecting glucose transport (Wojtaszewski *et al.*, 1999). Similarly, JNK does not regulate contraction-stimulated glucose transport or glycogen metabolism in skeletal muscle (Fujii *et al.*, 2004); (Witczak *et al.*, 2006).

2.2.5.2 Lipid Metabolism

AMPK has a number of roles in lipid metabolism including AICAR-mediated fatty acid uptake in skeletal muscle (Shearer *et al.*, 2004). AMPK activation is associated with FAT/CD36 translocation to the plasma membrane and a parallel increase in FA uptake. This processes has been suggested to be the regulatory step in contraction-mediated fatty acid uptake (Bonen *et al.*, 1999). Furthermore, it has been demonstrated that AICAR-stimulated FA uptake is severely blunted in mice that do not express FAT/CD36 (Bonen *et al.*, 2007). However, some studies have clearly demonstrated inconsistency between FA uptake, FAT/CD36 translocation and AMPK activation in response to contraction. Although AICAR stimulates an increase in FA oxidation, low-intensity muscle contraction increases glucose uptake, FA uptake, and total FA oxidation without increasing AMPK activity (Raney *et al.*, 2005); (Turcotte *et al.*, 2005). This suggests additional mechanisms regulate FA uptake in skeletal muscle and a potential role for calcium and MAPK signalling. Pharmacological activation of Ca^{2+} increases FA uptake in isolated rodent muscle (MacLean & Winder, 1995) as well as a decrease in malonyl-CoA levels in perfused muscle. In a recent report, caffeine and electrical stimulation were shown to increase FA uptake and FA oxidation (Raney & Turcotte, 2008). The increase in FA uptake and oxidation by contraction was associated with phosphorylation of AMPK and ERK1/2. Incubation with KN93 (a CaMK inhibitor) abolished caffeine-induced FA uptake, decreased contraction-induced FA uptake by 33%, and abolished both caffeine- and contraction-induced FA oxidation. The CaMK inhibitor KN93 reduced the contraction-induced increase in AMPK activation suggesting CaMK might be upstream of AMPK. These results suggest that CaMKII, in

part at least, regulates both Ca^{2+} and contraction-mediated FA uptake and oxidation (Raney & Turcotte, 2008). Other studies from the same group showed contraction-mediated increases in ERK1/2 phosphorylation, FA uptake, FA oxidation and an increase in plasma membrane FAT/CD36 (Raney *et al.*, 2005;Raney & Turcotte, 2007). These effects are decreased following incubation with PD98059, an inhibitor of the upstream kinase MEK1/2 which activates ERK1/2. These results suggest an important role for the ERK1/2 pathway in regulation of FA uptake and oxidation.

AMPK is also thought to regulate FA oxidation during exercise by controlling mitochondrial entry of FA by phosphorylating ACCβ at Ser^{218}. Phosphorylation of ACCβ reduces its activity by desensitising it to allosteric activation by cytosolic citrate (Vavvas *et al.*, 1997) and sensitising it to inhibition by palmitoyl-CoA (Rubink & Winder, 2005). Deactivation of ACC*β* will decrease the formation of malonyl-CoA, a potent inhibitor of carnitine palmitoyl transferase-I (CPT-I), the rate limiting enzyme in mitochondrial FA uptake (Winder & Hardie, 1996). 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). As noted earlier, caffeine stimulation decreases malonyl CoA activity in rat muscle (MacLean & Winder, 1995).

AMPK has also been suggested to be involved in the breakdown of intramuscular triglycerides (IMTG) during exercise by phosphorylation of HSL on Ser^{565} (Bangsbo *et al.*, 1990;Kiens, 2006). However, there is conflicting evidence surrounding AMPK activation and phosphorylation of HSL; thus, we are unable to come to any firm conclusions as to the role AMPK plays in IMTG breakdown during exercise (Roepstorff *et al.*, 2004a;Watt & Spriet, 2004). Regulation of IMTG breakdown by calcium signalling pathways appears to be negative as when intracellular calcium is increased by both caffeine and the Ca^{+}-ATPase inhibitor cyclopiazonic acid there is a decrease in HSL activity. This effect is abolished by the CaMK inhibitor KN93 (Watt *et al.*, 2003b). However, MAPK signalling may play a role in the breakdown of IMTG during exercise. Exercise at 60% peak pulmonary uptake increases HSL activity after 3 minutes and this coincides with an increase in ERK1/2 phosphorylation (Watt *et al.*, 2003c). Furthermore, HSL activity has been shown to increase after 1 minute of exercise at 30 and 60%

VO_2peak and a further increase is observed at 90% VO_2peak (Watt *et al.*, 2003a). This suggests that the regulation of HSL and IMTG lipolysis during exercise may be intensity-dependent. This is in keeping with the finding that ERK1/2 activation, which may mediate this response, is also regulated by exercise intensity (Widegren *et al.*, 2000).

This evidence highlights the importance of the signalling cascades in the regulation of metabolic processes in response to an external stimulus such as exercise. The activation of these kinases plays an important role in the substrate utilised for enrgy production during and after exercise. In this section, the immediate effects of the signalling cascades on metabolism such as the uptake, oxidation and storage of carbohydrate and/or fat are reviewed. However, these signalling pathways can also modulate the expression of a number of genes involved in these metabolic processes. Before reviewing the regulation of metabolic gene expression it is important to cover the basic science of gene transcription.

2.3 Regulation of Transcription

Gene transcription is the process resulting in the production of an mRNA "copy" of the DNA template that can be used for protein synthesis (Macfarlane, 2000). Transcriptional activity is regulated by the coordinated action of numerous transcription factors and transcriptional coregulators by transducing hormonal, nutrient and metabolite signals to a specific subset of genes. This is in response to physiological stimuli such as exercise and contractile activity for the regulation of metabolic homeostasis (Giguere, 2008); (Desvergne *et al.*, 2006).

Before transcription can occur, a specific transcription factor must be activated in response to a physiological stimulus such as exercise. Transcription factors are proteins that interact with the promoter sequence of DNA to modify the rate of transcription of a particular gene (Macfarlane, 2000). Transcription factor activation causes them to bind to complementary sequences on the promoter where their regulatory domains can regulate transcription (Alberini, 2009). This transcription factor can then either recruit and bind coregulating (coactivator/corepressor) proteins or act directly on the promoter to remodel chromatin. Both the direct and indirect mechanisms result in recruitment of

the transcriptional machinery to a core promoter to begin the process of transcription. This first step of the process, termed initiation, consists of the binding of RNA Polymerase II with the 'promoter' of the gene of interest. The promoter is defined as the shortest DNA sequence at which RNA pol II can initiate transcription and it must include a TATA box and an Initiator Element, which serves as the binding site for RNA pol II and overlaps with the transcriptional start site. The core promoter positions RNA pol II, in a state termed the preinitiation complex (PIC), to unwind the DNA helix and separate the strands so RNA pol II can begin mRNA synthesis. Elongation, the next step in transcription, involves RNA pol II using ATP to move along the unwound DNA molecule synthesising a complementary mRNA strand. It finally reaches a termination signal, the last step in the process, on the DNA template where it can no longer continue and separates from the DNA. These steps are repeated until the cell has synthesised all of the mRNA copies of that gene required (Macfarlane, 2000).

The model outlined above is the simplest form of transcription and is not very efficient. In reality, the promoter region of a gene will contain many DNA binding domains capable of binding transcription factors. Some genes also contain enhancer regions which work in combination with the promoter to enhance the efficiency of gene transcription (Macfarlane, 2000). After binding a transcription factor, the enhancer bends the DNA to increase the activity of RNA pol II in the PIC (Macfarlane, 2000). Negatively acting transcription factors repress transcription, either by binding in such a way as to block RNA pol II from binding to the DNA or by bending the DNA so as to minimise contact and decrease the activity of RNA pol II (Clark & Docherty, 1993). Many transcription factors exist as homo/heterodimers. Negatively acting transcription factors can also bind to these dimers to render these transcription factors inactive (Latchman, 1992).

As mentioned earlier, transcription factors can recruit coregulating proteins to the promoter. Transcription factors do not function alone and require coregulators to remodel chromatin and confer a second level of specificity to the transcriptional response (Feige & Auwerx, 2007). Coregulators are defined as proteins that alter transcriptional activity without binding to DNA (Spiegelman & Heinrich, 2004). Transcription factors bind to DNA in the promoter region of the gene of interest and mark it for activation or repression through the recruitment of coactivator or corepressor

proteins, these coregulators then serve as scaffolds for the recruitment of the necessary proteins for transcriptional (in)activation (Spiegelman & Heinrich, 2004).

Certain coactivators possessing acetyltransferase capability can modify histones to enhance transcriptional activity by allowing greater access of transcription factors to the DNA (Hermanson *et al.*, 2002). Acetylation of lysine residues within histone tails neutralizes their positive charge, thereby relaxing chromatin structure. This interferes with the generation of higher-order chromatin structures, increasing the accessibility of transcription factors to their target genes (Shahbazian & Grunstein, 2007). Conversely, corepressors have the opposite effect on chromatin, rendering it inaccessible to the binding of transcription factors. These corepressors are often associated with histone deacetylase activity (HDAC) (Ruthenburg *et al.*, 2007); (Spiegelman & Heinrich, 2004).

The activity of coregulators is also influenced by post translational modifications (PTMs). These PTMs exert control over the functional relationship between transcription factors and their coregulators and the subsequent regulation of transcription (McKenna & O'Malley, 2002). Hyperacetylation has been shown to increase transcriptional activity on a number of gene promoters (Deckert & Struhl, 2001). Numerous coregulators have been shown to be regulated by phosphorylation (Rowan *et al.*, 2000); (Akimoto *et al.*, 2005) and acetylation (Rodgers *et al.*, 2005); (Lerin *et al.*, 2006). In addition, methyltransferases have been shown to interact with coregulators to alter transcriptional activity and some coregulators have been shown to contain methylation sites (Teyssier *et al.*, 2005); (Mostaqul *et al.*, 2008).

Post translational modification of coregulators suggest that they may be targets of kinase-mediated cellular signalling pathways. Thus, kinase-mediated pathways may be exerting control over coregualtor activity and transcription factor binding, allowing the cell to transduce physiological stimuli into a tightly regulated transcriptional response. Finally, transcription factors and coregulators are themselves under transcriptional control. This means that genes encoding transcription factors may be regulated through the spatial and temporal control of their expression and activity in response to a physiological stimulus.

2.3.1 Regulation of transcription of metabolic genes by signalling cascades

The activation of intracellular signalling cascades, as outlined in an earlier section, result in dynamic and immediate changes in cellular metabolism, including the uptake, oxidation and storage of carbohydrate and/or fat. However, these same signalling cascades have been implicated in the regulation of gene expression in muscle cells. While the changes in transcriptional activity may not have a real-time impact on cellular metabolism, the gene expression profile activated by exercise helps the cell adapt to the physiological stress of an acute bout of exercise and to exercise training. It has been well documented that aerobic exercise training leads to increased mitochondrial number and size, but, for the purpose of this thesis, I will focus on the acute changes in gene expression. AMPK, CaMKIV and p38 are involved in GLUT4 expression by phosphorylating and altering the activity or cellular localisation of transcription complex proteins (Ojuka *et al.*, 2002; Lu *et al.*, 2000; Jager *et al.*, 2007; Zhao *et al.*, 1999). Other genes involved in metabolism that are regulated by AMPK and CaMK include: hexokinase (Jorgensen *et al.*, 2007); pyruvate dehydrogenase kinase 4 (PDK4) (Jorgensen *et al.*, 2005); CPT-1 (Winder & Hardie, 1996); and FAT/CD36 (Chabowski *et al.*, 2006). The regulation of gene expression following an acute bout of exercise by contraction-mediated signalling cascades appears to target genes involved in nutrient transport or oxidation. These findings guided the selection of gene targets chosen for analysis in my research experiments.

2.3.2 Transcriptional regulation of glucose metabolism

AMPK has been shown to act on GLUT4, the glucose transporter responsible for insulin- and contraction-mediated glucose uptake in the muscle (Ojuka *et al.*, 2002). AMPK is known to phosphorylate HDAC5, a transcriptional repressor that inhibits gene expression by deacetylating histone lysine residues making them inaccessible to transcription factors, on ser^{259} and ser^{498} (McGee & Hargreaves, 2008). Phosphorylation of HDAC5 dissociates it from MEF2, a transcription factor responsible for the regulation of GLUT4 gene expression, and provides binding sites for 14-3-3 which exports HDAC out of the nucleus thereby removing its transcriptional repression of MEF2. MEF2 binding to its site on the GLUT4 promoter is required for GLUT4 expression (Mora & Pessin, 2000). HDAC Ser ^{259} and Ser^{498} phosphorylation are

necessary for AMPK induction of GLUT4 (McGee & Hargreaves, 2008). Activation of CaMKII by electrical stimulation of cultured muscle cells causes subsequent nuclear exclusion of HDAC4, thereby relieving repression of MEF2 transcriptional activity (Liu *et al.*, 2005). Evidence to support this comes from another group , who have shown that constitutively active CaMKI/IV can dissociate HDAC5 from MEF2C; however, the mechanism for this is unknown. This model couples contraction-induced calcium signaling to an increase in the rate of transcription of MEF2 target genes such as GLUT4 (Lu *et al.*, 2000). The PKC pathway also promotes nuclear export of HDAC5 by stimulating phosphorylation of the 14-3-3 docking sites (Vega *et al.*, 2004). Further studies showed that PKCmu/protein kinase D (PKD) acts as a downstream effector kinase of PKC and stimulates the nuclear export of HDAC5 (Bassel-Duby & Olson, 2006). Therefore, PKC may also regulate GLUT4 expression. Ca^{2+} binding to Calcineurin, causes NFAT dephosphorylation and translocation of NFAT from the cytosol to the nucleus where it binds to the DNA of gene promoter regions and transcription factors including MEF2 (Chin *et al.*, 1998); (Derave *et al.*, 2000); (Wu *et al.*, 2001). Transgenic mice that overexpress activated calcineurin in fast-twitch fibres have been shown to increase GLUT4 protein content (Ryder *et al.*, 2003). Calcineurin can activate MEF2 either directly by MEF2 dephosphorylation (Wu *et al.*, 2001) or indirectly by NFAT dephosphorylation and subsequent interaction between activated NFAT and MEF2 (Youn *et al.*, 2000). p38 MAPK also acts on MEF2A by phosphorylating it on Thr^{312} and Thr^{319} to enhance transcription, thereby having an effect on GLUT4 expression (Zhao *et al.*, 1999). AMPK has been shown to phosphorylate GLUT4 enhancer factor (GEF) *in vitro* and to increase its binding to the promoter of GLUT4 (Holmes & Dohm, 2004). The AMPK induced expression of GLUT4 requires the gene transcription co-activator PGC-1α, and AMPK directly phosphorylates PGC-1α and activates the expression of PGC-1α (Jager *et al.*, 2007). AMPK is proposed to regulate PGC-1α by direct phosphorylation on Thr^{177} and Ser^{538}, releasing it from its bound repressor protein p160myb, which inhibits PGC-1α activation (Fan *et al.*, 2004). Inhibition of AMPKα does not affect GLUT4 expression in response to exercise and HDAC Ser^{259} and Ser^{498} can be phosphorylated by numerous kinases suggesting that AMPK's regulation of GLUT4 expression is important but not crucial (Holmes *et al.*, 2004). This evidence shows that both calcium- and AMP-activated pathways are in control of GLUT4 expression through the regulation of GEF activity, HDAC localisation and MEF2 DNA binding activity. A recent paper demonstrated that CaMKII, AMPK and calcineurin all play a role in the regulation of

GLUT4 (Murgia *et al.*, 2009). In this study, the researchers inhibited calcineurin and CaMKII with cain and KIIN, respectively while they used a KD-AMPK mouse model. GLUT4 enhancer activity was not affected by incapacitation of a single pathway – it is only by inhibition of two pathways that an effect on transcription is observed (Murgia *et al.*, 2009).

Hexokinase II (HKII) is an exercise responsive protein important for carbohydrate metabolism as it phosphoryates glucose in the first step of glycolysis. Several studies have shown induction of the HKII gene following acute bouts of exercise (Kraniou *et al.*, 2000); (Pilegaard *et al.*, 2003a); (Pilegaard *et al.*, 2002); (Yang *et al.*, 2005); (Pilegaard *et al.*, 2005). Low frequency stimulation causing contraction of rat muscle increased both HKII mRNA and protein (Hofmann & Pette, 1994). Phosphorylation of α2-AMPK induced by AICAR in rats results in the induction of HKII mRNA (Stoppani *et al.*, 2002). Similar results showed α2-AMPK activation by stimulation with AICAR as well as training increased HKII mRNA (Jorgensen *et al.*, 2007). Interestingly, the training-induced increases in HKII were not reduced in an α2-AMPK-KO mouse suggesting that other pathways may also be regulating HKII expression (Jorgensen *et al.*, 2007). Calcium signalling may represent the alternative pathway to upregulation of HKII as treatment of rat muscle cells with ionomycin, associated with an increase in calcium, increased HKII mRNA approximately 2-fold (Kusuhara *et al.*, 2007). AMPK and CaMKII are also thought to phosphorylate and activate CREB, which would theoretically enhance the transcription of genes with CRE binding sites in their promoter region, including HKII (Thomson *et al.*, 2008); (Wheeler *et al.*, 2008). These results demonstrate the influence of the signalling cascades activated by exercise on the adaptation of metabolic genes associated with glucose metabolism.

2.3.3 Transcriptional regulation of lipid metabolism

In addition to regulating the activity of certain enzymes associated with lipid metabolism in skeletal muscle, the exercise-activated signalling cascades can also regulate transcription of a number of genes involved in FA oxidation. AMPK activation by AICAR and exercise training in mouse muscle has been shown to increase the expression of both PDK4 and FOXO1 mRNA two genes central to the regulation of FA metabolism (Jorgensen *et al.*, 2005). Induction of the PDK4 gene may control the switch to fat metabolism after exercise by phosphorylation and inactivation of the PDC

complex to prevent the conversion of pyruvate to acetyl CoA, resulting in allosteric inhibition of glycolysis and suppression of glucose oxidation (Pilegaard & Neufer, 2004). In support of this, numerous studies demonstrate that an acute bout of exercise induces PDK4 expression in human skeletal muscle (Cluberton *et al.*, 2005); (Pilegaard *et al.*, 2005); (Mahoney *et al.*, 2005); (Coffey *et al.*, 2006); (Pilegaard *et al.*, 2002); (Civitarese *et al.*, 2005). FOXO1A promotes the expression of genes involved in energy metabolism resulting in the transition from carbohydrate oxidation to lipid oxidation in response to fasting and exercise (Bastie *et al.*, 2005) and FOXO1A mRNA has previously been shown to be induced by acute exercise in human muscle possibly driving this shift in metabolism (Pilegaard *et al.*, 2005); (Mahoney *et al.*, 2005); (Russell *et al.*, 2005). There is evidence to support the increase in FOXO1 mRNA seen with AICAR stimulation *in vivo*, however, in C_2C_{12} cells, AICAR and metformin stimulation decreases FOXO1 mRNA suggesting there are other regulatory factors at play (Nystrom & Lang, 2008). The effect of AICAR on PDK4 and FOXO1 is not seen in an α2-AMPK-KO mouse, but the training induced increases in expression are still evident suggesting alternative pathways may upregulate these proteins with exercise (Jorgensen *et al.*, 2005). This increase in FOXO1 and PDK4 mRNA with training cannot be explained by calcium signalling, as incubation of rat muscle cells with ionomycin, a cakcium activator, did not increase PDK4 expression (Kusuhara *et al.*, 2007). PDK4 mRNA is increased with activation of PPARδ by its agonist GW501516 and this is accompanied by an increase in AMPK activation and FA oxidation (Kramer *et al.*, 2007).

PPARδ is thought to be important to lipid metabolism, and activation of PPARδ in skeletal muscle cells promotes fatty acid oxidation and utilization (Wang *et al.*, 2003). Transgenic mice that overexpress calcineurin display increased protein content of PPARδ and PPARα suggesting that calcineurin may act through these proteins (Long *et al.*, 2007). These mice have increased lipid oxidation as well as increased expression of proteins involved in the breakdown and uptake of FA including LPL, FAT/CD36, and CPT-1 (Long *et al.*, 2007); (Ryder *et al.*, 2003). PPARδ activation also increases CPT-1 expression though this is thought to be mediated by AMPK rather than calcineurin (Kramer *et al.*, 2007). (Jorgensen *et al.*, 2005) also found that CPT-1 mRNA was increased through the activation of AMPK, as the AICAR-stimulated effect was blocked in α2-AMPK-KO mice. CPT-I is thought to be the rate-limiting enzyme in mitochondrial FA uptake (Winder & Hardie, 1996). The FA-binding protein FAT/CD36

controls the uptake of FA into the muscle at the plasma membrane. Activation of AMPK by AICAR has previously been shown to increase FAT/CD36 protein in cardiac muscle (Chabowski *et al.*, 2006). In contrast to the calcineurin-dependent effect on LPL expression observed by Long and colleagues (2007), LPL mRNA has also been shown to be increased by an AMPK-dependent pathway following incubation with either AICAR or metformin in L6 muscle cells (Long *et al.*, 2007); (Ohira *et al.*, 2009). LPL is responsible for the breakdown of fat and can increase FA oxidation by increasing FFA supply.

These findings outline the effect of activation of these signalling pathways on transcriptional-regulation skeletal-muscle metabolic proteins associated with FA oxidation. It is clear that many of these proteins are regulated by more than one of these cascades at any given time, a point which demonstrates the tight regulational control of their function and abundance.

2.3.4 PGC-1α-Transcriptional Co-activator

The role of transcription-factor co-regulators in skeletal metabolism is poorly understood. However, the identification of the co-activator peroxisome proliferator activated receptor gamma co-activator 1α (PGC-1α) has opened up new and exciting ways to study metabolic gene expression.

PGC-1α was first identified as a cold-inducible coactivator of nuclear receptors through its functional interaction with the transcription factor PPARα in brown adipose tissue (Puigserver *et al.*, 1998). It now transpires that PGC-1α co-activates the expression of many metabolic genes and is involved in the regulation of nutrient transport, substrate utilisation, mitochondrial oxidative metabolism and mitochondrial biogenesis. For the purpose of this review, I will focus on the regulation of PGC-1α expression and function, and the subsequent effects this may have on the aforementioned metabolic processes in skeletal muscle.

2.3.4.1 PGC-1α Structure

PGC-1α is highly expressed in oxidative tissues, particularly the heart and Type I fibers of skeletal muscle (Irrcher *et al.*, 2003a). PGC-1α does not possess histone-modifying activities itself; however, through a potent NH_2-terminal transcriptional activation domain, it interacts with cofactors containing histone acetyltransferase (HAT) activity to modify chromatin and induce transcription (Puigserver *et al.*, 1999). Nuclear hormone receptor coactivator signature motifs (LXXLL) adjacent to the activation domain are essential for coactivation of certain nuclear receptors. In addition, PGC-1α contains several arginine/serine rich domains within its carboxy-terminal region that couple pre-mRNA splicing with transcription (Monsalve *et al.*, 2000).

2.3.4.2 Description of PGC-1 promoter

There are many binding domains conserved within the PGC-1α promoter which are capable of binding transcription factors to regulate PGC-1α expression. The human PGC-1α promoter contains a GC box that has been shown to bind Sp1 (Esterbauer *et al.*, 1999). In addition, there is a cAMP Response Element (CRE) site which binds the cAMP Response Element Binding protein (CREB). Domains capable of binding the transcription factors MEF2, ATF2 and FOXO1 are also conserved within the PGC-1α promoter (Czubryt *et al.*, 2003); (Handschin *et al.*, 2003;Daitoku *et al.*, 2003). Furthermore, Irrcher *et al*. (2008) found three GATA sites, a consensus sequence for serum response element (SRE) and putative binding sites for p53 and NF-κβ on the promoter of PGC-1α (Irrcher *et al.*, 2008).

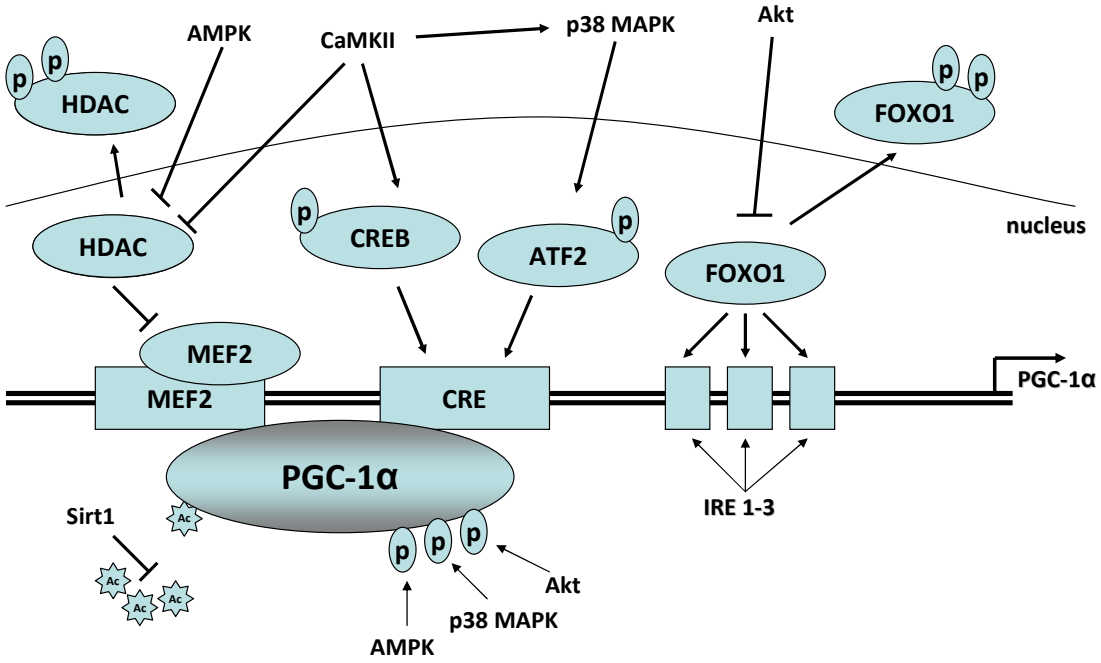

Figure 2.8 Illustration of the regulation of the PGC-1α promoter by intracellular signalling cascades.

The signalling pathways discussed previously regulate PGC-1α expression through transcription factors which bind to the PGC-1α promoter. As mentioned earlier, Czubryt *et al*. (2003) identified two MEF2 binding sites on the PGC-1α promoter and demonstrated that MEF2 was capable of increasing PGC-1α expression (Czubryt *et al.*, 2003). PGC-1α induction was blocked by MEF2 association with the histone deacetylase 5 (HDAC5). This is not surprising as HDAC is a transcriptional repressor that inhibits expression of MEF2 by deacetylating histone lysine residues making them inaccessible to transcription factors, on ser^{259} and ser^{498} (McGee & Hargreaves, 2008). Phosphorylation of HDAC dissociates it from MEF2 and provides binding sites for 14-3-3 which exports HDAC out of the nucleus thereby removing its transcriptional repression of MEF2 (McGee & Hargreaves, 2008). Interestingly, a number of the aforementioned signalling pathways also have an impact on MEF2. AMPK, CaMKII, CaMKI/IV and calcineurin signalling have all been shown to phosphorylate HDACs either directly or indirectly removing the inhibition of MEF2 by HDACs, allowing it to interact with PGC-1α (McGee & Hargreaves, 2008); (Liu *et al.*, 2005); (Lu *et al.*, 2000); (Wu *et al.*, 2001). In addition, a SIRT1 induced increase in PGC-1α mRNA expression in C2C12 cells was mediated by MEF2 (Amat *et al.*, 2009). p38 MAPK phosphorylates MEF2 directly on Thr^{312} and Thr^{319} to enhance transcription (Zhao *et al.*, 1999). This evidence suggests that a number, if not all these signalling pathways, are regulating

PGC-1α expression through MEF2. It is important to note that MEF2 is also involved in a positive autoregulatory loop whereby PGC-1α regulates its own expression (Handschin *et al.*, 2003).

Further evidence exists for a role of the signalling kinases in PGC-1α expression. AMPK activation by AICAR in rodent muscle and skeletal muscle cells increases the expression of PGC-1α mRNA and protein (Lee *et al.*, 2006); (Suwa *et al.*, 2003); (Irrcher *et al.*, 2008). Zong *et al*. (2002) found when AMPK was activated by beta-guanidinopropionic acid (GPA) that a creatine analog, that increases the AMP/ATP ratio, PGC-1α mRNA and CaMKIV protein, was increased in WT mice (Zong *et al.*, 2002). However, in dominant negative-AMPK mice the same treatment did not increase PGC-1α or CaMKIV. This suggests that AMPK is required for the AICAR-induced increase in PGC-1α expression but that CaMKIV may also be involved. However, transgenic mice displaying a constitutively active form of CaMKIV had increased PGC-1α protein expression (Wu *et al*., 2002) while adenoviral-mediated expression of a constitutively active form of CaMKIV and calcineurin increased PGC-1α mRNA expression in cardiac myocytes (Schaeffer *et al.*, 2004). Therefore, some CaMK isoforms are likely to be involved in the regulation of PGC-1α expression as well as calcineurin, which may also have important functions for PGC-1α. Raising cytosolic calcium in L6 myotubes increased PGC-1α protein (Ojuka *et al.*, 2003). Similarly, when cytosolic calcium was increased by ionomycin in primary rat muscle cells, PGC-1α mRNA was increased, but this effect was blocked by the CaMK inhibitor KN62 (Kusuhara *et al.*, 2007). The effect of Ca^{2+} signalling on PGC-1α expression is likely to act through the transcription factor CREB as it is a substrate of the CaM kinases (Sheng *et al.*, 1991) and it can bind to the CRE present on the PGC-1α promoter. Calcineurin is also a known activator of MEF2 by dephosphorylation either directly or through NFAT (Wu *et al.*, 2001); (Youn *et al.*, 2000).

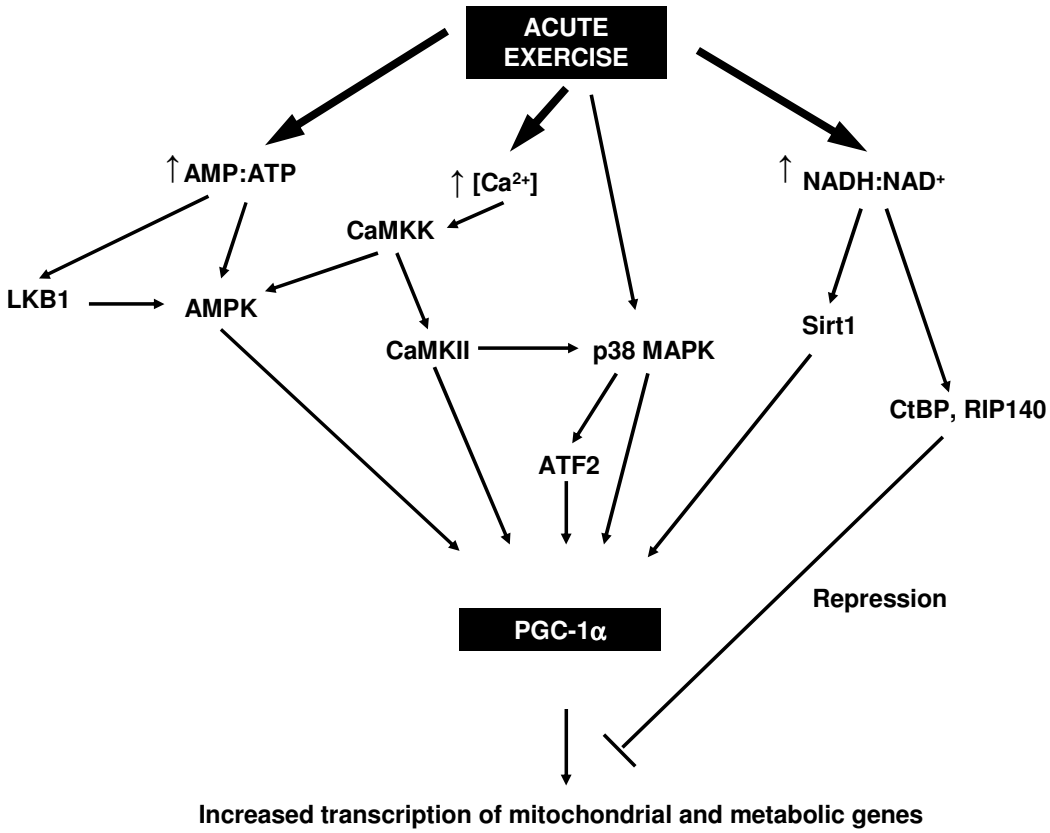

Figure 2.9 Schematic diagram of the regulation of PGC-1α by intracellular signalling cascades

There is also evidence that MAPK signalling can effect the expression of PGC-1α. ATF2, which has a consensus sequence on the PGC-1α promoter, is phosphorylated by p38 MAPK at Thr^{69} and Thr^{71} and activates ATF2 transcriptional activity (Zhao *et al.*, 1999); (bdel-Hafiz *et al.*, 1992). PGC-1α promoter activity is increased following the activation of p38 MAPK by a constitutively active form of its upstream kinases MKK3E and MKK6E in myocytes along with ATF2 mRNA expression (Akimoto *et al.*, 2005). Transgenic mice expressing a muscle specific constitutively active form of MKK6E display increased PGC-1α protein expression (Akimoto *et al.*, 2005).

The redox state of the cell also influences PGC-1α expression. PGC-1α expression is decreased in SIRT1-KO mice (Amat *et al.*, 2009). Conversely, overexpression or activation of SIRT1 in C_2C_{12} muscle cells increases PGC-1α mRNA and this occurs through MEF2 and MyoD. SIRT1 binds to the PGC-1α promoter and increases the recruitment of PGC-1α to its own promoter (Amat *et al.*, 2009). SIRT1 overexpression in PC12 cells also increases PGC-1α mRNA (Nemoto *et al.*, 2005). However, in

contrast to these findings, SIRT1 overexpression in rodent muscle leads to a 25% decrease in PGC-1α protein expression (Gurd *et al.*, 2009).

These studies demonstrate that PGC-1α expression is controlled by a number of signalling pathways including AMPK, CaMK, calcineurin, p38 MAPK and SIRT1. It is possible that a number of these cascades may be acting on PGC-1α through a similar mechanism

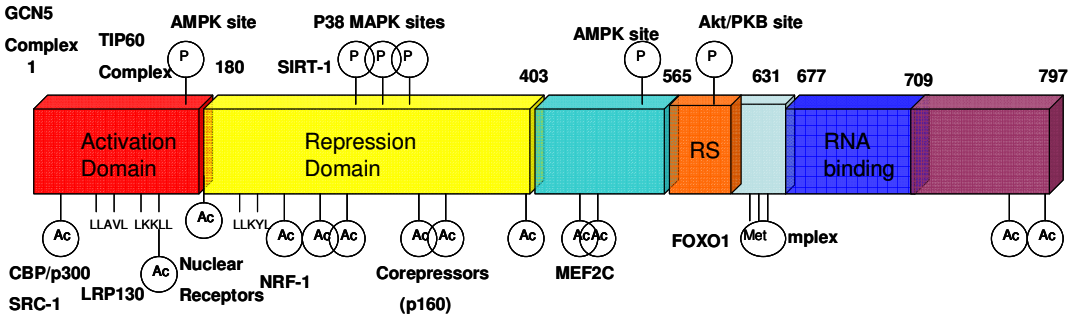

Figure 2.10 Illustration of main PGC-1α regulatory sites (adapted from (Rodgers *et al.*, 2008))

2.3.4.3 Regulation of PGC-1α activity

One of the interesting aspects of PGC-1α is that it co-activates its own promoter. Therefore, the protein content and activation of PGC-1α have a major impact on PGC-1α expression and function. This section will review the main post-translational modifications affecting PGC-1α function and how they are regulated.

PGC-1α can be phosphorylated, acetylated and methylated to modulate its activity (Knutti *et al.*, 2001); (Rodgers *et al.*, 2005); (Teyssier *et al.*, 2005). Three kinases directly phosphorylate PGC-1α, including p38 MAPK, AMPK and Akt/PKB. p38 MAPK phosphorylates PGC-1α on Thr^{262}, Thr^{298} and Ser^{265} in the repressor region of PGC-1α ,where phosphorylation disrupts the association of the p160myb binding protein, a protein that binds to PGC-1α and decreases its transcriptional activity (Fan *et al.*, 2004). This leads to PGC-1α being more stable and transcriptionally active (Knutti *et al.*, 2001); (Puigserver *et al.*, 2001;Fan *et al.*, 2004). AMPK phosphorylates PGC-1α at Thr^{177} and Ser^{538} stimulating PGC-1α activation of its own promoter (Jager *et al.*,

2007). Akt/PKB phosphorylation of PGC-1α at Ser^{570} reduces protein stability and transcriptional activity (Li *et al.*, 2007).

PGC-1α is acetylated by the histone acetyltransferase GCN5 at several residues and negatively regulates its transcriptional activity through nuclear sublocalisation (Lerin *et al.*, 2006). On the other hand, SIRT1 is an NAD^+-dependent deacetylase that increases the transcriptional activity of the coactivator (Rodgers *et al.*, 2005); (Gerhart-Hines *et al.*, 2007). Caloric restriction, pyruvate and resveratrol along with fluctuations in NAD^+ have been shown to deacetylate PGC-1α in a SIRT1-dependent manner (Rodgers *et al.*, 2005); (Lagouge *et al.*, 2006). PGC-1α is deacetylated in fasting conditions consistent with SIRT1 activation resulting in an increase in a number of genes involved in mitochondrial fatty acid oxidation (Gerhart-Hines *et al.*, 2007). PRMT1 has been shown to methylate PGC-1α at Arg665, Arg667, Arg669 (Teyssier *et al.*, 2005), but relatively little is known about the functional significance of methylation at this time.

2.3.4.4 PGC-1α-regulated genes

Mitochondrial biogenesis is a complex process that requires the coordination of genes from the nuclear and mitochondrial genomes. PGC-1α coactivates the transcription factors NRF-1, NRF-2 (Wu *et al.*, 1999b) and the orphan nuclear receptor ERRα to induce mitochondrial biogenesis (Schreiber *et al.*, 2003). Analysis by Ren and Dynlacht (2004) showed that NRF-1 binds to the promoters of a number of genes involved in mitochondrial biogenesis and metabolism (Ren & Dynlacht, 2004). PGC-1α can link nuclear regulatory events to the transcriptional machinery by coactivating NRF-1/2 to target their recognition sites on the promoter of Tfam, a nuclear encoded transcription factor that upon activation translocates to the mitchondria to activate transcripton of mitochondrial DNA (Gleyzer *et al.*, 2005). PGC-1α has been shown to bind directly with NRF-1 to increase its transcriptional activity and expression along with the promoter activity of Tfam (Wu *et al.*, 1999b). A dominant negative allele of NRF-1 blocks the effects of PGC-1α on mitochondrial biogenesis and Tfam, indicating NRF-1 is required for PGC-1α-induced mitochondrial biogenesis (Wu *et al.*, 1999b).

PGC-1α coactivates ERRα to promote mitochondrial biogenesis by a direct interaction via its AF-2 domain and sequence-specific leucine rich (LXXLL) nuclear receptor motifs to induce mitochondrial biogenesis (Schreiber *et al.*, 2003). A significant number

of gene promoters with mitochondrial functions upregulated by PGC-1α contain binding sites for ERRα (Mootha *et al.*, 2004). PGC-1α induction of genes encoding mitochondrial proteins was accompanied by an increase in mitochondrial DNA content and this could be blocked by siRNA against ERRα or mimicked by a constitutively active ERRα construct (Schreiber *et al.*, 2004). ERRα is also shown to interact with NRF-2 through a binding site on its promoter to facilitate PGC-1α stimulated induction of mitochondrial genes. Inhibition of ERRα abolishes the PGC-1α induced increase in NRF-2 in C2C12s, suggesting ERRα is necessary for NRF-2 induction (Mootha *et al.*, 2004).

The induction of NRF-1, NRF-2 and Tfam by ectopic expression of PGC-1α results in an increase in the mRNA of nuclear genes encoding proteins involved in mitochondrial oxidative phosphorylation including ATP synthetatse β, COXII, COXIV and cytochrome c (Wu *et al.*, 1999b). This is not surprising as NRF-1/2 have been shown to occupy the promoters of the mitochondrial proteins cytochrome c and COXIV in response to an increase in PGC-1α activity and protein expression (Wright *et al.*, 2007b). NRF-2 specifically binds to the COXIV promoter and is suggested as a general activator of gene expression of the cytochrome oxidases, essential respiratory chain proteins (Scarpulla, 1997); (Virbasius & Scarpulla, 1994). A similar increase in mitochondrial genes as well as mitochondrial content is seen in PGC-1α transgenic mice. MCK-PGC-1 transgenic mice display increased mRNA of the oxphos genes NADH-ubiquinone oxidoreductase, cytochrome c, COX5b and ATP synthetase (Choi *et al.*, 2008). Lin *et al.* (2002) showed similar increases in COXII and COXIV in the same mice (Lin *et al.*, 2002). Wende and colleagues observed comparable increases in the mRNA of the mitochondrial proteins COXII, COXIV, cytochrome c and ATP synthase β in a PGC-1 transgenic mouse model, but these increases were accompanied by an increase in mitochondrial function, depicted by an increase in the activity of citrate synthase (CS), a marker of mitochondrial activity (Wende *et al.*, 2007). In support of these findings, Calvo et al (2008) reported increased CS activity and increased expression of mitochondrial proteins in PGC-1α transgenic mice (Calvo *et al.*, 2008). Conversely, PGC-1α whole-body KO mice display decreased expression of a number of nuclear genes encoding proteins involved in mitochondrial electron transport and oxidative phosphorylation including COXIV, cytochrome c and ATP synthetase β (Leone *et al.*, 2005). Reductions in expression of these mitochondrial proteins were accompanied by decreased expression of Tfam and mitochondrial volume and density (Leone *et al.*,

2005). This evidence creates a strong argument for the regulation of the aforementioned genes and mitochondrial biogenesis by PGC-1α through its transcription factors NRF-1, NRF-2, ERR α and Tfam.

PGC-1α targets a number of genes involved in the regulation of substrate selection through co-activation of ERRα, an important regulator of mitochondrial energy-transduction pathways including fatty acid oxidation and oxidative phosphorylation. Forced expression of PGC-1α in myotubes has been shown to induce PDK4 mRNA and protein expression to promote glucose sparing and fatty acid oxidation through a nuclear receptor binding site occupied by ERRα, an effect that is lost in ERRα-null mice (Wende *et al.*, 2005). ERRα has been shown to target a number of lipid metabolic genes such as Medium-Chain Acyl-CoA Dehydrogenase (MCAD), which mediates the first step in β-oxidation of fatty acids, as well as CPT1, FABP3, FAT/CD36 and Acyl-CoA oxidase (Huss *et al.*, 2004). PGC-1α co-activates the nuclear receptor Peroxisome Proliferator Activated Receptor-α (PPARα) in the control of lipid metabolic genes such as MCAD, CPT1, FABP3, CD36 and Acyl-CoA oxidase (Vega *et al.*, 2000). PGC-1α co-activates FOXO1, which promotes the expression of genes involved in FA metabolism such as CD36 and ACC (Puigserver *et al.*, 2003;Bastie *et al.*, 2005).

Modest PGC-1α overexpression in rat muscle *in vivo* increases the expression of the FA transport proteins FAT/CD36 and FABPpm (Benton *et al.*, 2008). Similar results have been seen in PGC-1α transgenic mice where FAT/CD36, FATP and FABP3 were upregulated compared with controls (Calvo *et al.*, 2008). This increase in FA transport proteins was accompanied by the induction of CPT-1 and a number of markers of the mitochondrial FAO pathway including MCAD and LCAD (Calvo *et al.*, 2008). Wende *et al*. (2007) also observed similar increases in FAT/CD36, CPT-1 and MCAD mRNA, whereas, Choi *et al*. (2008) reported increases in the mRNA expression of CPT-1, CPT-2, LCAD, MCAD and the protein expression of CPT-1 and ACC in PGC-1α transgenic mice (Choi *et al.*, 2008). This outlines the function of PGC-1α expression in the control of the adaptation towards greater lipid metabolism.

PGC-1α expression affects the utilisation of carbohydrate by increasing glucose uptake and repressing glucose oxidation. Adenovirus-mediated PGC-1α expression in C_2C_{12} and L6 muscle cells co-activates MEF2 to increase the mRNA expression of GLUT4 to raise glucose uptake in the muscle (Michael *et al.*, 2001). The increased expression of

GLUT4 is dependent upon a binding site on its promoter for the transcription factor MEF2 (Thai *et al.*, 1998). The increase seen in GLUT4 is accompanied by increased glucose uptake in the cells (Michael *et al.*, 2001). Modest overexpression of PGC-1α in a single rat muscle *in vivo* increases GLUT4 protein expression (Benton *et al.*, 2008). In a PGC-1α transgenic mouse model, GLUT4 expression is increased accompanied by an increase in muscle uptake of glucose (Wende *et al.*, 2007). PGC-1α expression resulted in a reduction in the expression of the gene-encoding phosphofructokinase (PFK), a gene that catalyses a tightly regulated, rate-limiting step in the glycolytic pathway – the addition of a phosphate group to fructose 6-phosphate to create fructose 1,6-biphosphate (Wende *et al.*, 2007). Expression of PGC-1α in myotubes has been shown to promote glucose sparing and fatty acid oxidation by increased PDK4 mRNA and protein expression through a nuclear-receptor binding site occupied by ERRα (Wende *et al.*, 2005). Glucose-6-Phosphate levels, which is known to increase glycogen synthesis, were increased in PGC-1α transgenic mice in conjunction with increased muscle glycogen levels (Wende *et al.*, 2007). These findings prove that PGC-1α regulates a number of genes that act to conserve glucose oxidation but increase its uptake and storage in the muscle.

In this section, a diverse set of functions of the transcriptional coactivator PGC-1α have been outlined. PGC-1α controls mitochondrial biogenesis and the regulation of substrate metabolism through co-activation of its transcription factors to regulate the expression of its target genes. The multifunctional nature of PGC-1α gives reason to it being referred to as a 'master regulator' of metabolic processes.

2.3.5 Transcriptional response to exercise

Both acute and chronic exercise has been shown to modulate the expression of metabolic genes, but, for the purpose of this thesis, the effects of acute exercise on gene transcription will be discussed. This section will document and compare the effect of an acute bout of exercise on PGC-1α expression and the expression of its target genes previously outlined in this review. Exercise results in a number of adaptive changes to the metabolic pathways governing substrate utilisation including substrate availability, transport and oxidation. Exercise is also known to control the expression of a number of proteins involved in the oxidation of fuel in the mitochondria. This section will review the adaptation of the genes regulating these processes in response to acute exercise.

2.3.5.1 Regulation of transcription of PGC-1α by an acute bout of exercise

Numerous studies have shown that the expression of PGC-1α is increased in response to acute endurance exercise of varying mode, intensity and duration in human skeletal muscle (Mathai *et al.*, 2008); (Gibala *et al.*, 2009); (Coffey *et al.*, 2006); (Cluberton *et al.*, 2005); (Pilegaard *et al.*, 2005); (Russell *et al.*, 2005); (Mahoney *et al.*, 2005); (Cartoni *et al.*, 2005); (Vissing *et al.*, 2005); (Norrbom *et al.*, 2004). The exercise-mediated increase in PGC-1α expression also has clinical significance. PGC-1α mRNA was increased five hours after exercise in insulin-resistant muscle; however, this increase was significantly less than the response seen in healthy tissue (De Filippis *et al.*, 2008). Furthermore, Sriwijitkamol *et al*. (2007) found that PGC-1α expression can increase in obese and T2D subjects in response to 40 minutes exercise at both 50% and 70% VO_2max. Therefore, exercise may be important for increasing gene expression as well as energy expenditure in metabolic disease such as obesity and type 2 diabetes. Similar changes in skeletal muscle PGC-1α mRNA and protein have been reported in a variety of rodent models, including aerobic exercise of different intensities and duration (Spangenburg *et al.*, 2009); (Wright *et al.*, 2007b); (Terada *et al.*, 2005); (Baar *et al.*, 2002); (Terada *et al.*, 2002); (Terada & Tabata, 2004) as well as electrical stimulation in cell culture (Irrcher *et al.*, 2003b); (Atherton *et al.*, 2005). The expression and function of PGC-1α has been implicated in the regulation of metabolic gene expression. Therefore, the exercise-mediated increase in PGC-1α expression may be responsible for other exercise-mediated adaptations particularly those of PGC-1α-regulated genes.

2.3.5.2 PGC-1α and transcriptional control of glucose transport

Numerous studies have reported an increase in GLUT4 expression following an acute bout of exercise at different intensities and duration (Kraniou *et al.*, 2000); (Cluberton *et al.*, 2005); (Civitarese *et al.*, 2005); (Kraniou *et al.*, 2006). Similar increases in GLUT4 mRNA and protein have been observed in rats following exercise (Kuo *et al.*, 1999); (Smith *et al.*, 2007). In contrast to these findings, GLUT4 expression was unchanged following cycling- and knee-extensor exercise despite increased mRNA of the co-activator PGC-1α, which is thought to regulate GLUT4 expression (Pilegaard *et al.*, 2005); (Vissing *et al.*, 2005). However, Neufer and Dohm demonstrated an increase in the transcriptional activity of GLUT4 in response to swimming exercise in rats (Neufer

& Dohm, 1993). GLUT4 translocation to the plasma membrane, which correlates with glucose transport into the cell (Lund *et al.*, 1997), was increased following sixty minutes exercise at 60-70% VO_2peak (Kennedy *et al.*, 1999).

The AMPK-induced expression of GLUT4 requires PGC-1α (Zong *et al.*, 2002) and the increase in GLUT4 mRNA following exercise is thought to be associated with PGC-1α (McGee & Hargreaves, 2006). McGee and Hargreaves reported an increase in the DNA-binding activity of the MEF2 and the GLUT4 enhancer factor (GEF) promoters in response to a single bout of cycling exercise (McGee *et al.*, 2006). Transcription of GLUT4 by DNA-bound MEF2 is thought to be inhibited by the association of class II HDACs (McKinsey *et al.*, 2001). AMPK has been shown to phosphorylate class II HDACs and cause nuclear exclusion. McGee & Hargreaves (2004) found that HDAC-associated MEF2 and nuclear localisation were reduced following 60 minutes of cycling. AMPK has also been shown to phosphorylate GEF in vitro which binds to the promoter of GLUT4 and has been shown to increase its DNA binding activity (Holmes *et al.*, 2005). These findings suggest exercise can control GLUT4 expression by regulating the transcriptional activity of GEF and MEF2, through modulation of MEF2 interaction with HDACs and the coactivator PGC-1α, which are regulated by the exercise-responsive signalling cascades.

2.3.5.3 Mobilisation and transport of fatty acids

Hormone sensitive lipase (HSL) is a key enzyme in the mobilisation of fatty acids from intracellular lipid stores. HSL is transiently activated during exercise at 60% VO_2peak, reaching maximal activation after 60 minutes but returning to near basal activity by 120 minutes (Watt *et al.*, 2003a). Roepstorff showed that AMPK-dependent phosphorylation of HSL on Ser^{565} had no effect on HSL activity (Roepstorff *et al.*, 2004b). In support of these findings, endurance training had no effect on HSL activity or protein expression in rat skeletal muscle (Donsmark *et al.*, 2004). Lipoprotein Lipase is a key enzyme in the hydrolysis of triacylglycerol rich chylomicrons and VLDL. LPL activity has been shown to increase following 60 minutes dynamic knee-extensor exercise (Kiens *et al.*, 1989). Eight weeks of single-leg exercise training increased LPL activity in skeletal muscle compared with the untrained leg (Kiens & Lithell, 1989). Numerous studies have demonstrated an increase in the transcriptional activation of LPL in response to an acute bout of exercise (Pilegaard *et al.*, 2005) (Pilegaard *et al.*, 2003a); (Seip *et al.*,

1997); (Hildebrandt *et al.*, 2003); (Kiens & Richter, 1998). Pilegaard and colleagues observed an increase in LPL transcriptional activity stimulated by 75 minutes cycling at 75% VO_2peak (Pilegaard *et al.*, 2005). However, these changes in transcriptional activity were not matched by an increase in mRNA or protein expression (Pilegaard *et al.*, 2005). Cycling for 90 minutes at 60% VO_2peak increased LPL mRNA in both untrained and trained individuals (Kiens *et al.*, 2004). Similarly, 3 hours of knee-extensor exercise increased LPL mRNA expression 1 hour post-exercise and remained elevated during 8 hours of recovery (Vissing *et al.*, 2005). This evidence suggests that exercise plays a role in the adaptation to substrate utilisation by increasing the enzymes responsible for lipolysis of TGs and therefore the subsequent availability of circulating FA for oxidation.

The transport of FA into the mitochondria for oxidation provides a number of significant points of regulation in the supply of FA. The fatty acid binding protein (FABP) which is bound to the plasma membrane regulates FA uptake into the skeletal muscle cell. An acute bout of knee-extensor exercise increased FABP expression 3 h after exercise and remained elevated during 20 h of recovery (Vissing *et al.*, 2005). In support of this evidence, an acute bout of bicycle exercise increased FABPpm (FABP located on the plasma membrane) and FABPc (FABP located in the cytoplasm) mRNA. This suggests an increase in FABP localised to the plasma membrane to allow FA uptake into the cell. However, the induction of FABP in this case seems to be gender specific as FABPpm mRNA increases in untrained males and trained females whereas FABPc is increased only in untrained males (Kiens *et al.*, 2004). The authors suggest that women express higher basal levels of FABPc mRNA pre-exercise than men; hence the lack of improvement with exercise. In contrast to these findings, 60 minutes of intense exercise was shown to have no effect on FABP expression (Roepstorff *et al.*, 2004a).

Membrane-bound FAT/CD36 also seems to be responsive to an acute bout of exercise in human skeletal muscle. Moderate intensity aerobic exercise has been shown to increase FAT/CD36 protein content in some: (Roepstorff *et al.*, 2004a); (Holloway *et al.*, 2006), but not all studies (Civitarese *et al.*, 2005). There may be an intensity-dependent response for FAT/CD36 expression as there were no changes in protein content following high-intensity aerobic and resistance exercise (Yang *et al.*, 2005). As with FATP there may also be a gender specific response. Kiens *et al*. (2004) reported an

increase in FAT/CD36 expression following an acute bout of exercise but only an increase in mRNA in males (Kiens *et al.*, 2004). The authors speculate that women express higher levels of FAT/CD36 mRNA than men and this is supported by the findings of (Steffensen *et al.*, 2002).

2.3.5.4 Transcriptional control of substrate utilisation

Substrate selection is regulated at a number of points in the cell and is dependent on nutrient and oxygen availability. Exercise increases the expression of a number of genes that influence substrate selection. The exercise-mediated regulation of these genes may form part of the adaptive response to exercise training, facilitating lipid oxidation at rest and during submaximal exercise.

In the post-exercise period the contribution of lipid oxidation to energy expenditure is increased (Kiens & Richter, 1998). PDK4 may be one of the important components that control the switch to fat metabolism by phosphorylation and inactivation of the PDC complex to prevent the conversion of pyruvate to acetyl CoA, resulting in allosteric inhibition of glycolysis and suppression of glucose oxidation (Pilegaard & Neufer, 2004). PDK4 could promote carbohydrate sparing in the post-exercise period in an attempt to replenish muscle glycogen content. Kimber et al (2003) found that following glycogen depleting exercise there was a decrease in glucose oxidation, an increase in glycogen synthesis and an increase in mitochondrial fatty acid oxidation (Kimber *et al.*, 2003). The expression of PDK4 is highly sensitive to alterations in metabolic status such as acute exercise, fasting and high-fat/low-carbohydrate diets, and may form part of the adaptive response to exercise (Pilegaard *et al.*, 2000;Peters *et al.*, 2001;Pilegaard *et al.*, 2003b;Tsintzas *et al.*, 2006). A number of studies have reported increased PDK4 expression in human skeletal muscle following an acute bout of exercise (Cluberton *et al.*, 2005); (Pilegaard *et al.*, 2005); (Mahoney *et al.*, 2005); (Coffey *et al.*, 2006); (Pilegaard *et al.*, 2002); (Civitarese *et al.*, 2005). Pilegaard et al (2000) showed that PDK4 mRNA and transcription were increased immediately following 75 minutes and 4 hours of exercise and remained elevated for at least 4 hours after exercise (Pilegaard *et al.*, 2000). PDK4 mRNA is increased following aerobic exercise at 50% and 75% VO_2max (Hildebrandt *et al.*, 2003) and following resistance exercise (Yang *et al.*, 2005). The increase in PDK4 mRNA is sustained for longer following aerobic (8-12 h) than resistance exercise (2-8 h) (Yang *et al.*, 2005).

PDK4 expression is under the control of a number of transcription factors and co-activators, including PGC-1α. FOXO1 binds directly to the PDK4 promoter in C_2C_{12} muscle cells and increases PDK4 expression (Furuyama *et al.*, 2003). FOXO1 mRNA is increased in response to an acute bout of intermittent exercise in the skeletal muscle of mice suggesting it may be involved in the exercise-induced increase in PDK4 (Huang *et al.*, 2007). As stated previously, PGC-1α expression is increased by exercise. PGC-1α is known to coactivate PPARα and the orphan nuclear receptor ERRα (Vega *et al.*, 2000); (Wende *et al.*, 2005). The expression of ERRα increases concurrently with PGC-1α following an acute bout of exercise (Cartoni *et al.*, 2005). ERRα cooperates with PPARα to amplify the PGC-1α-mediated regulation of metabolic gene expression as they have been shown to target MCAD, an enzyme that mediates the first step in β-oxidation of fatty acids (Huss *et al.*, 2004). PGC-1α increases PDK4 mRNA and activates the PDK4 promoter possibly encouraging glucose sparing and fatty acid oxidation (Wende *et al.*, 2005). PGC-1α co-activates PDK4 through a nuclear receptor binding site occupied by ERRα and this effect is lost in ERRα-null mice (Wende *et al.*, 2005). The transcription factor PPARα may also play a role as PDK4 mRNA and protein are increased in rat skeletal muscle after feeding the PPARα analogue WY14,643 (Wu *et al.*, 1999a). Similar increases in PDK4 mRNA levels were seen in liver cells stimulated by WY14,643 (Huang *et al.*, 2002). This evidence suggests that the PGC-1α/ERRα complex along with PPARα may be involved in the control of substrate selection after exercise by inducing PDK4 expression.

Another important step in substrate selection is the regulation of LCFA-CoA transport across the inner mitochondrial membrane by CPT1. The transcriptional activity of CPT1 increases after a single bout of exercise in human skeletal muscle (Holloway *et al.*, 2006). Pilegaard et al (2005) saw an increase in the transcriptional activity of CPT1 following bicycle exercise at 75% VO_2peak; however, this increase was not seen at the mRNA level (Pilegaard *et al.*, 2005). Against this, other studies reported no change in CPT1 expression following resistance exercise and aerobic exercise at both high and low intensity ((Yang *et al.*, 2005); (Hildebrandt *et al.*, 2003).

These results suggest that PGC-1α co-activated genes play a major role in the adaptive response to an acute bout of exercise. However, PGC-1α is also known to be an important regulator of nuclear encoded mitochondrial genes and mitochondrial

biogenesis. Exercise training has also been shown to increase mitochondrial content, but little is known about the response of mitochondrial genes to an acute bout of exercise.

2.3.5.5 Transcriptional response to exercise in mitochondria

Mitochondrial biogenesis is a complex process requiring the coordinated expression of nuclear and mitochondrial genes, protein translation, mitochondria targeting and importation followed by protein or subunit complex assembly. As part of this review, I will focus on mitochondrial proteins known to be regulated by PGC-1α in response to a single bout of exercise, while acknowledging that mitochondrial biogenesis is only likely to occur following repeated bouts of exercise.

Citrate synthase (CS), a marker of mitochondrial activity, is an important enzyme in the metabolism of carbohydrates and fats as it catalyses the first reaction in Kreb's cycle. Acetyl CoA is combined with oxaloacetatic acid (OAA), and catalysed by the enzyme CS to produce citric acid. CS activity is increased in PGC-1α transgenic mice, suggesting it is a target of the coactivator (Wende *et al.*, 2007); (Calvo *et al.*, 2008). CS expression is upregulated after acute exercise. A single bout of knee-extensor exercise resulted in an increase in CS mRNA in human skeletal muscle (Vissing *et al.*, 2005). Supporting evidence for these results was seen in rodent models where both the transcriptional activity and mRNA expression of CS were increased by exercise (Neufer & Dohm, 1993); (Wright *et al.*, 2007b).

Exercise is known to influence the expression and transcriptional activity of a number of transcription factors involved in the control of nuclear genes encoding mitochondrial metabolic proteins. Nuclear respiratory factor-1 (NRF-1) is one such transcription factor implicated in the PGC-1α-mediated control of muscle metabolism with particular influence on mitochondrial biogenesis as discussed earlier. PGC-1α binds NRF-1 and can activate transcription of NRF-1 target genes involved in mitochondrial respiration (Wu *et al.*, 1999b). Exercise has been shown to increase the expression of NRF-1 (De Filippis *et al.*, 2008); (Sriwijitkamol *et al.*, 2007) and NRF-2 (Cartoni *et al.*, 2005), accompanied with a simultaneous increase in PGC-1α expression in human skeletal muscle. This evidence is supported in C_2C_{12} muscle cells where stimulation of contractile activity is shown to upregulate both NRF-1 and PGC-1α (Irrcher *et al.*, 2003b). In contrast, various other exercise studies reported an increase in PGC-1α

expression with no change in either of these transcription factors in human muscle (Pilegaard *et al.*, 2003b); (Norrbom *et al.*, 2004).

Both NRF-1 and 2 have binding sites in the promoter region of Tfam (Virbasius & Scarpulla, 1994); (Larsson *et al.*, 1998); (Rantanen *et al.*, 2001). Overexpression of PGC-1α in myotubes coactivates NRF-1 and 2 (Wu *et al.*, 1999b) to regulate expression of Tfam (Fisher *et al.*, 1992); (Garesse & Vallejo, 2001); (Larsson *et al.*, 1998). As mentioned previously, stimulation of contractile activity is shown to upregulate both NRF-1 and PGC-1α, and this is coincident with an increase in Tfam protein expression in C2C12 muscle cells (Irrcher *et al.*, 2003b). An acute bout of exercise in rats increases Tfam protein content (Kang *et al.*, 2009). PGC-1α can link nuclear regulatory events in response to exercise to the transcriptional machinery by targeting the NRF-1/2 binding sites on the Tfam promoter leading to increased mRNA expression (Gleyzer *et al.*, 2005). This evidence indicates NRF-1 and NRF-2, coactivated by PGC-1α, are important for control of the expression of genes involved in mitochondrial function and metabolism in response to exercise.

There is evidence to suggest that an acute bout of exercise results in an adaptive response to a number of the enzymes involved in oxidative phosphorylation in the mitochondria. Exercise has been shown to augment the DNA binding activity of NRF-1 and NRF-2. NRF-2 specifically binds to the COX IV promoter (Scarpulla, 1997); (Virbasius & Scarpulla, 1994). In addition, COX IV is also increased by a single-exercise session. COX IV mRNA was increased 24 hours after a 10km bike trial (Cartoni *et al.*, 2005). Intermittent cycling exercise at high intensity also increased COX VI mRNA (De Filippis *et al.*, 2008). Swimming was shown to increase the binding of the transcription factor NRF-2 to the promoter region of COX IV after a single bout of exercise in rats (Baar *et al.*, 2002); (Wright *et al.*, 2007b). Cytochrome c expression is also upregulated following a 6-h swimming exercise in rats (Wright *et al.*, 2007b). This increase in mRNA is accompanied by binding of the transcription factor NRF-1 to the promoter of cytochrome c. Pharmacological activation of AMPK, a kinase activated by exercise, also results in increased expression of cytochrome c (Jager *et al.*, 2007). Furthermore, uncoupling protein 3 (UCP3), a mitochondrial carrier protein, is transcriptionally activated by acute exercise in human skeletal muscle (Hildebrandt *et al.*, 2003); (Pilegaard *et al.*, 2005). UCP3 mRNA increased after 3 hours of knee-

extensor exercise (Pilegaard *et al.*, 2002). Similar results were observed following only 60 minutes of cycling in a separate study (Cluberton *et al.*, 2005).

2.4 Effect of exercise on muscle fiber morphology

The adaptive response to exercise in human skeletal muscle is not limited to the molecular level. Several morphological and structural adaptations occur in the muscle fiber as part of exrcise response. Following an acute bout of exercise myofibrillar damage has been observed in the form of disrupted Z-line streaming where Z-lines are out of register, loss of thick myofilaments, myosin and titin, as well as disruption of the filaments (myosin) in the A-band (Friden *et al.*, 1981); (Friden *et al.*, 1983). Friden et al also observed disruption to the localisation of the cytoskeleton protein desmin, which links Z-lines together, after a bout of eccentric exercise (Friden *et al.*, 1984). This evidence suggests that exercise initially causes damage to the ultrastructure of the muscle cell.

An acute bout of exercise has also been shown to affect the extracellular matrix. Stauber et al. carried out muscle biopsies following eccentric exercise and observed mast cell degranulation in the perimysial area, mononuclear cells in the perimysial and endomysial regions, and that the extracelluar matrix had become separated form the myofibers (Stauber *et al.*, 1990). Fibrinogen and albumin (plasma constituents) were also found in the extracellular space suggesting damage to the capillaries as well as the extracellular matrix (Stauber *et al.*, 1990).

In response to exercise, skeletal muscle undergoes capillarisation to allow greater oxygen flow to working muscles. An increase in capillary density in the working muscle has been observed in both cycling and rowing (Andersen & Henriksson, 1977); (Larsson & Forsberg, 1980). This increase in muscle capillarity, is thought to be important for improving blood-tissue exchange properties by increasing the surface area for diffusion, shortening the average diffusion length within the muscle and increasing the length of time for diffusive exchange between blood and muscle (Bloor, 2005).

Exercise training is known to increase both the size and number of mitochondria in skeletal muscle thought to be an effort to allow greater ATP supply to the working

muscles. A standard exercise bicycle training program lasting 6 weeks has been shown to increase mitochondrial density by ~40% in the vastus lateralis of subjects (Hoppeler *et al.*, 1985). Endurance training has also been shown to preferentially increase subsarcolemmal mitochondria as opposed to intermyofibrillar mitochondria (Hoppeler *et al.*, 1985). Adaptatiopns in the content of mitochondrial nzymes alsooccur in response to exercise but these adaptations are discussed under the heading mitochondrial biogenesis elsewhere in the text.

Four types of muscle fiber exist in skeletal muscle (Type I, Type IIa, Type IIb and Type IIx). Individual fiber types can be identified by the isoform of Myosin Heavy Chain (MYH) present as well as by their oxidative capacity (oxidative/glycolytic). Type I fibers possess MYH7 and have a high oxidative capacity and appear red in colour due to a high concentration of the oxygen binding protein myoglobin. Type I fibers have low glycolytic and slow contraction capabilities but are resistant to fatigue. Type IIa can be identified by the MYH2 isoform and possess high glycolytic and oxidative capabilities. Type IIx fibers possess the MYH1 isoform and are high in glycolitic but low in oxidative properties but capable of fast contractions. Finally, Type IIb fibers are identified by the MYH4 isoform and possess the ability to produce rapid contractions but have very little fatigue resistance, These fibers tend to be white in appearance as they have very low levels of myoglobin or capillaristaion.

In response to exercise there is a shift in the fiber type within the muscle depending on the nature of the exercise. Following endurance training or aerobic exercise there is a shift from Type IIb fibers to the more oxidative Type I (Howald *et al.*, 1985;Andersen & Henriksson, 1977;Simoneau *et al.*, 1985) and Type IIa fibers (Andersen & Henriksson, 1977);(Ingjer, 1979). Conversely, there is an increase in the proportion of fast fiber types (32-38%) following sprint training accompanied by a concurrent decrease in Type I fibers (57-48%) (Jansson *et al.*, 1990). This data suggests the switch in fiber types is specific to the exercise stimulus.

The evidence presented in this section demonstrates that the muscle fiber undergoes several structural and functional morphological changes in response to both a single bout of exercise as well as exercise training.

2.5 Summary

It is apparent from the literature that significant adaptations to the factors governing metabolism in the muscle occur in response to an acute bout of exercise. This adaptation begins with the activation of a number of signalling kinases with AMPK, CaMKII, p38 MAPK and NAD^+ acting as the protagonists in a tightly regulated response to this metabolic perturbation (Bassel-Duby & Olson, 2006). As part of the response, these signalling kinases regulate the activity of a number of transcription factors and transcriptional co-regulators which interact to translate the physiological stimulus of exercise into a transcriptional adaptation in a bid to return to homeostasis (Desvergne *et al.*, 2006). In turn, this affects the expression and activity of a number of enzymes that regulate metabolic processes such as substrate utilisation and mitochondrial function.

In reviewing the literature it is impossible to ignore the significant role of the co-activator PGC-1α in the exercise-mediated transcriptional adaptations. It is not surprising given that the signalling kinases discussed all converge to regulate its expression and acivity, added to it's interactions with several transcription factors and the control it exerts over so many metabolic gene targets that it has been termed the 'master regulator' by some researchers (Czubryt *et al.*, 2003;Wu *et al.*, 2002).

In conclusion, it is clear from the literature that a single bout of exercise leads to a transient increase in the mRNA content of a plethora of genes involved in mitochondrial function, carbohydrate and lipid metabolism in human skeletal muscle as described above. Enhanced levels of these gene transcripts can lead to the synthesis of proteins and provoke remodelling of the muscle in the long term (Fluck, 2006). These changes in mRNA in response to exercise can lead to structural and functional adaptations in the muscle specific to the demands being placed on the system (Dufour *et al.*, 2006). However, not enough is known about the mechanisms involved in these adaptations. Furthermore, the role of exercise and muscle contraction in terms of the mode, frequency, intensity and duration in transcriptional regulation is not fully understood. This information may have implications for the prevention and treatment of diseases such as Type 2 Diabetes Mellitus and obesity, therefore, further investigation into the adaptive response of human skeletal muscle to acute bouts of exercise is warranted.

Chapter III Methodologies

3.1 Introduction

The overall aim of this thesis was to examine the regulation of metabolic gene expression in human skeletal muscle following an acute bout of exercise. For this purpose experiments were designed to investigate the impact of altered contraction force and frequency on metabolic gene expression.The experimental design for each study has many common elements to allow for comparison. This section will describe the experimental design and methodologies for both experiments. The development of novel techniques and procedures which had not previously been set up in the Metabolic Physiology Research Unit at DCU will also be described.

3.1.1 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}. A pedal frequency of 75-80RPM was maintained throughout both trials. Skeletal muscle biopsies from the *m. vastus lateralis* were taken at rest and at +0 h, +3 h and +19 h after exercise.

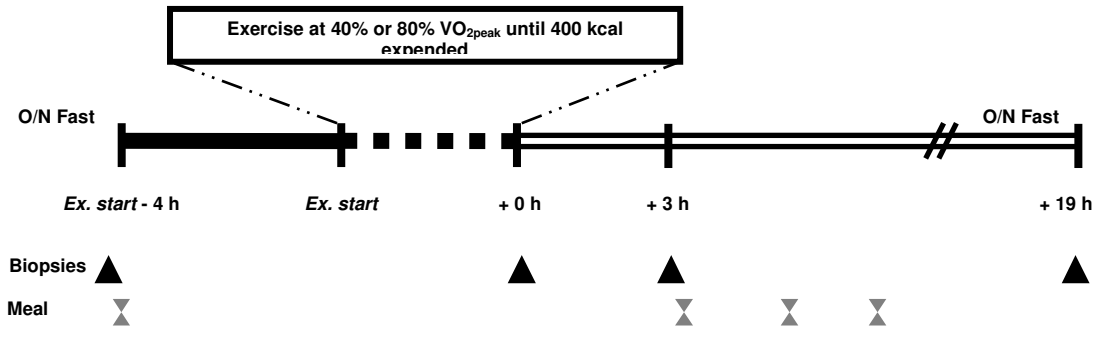

Figure 3.1 Schematic of experimental design for experiment I

3.1.2 Experiment II:

Eight healthy, sedentary males performed two isocaloric (400 kcal) cycle exercise trials at identical power outputs, eliciting approximately 50% VO_{2peak}, pedalling at a cadence

of either 50 RPM or 80 RPM. Skeletal muscle biopsies from the *m. vastus lateralis* were taken at rest and at +0 h, +3 h and +19 h after exercise.

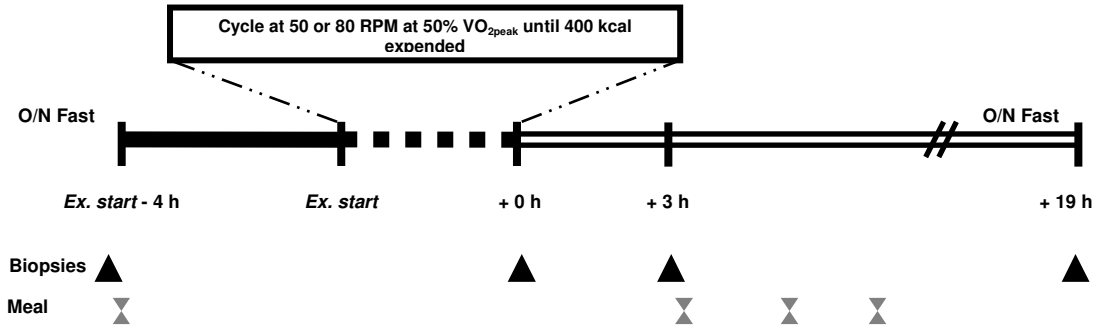

Figure 3.2 Schematic of experimental design for experiment II

3.1.3 Participants

Eight healthy young, sedentary, males participated in each study. In all cases the participants provided written informed consent, after an explanation of the experimental procedures, which were approved by the Dublin City University Research Ethics Committee and conformed to the Declaration of Helsinki. Each participant completed a health history questionnaire underwent a medical screening examination and electrocardiogram to confirm their suitability for the experiment. Subjects were excluded if they exercised regularly, smoke, had a BMI >27, had diabetes, suffered from other acute or chronic diseases or used drugs that the physician and investigators decided would interfere with the normal adaptation to the proposed intervention.

3.1.4 Peak Oxygen Uptake (VO_2peak) and verification of submaximal intensity

All exercise tests were carried out under standard laboratory conditions (19-21°C, 40-55% relative humidity). Participants performed an incremental exercise test to volitional fatigue on an electronically braked cycle ergometer (Ergoline 900, SensorMedics, Yorba Linda, CA) to determine VO_{2peak}. Subjects began cycling at 100 W for five minutes to warm-up and the power output was increased by 50 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, sampled from a mixing chamber and analysed by the Vmax 29C indirect calorimetry system (SensorMedics, Yorba Linda, CA). Heart rate was continuously monitored during exercise by radiotelemetry (Polar Vantage NVTM; Polar, Port Washington, NY).

Between four and seven days later, each participant returned to the laboratory to perform sub-maximal exercise to verify the power outputs required to elicit 40% and 80% VO_{2peak} in Experiment I, and 50% VO_{2peak} in Experiment II. The power output required to elicit a given percentage of VO_{2peak} was estimated 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 was 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. Subjects cycled for 10 min at a number of different power outputs, starting at 15 W below the predicted power output and adjusted thereafter until the correct percentage of VO_{2peak} was maintained in steady state. This trial took approximately 30-40 minutes.

3.2 Experimental trials

Exercise trials were carried out in random order and separated by exactly seven or fourteen days depending on the availability of subjects and the research team. The experimental design required the participants to complete two main experimental trials consisting of cycle exercise at either high (80% VO_{2peak}, HI) or low (40% VO_{2peak}, LO) intensity in experiment I or at identical power outputs, eliciting approximately 50% VO_{2peak}, pedalling at a cadence of either 50 RPM or 80 RPM in experiment II. The exercise bouts were isocaloric in both experiments and required each participant to expend 400 kcal. The experimental protocol was identical for the two trials in every aspect except for the exercise intensity in experiment I and the contraction frequency in experiment II. The isocaloric energy expenditure resulted in a different duration of exercise between trials. All experimental trials began between 0730 and 0930 to preclude the influence of circadian variation. Participants reported the Metabolic Physiology Research Unit after an overnight (8-10 h) fast and were requested to rest quietly in a supine position for approximately ten minutes. A resting venous blood sample (12 ml) was collected by venipuncture of an antecubital vein and 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 to allow for nutrient digestion, absorption and disposal. Immediately prior to exercise, an indwelling catheter (Insyte-W 20/22G, Becton Dickinson, Franklin Lakes, NJ) was introduced into an antecubital vein. Blood samples (8 ml) were taken at rest, at 10 min intervals throughout exercise and at the termination of exercise. Expired air was collected continuously throughout exercise and analysed using the Vmax 29C gas analysis system (SensorMedics, Yorba Linda, CA). The intensity of exercise was monitored and the minute averages for oxygen consumption and carbon dioxide production were used to calculate the rate and total amount of energy expenditure (CONSOLAZIO *et al.*, 1963). Participants were required to maintain the cycle cadence between 70 and 75 rpm in experiment I and at either 50 or 80 rpm in experiment II. A second muscle biopsy (#2) was taken immediately after exercise. In the 3-hrs post exercise participants remained in the laboratory with minimal ambulation and were permitted to consume only water ad libitum. Another muscle biopsy (#3) was taken 3-hrs after exercise and a standardised meal was consumed after which participants were free to leave the laboratory. Subjects were provided with a standardised evening meal and snack, and water was permitted ad libitum. The following morning participants reported to the laboratory after a similar overnight fast and were requested to rest quietly for approximately ten minutes. A resting venous blood sample was collected and a fourth muscle biopsy (#4) was taken at 19 h after the termination of exercise.

3.2.1 Muscle Biopsies

A biopsy was then taken from the middle portion of the vastus lateralis using a percutaneous muscle biopsy needle (United HealthCare), as previously described (Bergstrom et al., 1962).

After the area of the skin above the muscle to be biopsied was identified the area was shaved and cleaned by the application of a fluid antiseptic (Betadine) which was by an alcohol swap. After cleaning, the muscle was anaesthetised with 2% w/v Lidocaine HCl. This was done by injecting a small amount (1 ml) of the anaesthetic to raise a bleb under the skin and more (3-5 ml) into the covering of the muscle (fascia).

The anaesthetic was given 5–minutes prior to biopsy to desensitise the muscle and then a small incision (approx 0.5-1 cm) was made with a #11 scalpel. The incision was made through the skin and the covering (fascia) of the muscle. The biopsy needle was then

introduced through the fascia and a number of muscle pieces were cut after brief suction. The needle was removed from the leg and the muscle sample (~100-150 mg) was immediately snap frozen in liquid nitrogen and stored at -80°C for subsequent analysis. The incision was then closed with steri-strips and wrapped in a large pressure bandage.

Biopsies #1 and #4 were taken from the right leg; biopsies #2 and #3 were taken from the left leg. For each biopsy, a fresh incision was made at the biopsy site, at least 2 cm distal or proximal to any other site. In the case of biopsy #2, local anaesthetic was administered immediately prior to exercise during the high intensity trial of experiment I. During the low intensity trial and both trials of experiment II, exercise was interrupted briefly approximately fifteen minutes prior to the estimated end of exercise and the anaesthetic was administered. The post-exercise muscle biopsies were completed within 90 seconds.

Ideally, we would have liked to investigate a time course of metabolic signalling and gene expression over a 24 h period with more time points but as the muscle biopsy is an invasive procedure ethical considerations limited the number of biopsies performed. Due to the transient nature of contraction signalling, transcriptional activation and mRNA and protein expression, it was important to time the biopsies to coincide with critical events in the metabolic response to exercise. A fasting muscle biopsy was performed on the morning of each trial to provide a resting/control sample. Activation of various signalling kinases has been shown during exercise but this decreases rapidly upon cessation of contraction (Wojtaszewski *et al.*, 2000); (Chen *et al.*, 2003); (Rose *et al.*, 2006); (Widegren *et al.*, 1998). This evidence prompted us to carry out a biopsy immediately post exercise. Numerous studies have shown that the mRNA of a number of exercise responsive genes are upregulated between 2-4h after exercise in humans (Civitarese *et al.*, 2005);(Kraniou *et al.*, 2000);(Yang *et al.*, 2005);(Cluberton *et al.*, 2005);(Vissing *et al.*, 2005). It is for this reason we elected to perform a biopsy 3 h post exercise. The final biopsy at 19 h post exercise was performed to determine if there had been any changes in protein content following the exercise bout.

3.2.2 Dietary Control

It was important to control dietary intake during the experimental trials as nutrient availability has been shown to alter the activity of signalling kinases and the expression

of some metabolic genes (Pilegaard *et al.*, 2003a); (De Lange *et al.*, 2006). Pre-exercise preparation was the same for each exercise test. Subjects were instructed 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 trials and to replicate their dietary intake for the second trial. The dietary intake during each experimental 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 (1918) equation (see below) multiplied by an appropriate activity factor. As the subjects performed very little activity throughout the day of the experimental trial an activity factor of 1.4 was adopted based on a paper by (Durnin, 1996), plus 400 kcal added for energy expended during the exercise trial, therefore, the total energy expended was estimated at (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, carbohydrate, fat and protein intake was 36 kcal.kg^{-1} BM, 6.0 g.kg^{-1} BM, 0.8 g.kg^{-1} BM, and 1.2 g.kg^{-1} BM, respectively. Hence, the percentage contribution of each macronutrient was 67% CHO, 20% fat and 13% protein. No consumption of other food or beverage other than water was permitted.

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

3.3 Laboratory analysis

3.3.1 Blood analysis

Blood samples (4 ml) were collected in vacutainers (FX Plus, Becton Dickinson, Franklin Lakes, NJ) and stored on ice until centrifugation at 3000 rpm for 15 min at 4°C. Plasma glucose and lactate was determined in duplicate using an automated analyser (YSI 2300 Stat Plus, Yellow Springs Instruments, OH). In addition, blood samples (4 ml) were collected into vacutainers (Z, Becton Dickinson, Franklin Lakes, NJ) and centrifuged as above. The serum was stored at -80°C for later analysis.

3.3.2 RNA isolation and qPCR

Total RNA was isolated from ~20 mg crude tissue based on the acid guanidinium thiocyanate-phenol-chloroform extraction method of Chomczynski & Sacchi (1987) using TRI reagent (Sigma-Aldrich, UK; T9424) as per the manufacturer's instructions. Total RNA was determined spectrophotometrically at 260 nm and the integrity of each RNA sample was verified by measuring the spectrophotometric 260/280 nm ratio (>1.8) (RNA 6000 Nano Lab Chip & 2100 Bioanalyzer, Agilent Technologies, Palo Alto, CA). Following DNase digestion (RQ1 RNase-free DNase; Promega, Madison, WI; M6101), 2 μg of RNA was reverse transcribed to cDNA using the Reverse Transcription System (Promega, Madison, WI; A3500) primed with oligo-$dT_{(15)}$ as per the manufacturer's instructions. The cDNA template was stored at -20°C until further analysis.

Real-time PCR was performed using the ABI Prism 7900 high throughput sequence detection system and software package (version 1.1; Applied Biosystems, Foster City, CA) and Assay-on-Demand pre-designed gene-specific primer and probe sequences (P/N 4331182; Taqman® Gene Expression Assays, Applied Biosystems, Foster City, CA). 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, Foster City, CA) and nuclease-free water up to 20 μl. 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 analysed in duplicate and the mRNA content was calculated from a standard curve (critical threshold cycle number vs. log dilution) run 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 RT-PCR reaction. The average C_T value of the unknown samples was converted to relative expression data using the appropriate standard curve. mRNA data was expressed as the ratio between the gene of interest and the housekeeper gene, GAPDH. We tested a number of housekeeping genes, based on previous papers that measured mRNA following exercise. Our analysis showed that GAPDH was constitutively expressed and stable at all four sampling points for both studies (Figure 3.3). In contrast, we found considerable variation in the expression of

other housekeeper genes, β_2-microglobulin and cyclophilin, at the same time points (data not shown).

Table 3.1 Gene targets and assay ID for the pre-designed primer and probe sequences used for the experiments.

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.

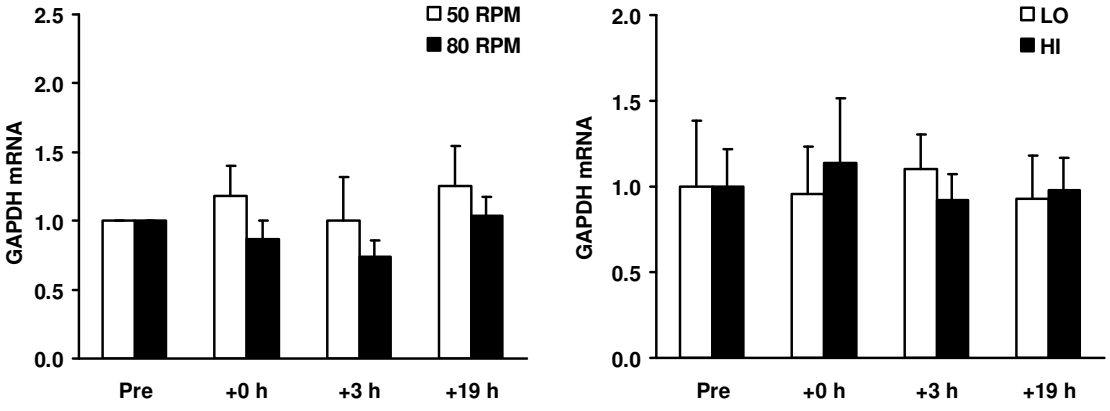

Figure 3.3. Verification of GAPDH as a stable reference gene

3.3.3 Western Blot Analysis

Approximately 20-25 mg of crude muscle was homogenised in 1 ml of ice-cold homogenisation buffer for the determination of protein content (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. Muscle homogenates were rotated end over end for at least 60 min at 4°C. Samples were centrifuged (12,000 *g* for 15 min at 4°C), and protein concentration of the supernatant was determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA; 500-0002), which is based on the Bradford method of protein determination (Bradford, 1976). An aliquot of muscle homogenate (50 μg protein) was mixed with Laemmli buffer containing β-mercaptoethanol (protein concentration, 2 μg/μl) and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples for each subject from the respective exercise trials were compared in parallel. Proteins were separated by SDS-PAGE (7.5-10% resolving gel) by running overnight (~13 hours) at 35mA in electrophoresis buffer (Tris base 25mM, Glycine 192mM, 1% SDS). Proteins were then transferred to polyvinylidine fluoride (PVDF) membranes (Immobilon P-IPVH00010, Millipore, Billerica, MA) by running for 4 hours at 500mA in transfer buffer (Tris base 25mM, Glycine 192mM, 10% Methanol). Membranes were 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. Membranes were incubated overnight with specific antibodies at concentrations listed in Table 3.3. Membranes were washed in TBS-t (4 X 15min) and incubated with appropriate secondary horseradish peroxidase-conjugated antibodies for 2 hours and the wash step was repeated (1:20000; Bio-Rad Laboratories, Hercules, CA). Immunoreactive proteins were visualized by enhanced chemiluminescence (incubated for 5 mins, dried and placed in cassette) (ECL; Amersham Biosciences, Arlington Heights, IL) on developing film and quantified by densitometry (GS710 Calibrated Imaging Densitometer, Biorad Laboratories, Hercules, CA) using the Quantity One software package (BioRad Laboratories, Hercules, CA).

Table 3.2 Resolving and stacking gels for SDS-PAGE separation and analysis by Western blot

Resolving gels	**6%**	**7.5%**	**10%**	**12%**	**Stacking gel**	
ddH2O (ml)	21.8	19.4	16.4	14	ddH2O (ml)	6.2
1.5M Tris pH 8.8 (ml)	10	10	10	10	0.5M Tris pH 6.8 (ml)	2.5
30% Acrylamide (ml)	8	10	13.2	16	30% Acrylamide (ml)	1.3
10% SDS(ul)	400	400	400	400	10% SDS(ul)	100
10% APS (ul)	180	200	180	200	10% APS (ul)	100
Temed (ul)	16	20	16	20	Temed (ul)	10

Table 3.3 Antibodies directed against target proteins for immunoblot analysis

Target	Supplier	Code	Source	Molecular Weight	Concentration
COXIV	Cell Signaling	4844	Polyclonal Rabbit IgG	17 kDa	1:1000
CPT1	Santa Cruz	sc-20670	Polyclonal Rabbit IgG	~75 kDa	1:500
ERRα	Santa Cruz	sc-32972	Polyclonal Goat IgG	~50 kDa	1:500
FOXO1A	Cell Signaling	9454	Polyclonal Rabbit IgG	78-82 kDa	1:1000
GAPDH	Santa Cruz	sc-25778	Polyclonal Rabbit IgG	37 kDa	1:500
HKII	Cell Signaling	2106	Polyclonal Rabbit IgG	102 kDa	1:1000
PDK4	Abgent	AP7041b	Polyclonal Rabbit IgG	46 kDa	1:500
PGC-1α	Santa Cruz	sc-13067	Polyclonal Rabbit IgG	75 kDa	1:500
PGC-1α	Calbiochem	ST1202	Polyclonal Mouse IgG	90-95 kDa	1:1000
phospho-Ser^{133}-CREB	Cell Signaling	9191	Polyclonal Rabbit IgG	43 kDa	1:1000
phospho-Ser^{79}-ACC	Cell Signaling	3661	Polyclonal Rabbit IgG	280 kDa	1:500
phospho-Thr^{172}-AMPK	Cell Signaling	2531	Polyclonal Rabbit IgG	62 kDa	1:1000
phospho-	Cell Signaling	3361	Polyclonal	50-75	1:1000

Thr^{286}-CaMKII	Rabbit IgG	kDa

3.3.4 PGC-1α Acetylation

Aliquots of muscle homogenates (200 μg protein) were used to determine PGC-1α acetylation. 20 μl of recombinant Protein A Agarose beads (#20365, Thermo Scientific) were added to the muscle homogenates and left to incubate for 1 hour at 4°C to pre-clear the lysate. The samples were spun at 14,000 rpm for 1 minute and the supernatant was transferred to another tube. 4μg of antibody (PGC-1α H-300, Santa Cruz Biotechnology) was added to 200μg of muscle homogenate and made up to 200μl with homogenate buffer. Samples were rotated for 8 hours at 2-4^{0}C. 50μl of Protein A Agarose beads (Pierce) were added to each sample and allowed to rotate overnight at 2-4^{0}C. Samples were spun at 14000 RPM for 1 minute to pellet the beads. The supernatant was removed and discarded (or kept as control during optimisation of technique). 1ml of homogenate buffer was added to each sample and samples were agitated briefly. Samples were spun at 14000 RPM for 1 minute. The supernatant was removed and this wash step was repeated 3 times with homogenate buffer and twice with TBS. The supernatant was removed and discarded. Samples were resuspended in Laemmli buffer and heated to 95^{o}C for 5 minutes and disturbed occasionally. Samples were spun at 14000 RPM for 1 minute. The supernatant was separated by SDS-PAGE, transferred to polyvinylidine fluoride (PVDF) membranes (Immobilon PIPVH00010, 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. Membranes were incubated overnight in Acetyl-Lysine antibody (#9441 Cell Signalling) or PGC-1α (H-300 Santa Cruz Biotechnology) at a concentration of 1:1000. 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 (GS710 Calibrated Imaging Densitometer, Biorad Laboratories, Hercules, CA) using the Quantity One software package (BioRad Laboratories, Hercules, CA).

3.3.5 Glycogen determination

Frozen muscle samples (~10 mg) were lyophilised for 17-19 h and dissected free of blood and connective tissue. 0.5ml 2N HCl was added to ~2mg of muscle, the volume of tubes were weighed and noted. Samples were covered with marbles (to allow some ventilation during boiling) and hydrolysed in 2N HCL by incubation at 100°C for 2 h with agitation every 15 min. Once cooled samples were weighed and reconstituted to original volume with ddH_2O. Samples were then neutralised by adding 1.5ml 0.67N NaOH and agitated vigorously to ensure muscle was in solution. . 1 ml of reagent mix containing Tris base, HCl, $MgCL_2$, DTT, ATP, NADP, HK and G-6-PDH was added to each sample and a glucose standard. The reaction was allowed to proceed for 10 minutes and samples were excites at 340nm on a fluorometer. A blank value was calculated on the flourometer by using the reagent mix and this was then subtracted from sample values (Passonneau & Lauderdale, 1974).

Glycogen Formula

ΔSample = Sample – Blank

ΔStd = Standard – Blank

C = mM concentration of standard

V = ml volume of standard

D = Dilution

W = weight of muscle

((ΔSample/ ΔStd)*(C*V)*(D)) = Glycogen $mmol.kg^{-1}.dw$

3.3.6 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 by a member of the research team. This data was used to calculate the rate of energy expenditure, carbohydrate and fat oxidation in real time. This enabled us to determine when 400 kcal had been expended during the exercise trials along with providing data on substrate utilisation during exercise. (CONSOLAZIO *et al.*, 1963).

3.3.7 Statistical analysis

Experimental data are presented as mean ± SE. Data were evaluated using the SigmaStat for Windows v3.11 software package (Systat Software, Inc, San Jose, CA). 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. The significance level was set at α=0.05 for all statistical tests.

3.4 Methodological Development

It is important to note that none of these analytical techniques had been developed in the laboratory prior to these experiments. As a result a significant amount of time and effort was dedicated to developing, optimising and validating these methodologies. As expected with the setting up of any technique there was much trial and error involved but I had to ensure the data analysis was reliable. This proved to be an invaluable experience in terms of the understanding I have gained and an appreciation of the challenges faced in establishing techniques.

As part of the PhD process I sought the guidance of more experienced researchers in other departments within DCU such as the National Institute for Cellular Biotechnology (NICB), and the Vascular Health Research Centre and at other institutions. I spent two months at the Karolinska Institutet, under the guidance of Prof. Juleen Zierath learning to perform western blots and qPCR.

In the process of developing the methods and optimising assays we identified a number of issues that are worthwhile noting in this section. In particular the evaluation of PGC-

1α protein and acetylation were particularly challenging. As PGC-1α was such a criticial element of the experiments, a significant amount of time was spent optimising these assays. Some of the issues are now highlighted. The PGC-1α acetylation method described earlier had never been performed with human tissue before so this required the development of a novel technique. There were many issues encountered in the development of the assay including antibody specificity and quantitiy, protein quantity, Protein A/G agarose beads, and the make up of the homogenisation buffer including protease and acetylase inhibitors. In all, the development of this technique took approximately six months.

3.5 Methodological issues

3.5.1 PGC-1α protein

A number of commercial antibodies are available for the detection of PGC-1α protein by immunoblot techniques and we have tried all of them in an attempt to determine their specificity and validity. There have been reservations about the specificity of a number of these antibodies among researchers in the field which have been voiced at conference proceedings. We have learnd from our collaborators that one of the antibodies we had tried detected a band in PGC-1α-null mice (Brendan Egan, Alexander Chibalin, Karolinska Institutet, personal communication). Therefore, we spent a lot of time optimising this assay and interpret published data using PGC-1α antibodies with caution.

Initially we used the PGC-1α H-300 antibody (sc-13067, Santa Cruz Biotechnology), based on the fact that it had been used in previously published papers (Taylor *et al.*, 2005;Akimoto *et al.*, 2005;Hancock *et al.*, 2008). However, an issue arose as this antibody repeatedly produced a band at a molecular weight of ~75 kDa as opposed to the 92-105 kDA molecular weight of the full-length protein. To troubleshoot these problems we used samples from separate protein extractions to ensure there had been no degradation of the protein to no avail. We also used a rat tissue sample we had received as a gift from our collaborators at the Karolinska Institutet. Many different concentrations of antibody were used as well as alternative blocking solutions such as milk protein and Bovine Serum Albumin to minimise the background and possibility of non-specific antibody binding. We sought assistance from Santa Cruz Biotechnology

and they assured us of the specificity of the antibody. While other studies report PGC-1α protein using this antibody, none have reported the molecular weight of the detected band. Recently, we were informed of an antibody (PGC-1α, ST1202, Calbiochem) (Puigserver *et al.*, 1998), which was detecting a band at ~90-95 kDa in human skeletal muscle samples by a collaborator (Brendan Egan, Karolinska Institutet, Personal communication). We have since used this antibody with greater success, producing a blot with much less background and less non-specific bands. The band also appears at an appropriate molecular weight of approximately 90-95 kDa. The protein data presented in this document were obtained using this antibodyand the bands shown appeared at the correct molecular weight.

3.5.2 PGC-1α acetylation

This area of research has been evolving at a fast rate during our experiments. It became clear that not only the protein content, but the post-translational modification of PGC-1α, in particular protein acetylation, was very important. In light of this, and the finding that SIRT1 which is known to deacetylate PGC-1α was increased in response to exercise we decided to investigate the acetylation status of PGC-1α. However, this had not previously been done in human skeletal muscle samples so required the development of a new technique.

(Rodgers *et al.*, 2005) had previously demonstrated PGC-1α acetylation in mouse liver tissue so we used a modified version of their protocol to begin with. An anti-PGC-1α (H-300 Santa Cruz Biotechnology) antibody was used as this had previously been shown to immunoprecipitate PGC-1α and measure acetylation (Rodgers *et al.*, 2005). We have subsequently found that there are specificity issues with this antibody as discussed, but it was the only one to have been reported in the literature at that time. When we used this antibody to immunoprecipitate PGC-1α several non-specific bands appeared at ~50, ~60 and ~150 kDa, whereas a band appeared at ~75 kDa in the supernatant which was run as a control. This suggests the antibody did not bind to its antigen. As this antibody had been used previously we persevered with it and decided to increase the quantity of antibody used until this was optimised at 4 µg. We also optimised the quantity of protein at 200 µg. Initially, muscle was homogenised as described (general methodology). The quantity of beads used also had to be optimised.

Following a number of unsuccessful attempts, doubts were cast as to the constituents of the homogenate buffer. We experimented with a modified RIPA buffer and the buffer used by (Rodgers *et al.*, 2005) and found that the latter produced the best results, probably due to the addition of nicotinamide, an acetylase inhibitor. Recently, another paper has been published with PGC-1α acetylation data (Canto *et al.*, 2009). In this study, the researchers used an extra deacetylase inhibitor (sodium butyrate) that we were unaware of at the time of analysis which may have improved the measurement of acetylation in our samples if we had included it in our buffer. After much optimisation we eventually produced blots displaying a band at ~75 kDa where no band was detected in the corresponding supernatant which was used as a control. It is difficult to state that this blot actually represents the true acetylation status of PGC-1α as the antibody used in this experiment has since been proved to be questionable.

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

It has been well established that skeletal muscle is a highly malleable tissue, capable of metabolic and morphological adaptations in response to contractile activity (i.e. exercise) (Hood *et al.*, 2006). This ability to adapt to physiological cues is termed muscle plasticity and allows the muscle to adjust its structure and function to the demands being placed on it. A single bout of exercise leads to a transient increase in the mRNA content of a plethora of genes involved in metabolic function in human skeletal muscle Enhanced levels of these gene transcripts can lead to the synthesis of proteins and provoke remodelling of the muscle if the stimulus is repeated frequently (Fluck, 2006). These changes in mRNA in response to exercise may gradually accumulate and alter muscle structure or function (Hoppeler & Fluck, 2002).

An important feature of skeletal muscle plasticity is the specificity of the adaptive response to a given stimulus (Fluck & Hoppeler, 2003). Stimulation of rat muscle mimicking endurance and resistance exercise resulted in the upregulation of two different gene programs (Atherton *et al.*, 2005). The specificity of the response is dependent on the intracellular signalling cascades that are activated and the magnitude of their activation. The signalling pathways involved are activated by alterations in ATP turnover, calcium flux, cellular stress and the redox state in the muscle cell. These metabolic pathways act as a coordinated network to (i) regulate substrate utilisation during after exercise and (ii) alter the expression of genes that will assist muscle adaptation to te physiological stress of exercise. As outlined in Chapter 2, the identification of the transcription factor co-activator, PGC-1α, is particularly important for co-ordinating the expression of metabolic genes.

The purpose of this project was to determine the impact of exercise intensity on the activation of contraction-mediated signalling cascades and PGC-1α gene expression. In addition, I sought to determine if the intensity of exercise altered the expression of PGC-1α co-activated genes and if these changes were associated with an increase in PGC-1α mRNA, protein content or protein function. We hypothesised that a single bout of high intensity exercise would result in greater activation of the AMPK and calcium

signalling cascades when compared to a single bout of isocaloric low intensity exercise, and that this would result in a differential transcriptional response of the metabolic proteins.

4.2 Experimental design

A detailed overview of the experimental design was provided in Chapter 3. Briefly, eight healthy, sedentary males performed two isocaloric (400 kcal) cycle exercise trials, 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.

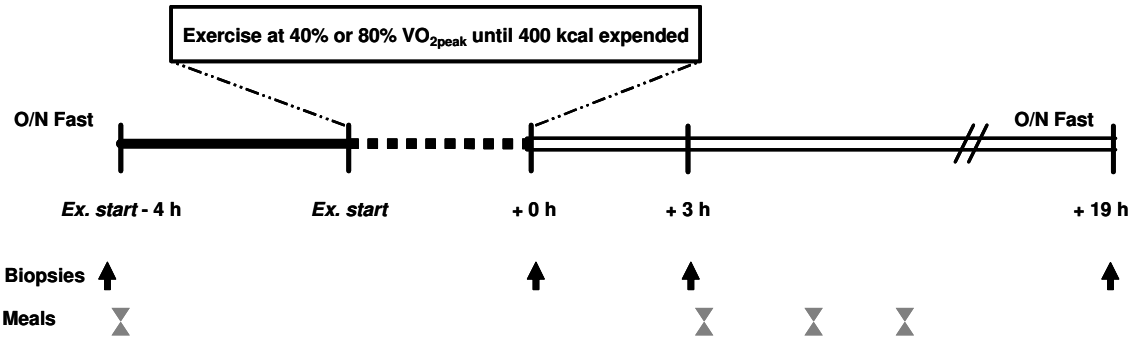

Figure 4.1 Schematic representation of experimental design for experiment I

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

(Values are mean±SE)

4.3 Results for Experiment I

4.3.1 Whole body energy expenditure and substrate utilization

In both exercise trials subjects exercised at the target intensity and expended a similar amount of energy (Table 4.2). As a result the 400 kcal energy expenditure target was achieved significantly quicker in the high intensity trial ($p<0.05$). The rate of energy expenditure was greater during the high intensity trial ($p<0.05$) and this resulted in a greater reliance on the relative ($p<0.05$) and absolute ($p<0.05$) contribution of carbohydrate to energy expenditure . As expected the contribution of fat oxidation to total energy expenditure was lower during the high intensity trial ($p<0.05$).

Table 4.2. Energy expenditure and substrate utilization during isocaloric low and high intensity exercise trials.

	Low Intensity	High intensity
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**

Data are presented as mean±SE. ** significantly different compared to low 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 significantly decreased following both exercise trials (176±22 vs. 128±34 mmol•kg^{-1} dw for the low and high intensity trials,

respectively; $p<0.05$), but returned to baseline the following morning. As anticiated the rate of glycogen utilization was greater during the high intensity trial (Table 4.2). Plasma lactate concentration was unchanged from baseline during low intensity exerccise, but increased to 7.23±1.07 mM at the end of the high intensity trial ($p<0.001$ compared to both baseline and low intensity exercise). This reflects the high intensity nature of the exercise.

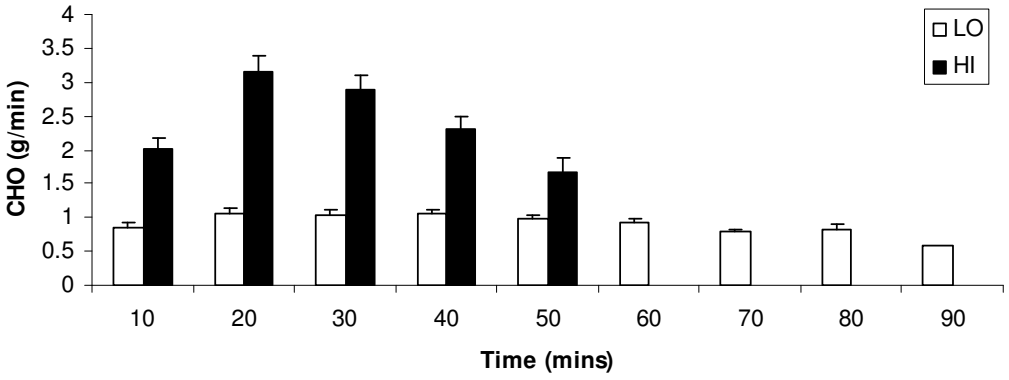

Figure 4.2 Breakdown of the rate of carbohydrate utilisation by time over the course of each exercise protocol. Data are presented as mean±SE.

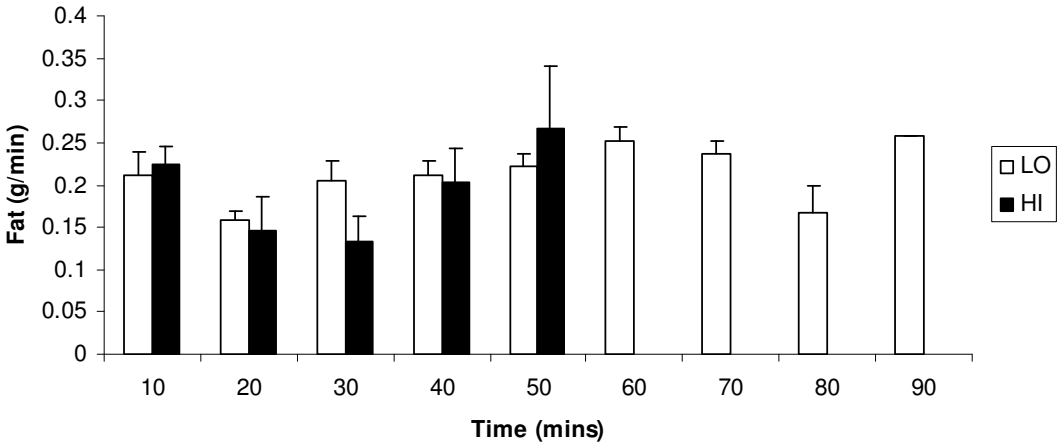

Figure 4.3 Breakdown of the rate of fat utilisation by time over the course of each exercise protocol. Data are presented as mean±SE.

4.3.2 Contraction-activated signalling cascades

AMPK phosphorylation (Fig. 4.2A) was similar at baseline between trials, and increased 4.1-fold immediately following the high intensity exercise trial ($p<0.05$). However, there was no significant change in AMPK phosphorylation following isocaloric low intensity exercise, creating an intensity dependent difference between trials ($p<0.05$). AMPK phosphorylation is important but does not completely represent AMPK activity so it was decided to determine if a downstream marker of AMPK activation, acetyl CoA carboxylase β (ACCβ) phosphorylation. ACCβ phosphoylation was increased 3.6-fold ($p<0.05$) following low intensity exercise and 7.9-fold ($p<0.05$) after the high intensity trial. The intensity dependent difference in AMPK phosphorylation was also present with ACCβ phosphorylation. This corroborates the contention that there is greater activation of the AMPK signalling cascade following high intensity exercise compared with low intensity.

Total CaMKII phosphorylation was defined as the summation of β_M and γ/δ isoforms as previously describted (Rose *et al.*, 2006;Rose *et al.*, 2007)]. CaMKII phosphorylation increased immediately following the high intensity (42%, $p<0.05$), but not low intensity trial. There was an intensity dependent difference between trials (Fig. 4.4C; $p<0.05$). This suggests activation of the calcium signalling cascade during high intensity exercise but that low intensity is not sufficient to induce activation of this pathway. CREB is a downstream target of CaMKII and I determined if either exercise trial increased CREB phosphorylation. Phosphorylation of CREB (Fig. 4.4D) was unaltered immediately or 3 h after exercise, but tended to increase after 19 h of recovery. Phosphorylation of other CaMKII substrates phospholamban and SRF was unaltered either immediately after exercise or in the recovery period.

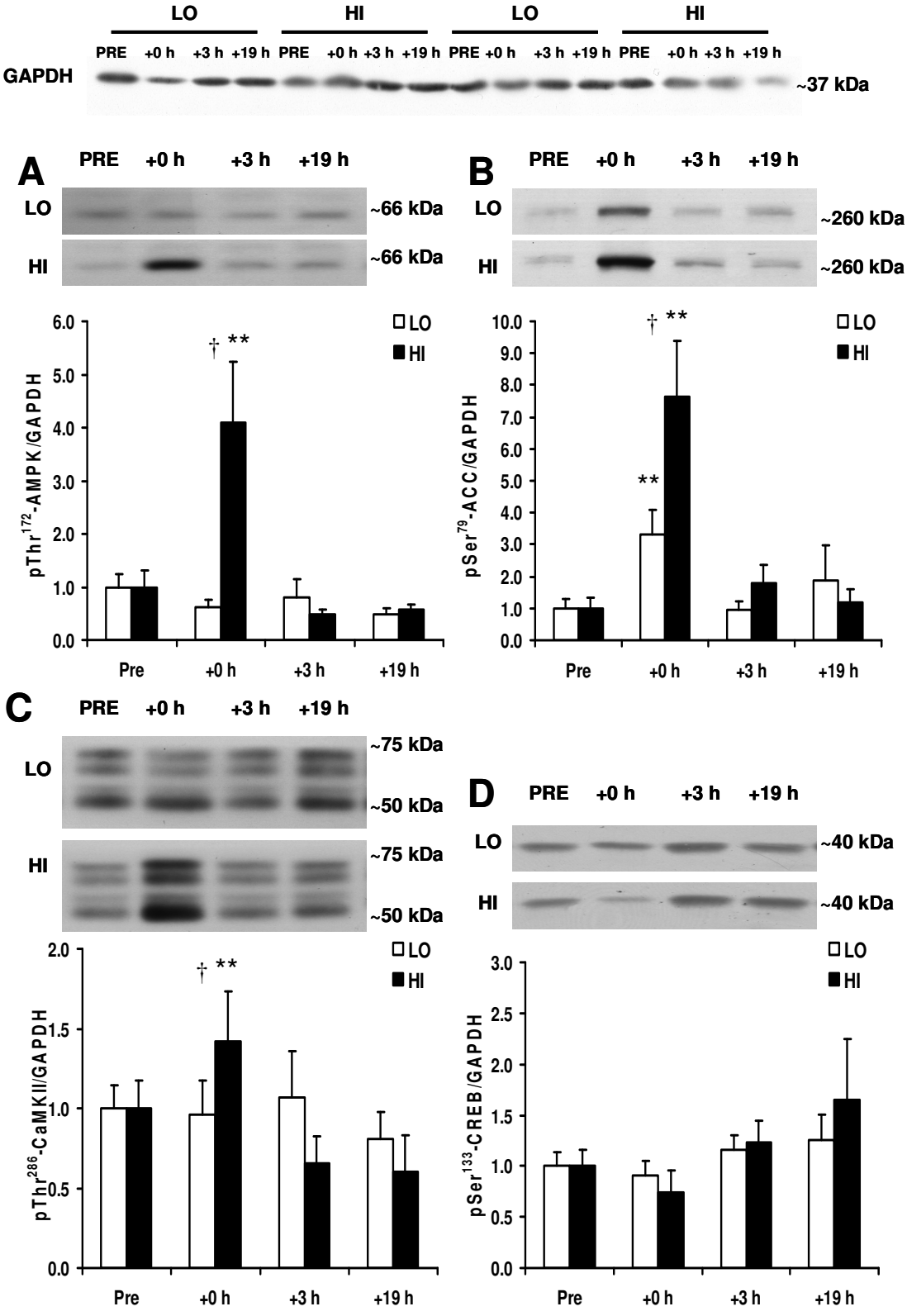

Figure 4.4 Protein phosphorylation of (A) AMPK, (B) ACCβ, (C) CaMKII and (D) CREB, normalised to GAPDH protein content. Representative blots for each protein are included. Values are mean±SE. ** significantly different to Pre ($p<0.05$); † significant difference between trials ($p<0.05$).

4.3.3 Gene expression

Transient changes were observed in a number, but not all, of the metabolic genes during the recovery from exercise. PGC-1α mRNA increased 3-hrs after both exercise trials in an intensity dependent manner and with a similar pattern to the intracellular kinases. (Fig. 4.5). PGC-1α mRNA was elevated by 3.8- and 10.2-fold 3 h after the low intensity and high intensity trials, respectively ($p<0.05$). There was a significant difference in PGC-1α mRNA between trials ($p<0.05$).

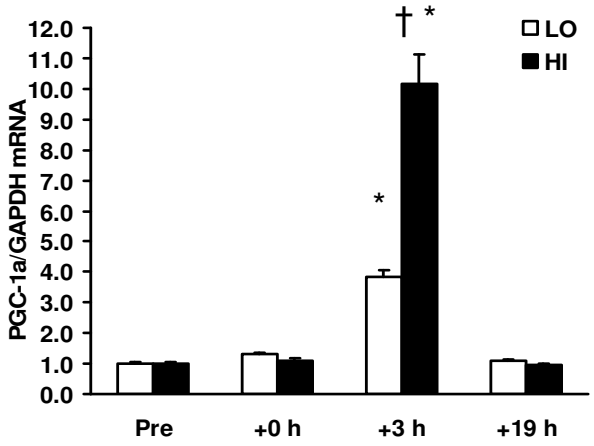

Figure 4.5 PGC-1α mRNA (Values are mean±SE, normalised to GAPDH mRNA. * significantly different to Pre ($p<0.05$); † significant difference between trials ($p<0.05$).

We then decided to quantify the expression of known PGC-1α targets to determine if the intensity of exercise differentially expressed these genes, indicating a PGC-1α-mediated response. Firstly, I quantified the mRNA of PGC-1α co-activated transcription factors NRF-1, NRF-2 and ERRα. I did not find an exercise or intensity dependent response for any of these target genes. As these transcription factors are primarily involved in the expression of nuclear encoded mitochondrial genes and that this process is unlikely to be responsive to an acute bout of exercise I decided to focus on genes that regulate substrate utilisation.

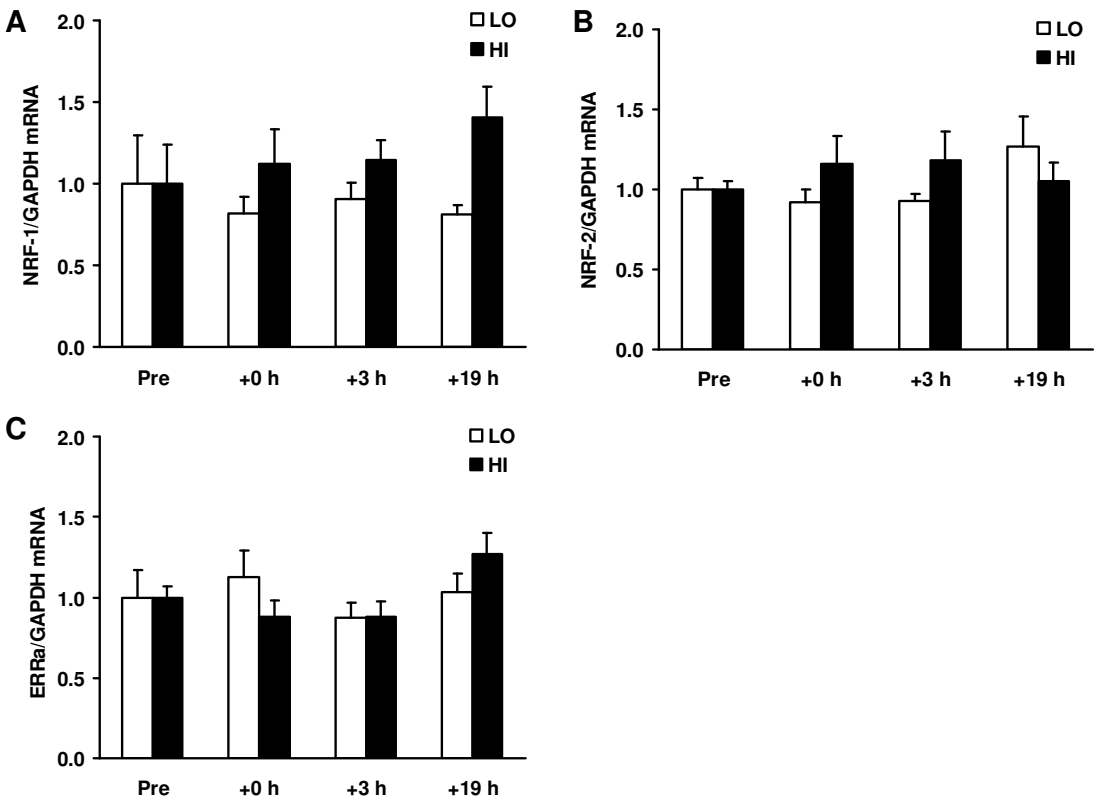

Figure 4.6 mRNA expression of selected genes (A) NRF-1, (B) NRF-2, (C) ERRα mRNA. (Values are mean±SE, normalised to GAPDH mRNA.

The expression of FOXO1A mRNA (Fig. 4.7A) increased immediately and 3 h following the low and high intensity exercise trials ($p<0.05$). FOXO1A mRNA was greater 3 h following the high intensity compared to the low intensity trial ($p<0.05$). FOXO1A has been implicated in the expression of PGC-1α and the timeframe of these changes would suggest an increase in FOXO1A mRNA prior to a change in PGC-1α. However, there was no change in the protein expression of FOXO1 after exercise. The expression of PDK4 mRNA (Fig. 4.7B) was increased similarly 3 h after both high and low intensity exercise with no difference between trials ($p<0.05$). Therefore, the PDK4 response to exercise may be dependent more on caloric expenditure and not the intensity of exercise. I also looked at the expression of PPARδ and found that mRNA expression was increased 3 h after high but not low intensity exercise ($p<0.05$). I did not find a change in the expression of CPT-1, the mitochondrial regulator of substrate utlisation or the uncoupling protein, UCP3.

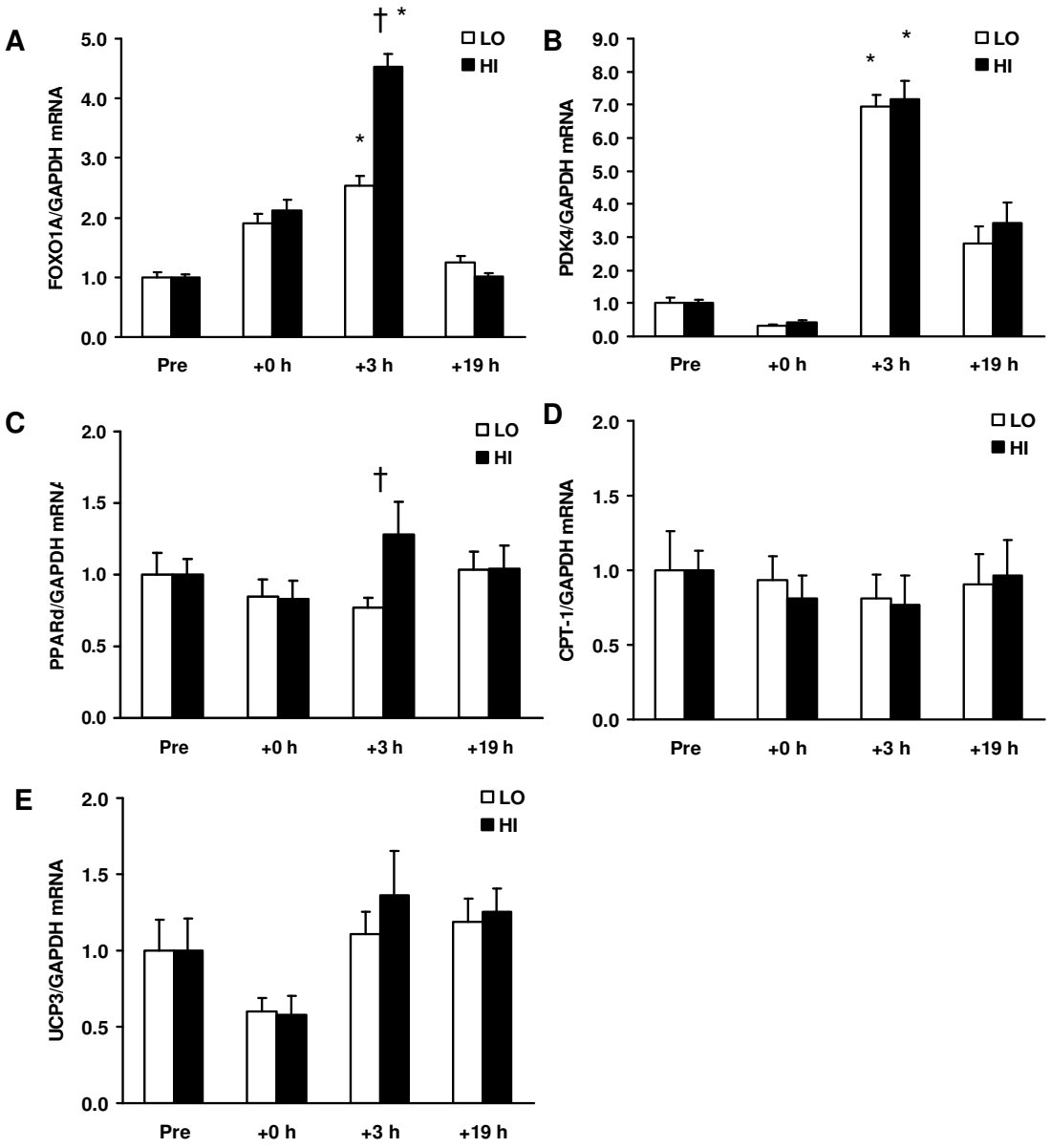

Figure 4.7 mRNA expression of selected genes (A) FOXO1A, (B) PDK4, (C) PPARδ, (D) CPT-1, (E) UCP3 mRNA. Values are mean±SE, normalised to GAPDH mRNA. * significantly different to Pre ($p<0.05$); † significant difference between trials ($p<0.05$).

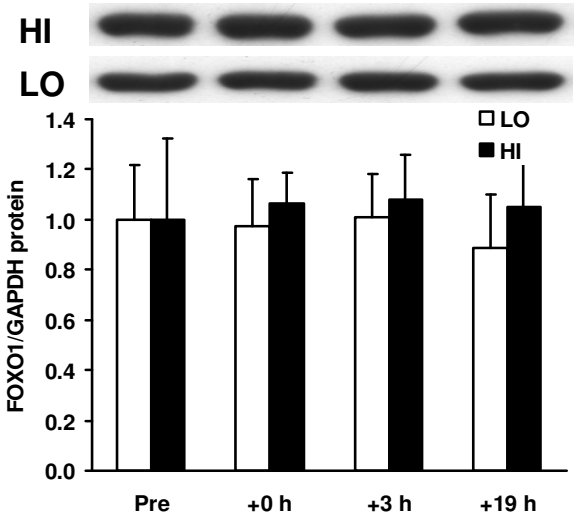

Figure 4.8 Protein expression of FOXO1. Representative blots are included. Values are mean±SE, normalised to GAPDH protein content.

Finally, I decided to investigate GLUT-4 mRNA, which has previously been shown to increase following exercise. To our surprise I did not find a change in GLUT-4 mRNA following the high or low intensity exercise trials (Figure 4.9A). I performed further analysis of GEF and MEF2A&D expression, the enhancer and transcription factor for both PGC-1α and GLUT-4. I did not observe a change in the expression of any of these genes. These results suggested to us that changes in PGC-1α protein content or function is required before the regulation of down-stream genes is seen in the post-exercise period.

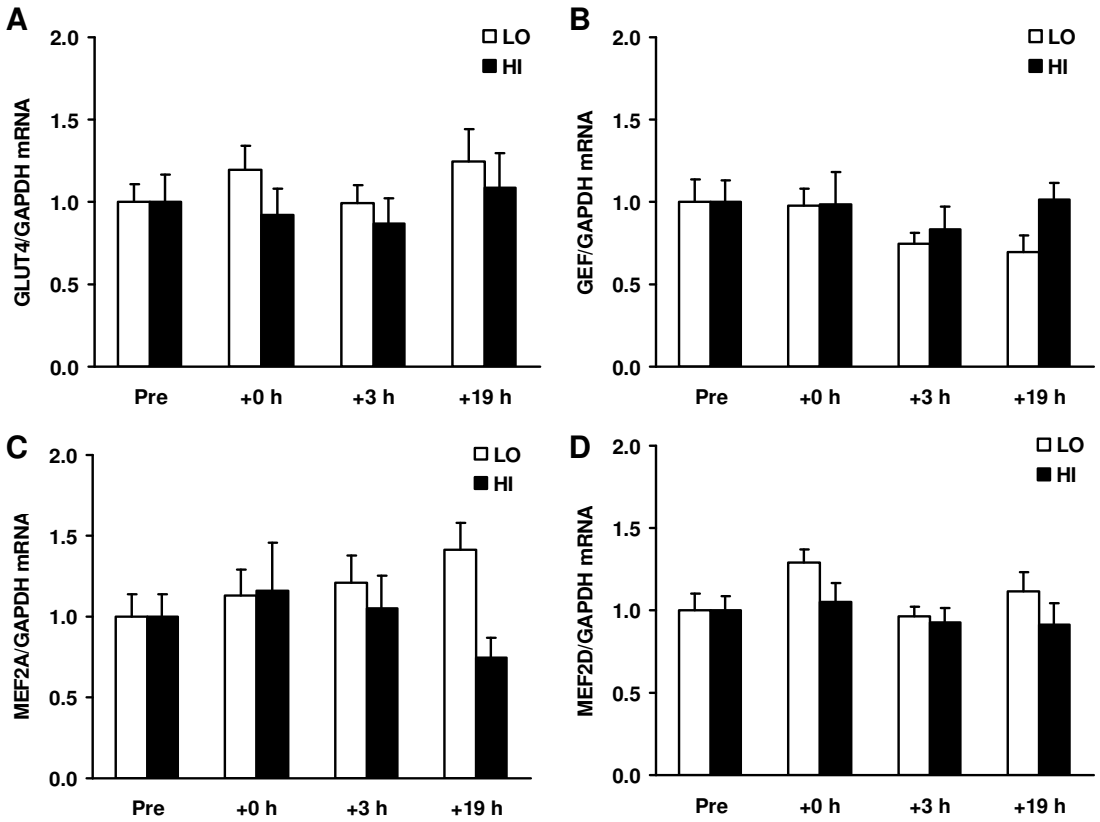

Figure 4.9 mRNA expression of selected genes (A) GLUT4, (B) GEF, (C) MEF2A, (D) MEF2D. (Values are mean±SE, normalised to GAPDH mRNA.

4.3.4 PGC-1α protein content and acetylation

I then decided to investigate the possibility that the activation of PGC-1α by postranslational modification, as well as its abundance, would be regulated by exercise. To examine this, I measured PGC-1α acetylation and protein content at each time point under both exercise conditions. I found that both PGC-1α protein content and acetylation were unchanged at any time point following exercise.

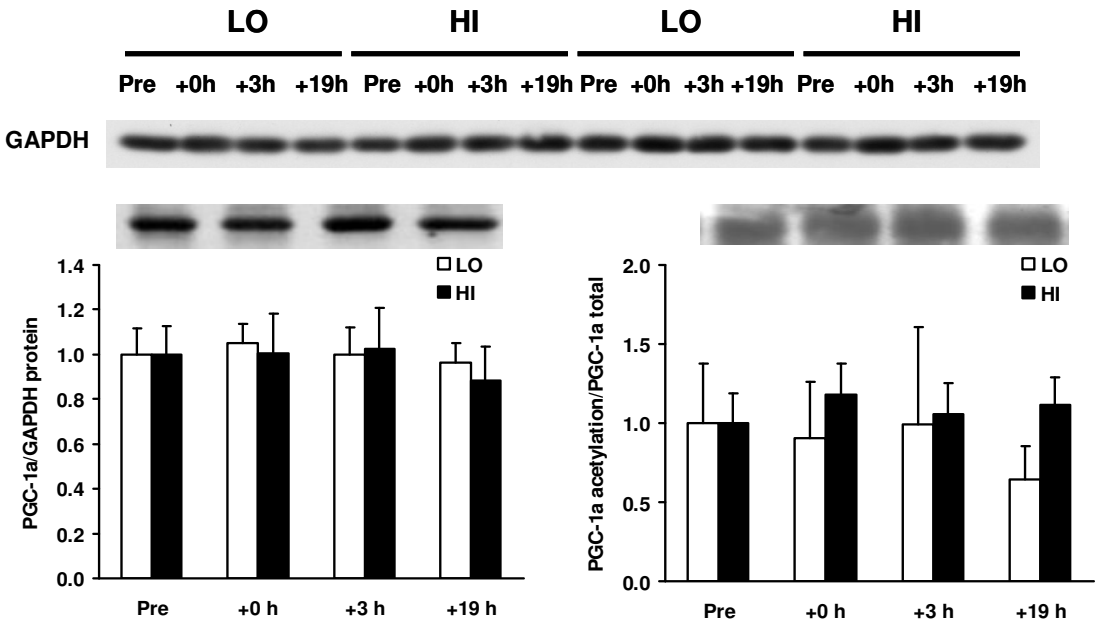

Figure 4.10 PGC-1α total protein and acetylation. Values are mean±SE, normalised to total PGC-1α protein expression.

4.3.5 Transcription factor co-repressor Receptor Interacting Protein 140 (RIP140)

After this study had been completed I became aware of a recently identified transcription co-factor. This was RIP140, a co-repressor of metabolic gene expression and a negative regulator of PGC-1α mediated gene expression. A more detailed explanation of RIP140 function is provided in the discussion but our subsequent analysis identified for the first time in human skeletal muscle that RIP140 mRNA increased following an acute bout of exercise. I found increased RIP140 mRNA immediately and 3-hrs following both exercise trials but no differences between trials ($p<0.05$). (Fig. 4.11).

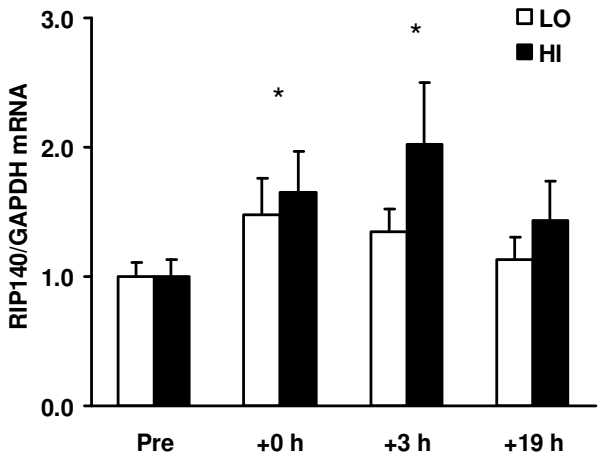

Figure 4.11 RIP140 mRNA. (Values are mean±SE, normalised to GAPDH mRNA. * significantly different to Pre ($p<0.05$); † significant difference between trials ($p<0.05$).

4.4 Summary

In summary, high intensity exercise caused a greater upregulation of AMPK and CaMKII phosphorylation compared with low intensity. This suggests that high intensity exercise produces greater activation of the signalling cascades in control of metabolic regulation. This was accompanied by a similar intensity-dependent effect on the mRNA expression of the transcriptional co-activator PGC-1α. This may have implications for a number of PGC-1α co-activated genes and downstream gene targets. A similar intensity effect was seen in the expression of the transcription factor FOXO1A. PDK4 mRNA expression was increased after both exercise conditions and no change was observed in GLUT4 expression. These results suggest that exercise regulates substrate utilisation and that the intensity of exercise may play a role in that regulation. Finally, I observed an increase in the expression of the transcriptional repressor RIP140 after exercise, which was a novel finding. The significance and implications of these results will be discussed in detail in Chapter 6.

Chapter V The impact of contraction frequency during an acute bout of exercise on the expression of genes involved in metabolism and substrate selection in human skeletal muscle.

5.1 Introduction

The intensity of exercise is often used to differentially regulate skeletal muscle function. However, the frequency of contaction is also an important determinant of exercise outcomes. 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 the adaptive response to exercise (Chin, 2004;Chin, 2004;Ojuka, 2004;Ojuka, 2004). It is thought that the amplitude and duration of Ca^{2+} transients in response to activity can determine the gene transcription profile, coupling the extent of muscle excitation to transcription and allowing muscles to adapt specifically to the demands placed on them (Chin, 2005). The specificity of signal transduction by calmodulin is determined by the specific CaMK isoform activated, their localisation and the duration, amplitude and frequency of Ca^{2+} flux (Chin, 2005).

We sought to alter the calcium transient by varying the frequency of contraction while maintaining the power output during exercise. We believed that a higher frequency of contraction would result in a greater number of muscle contractions with a lower amplitude and duration of calcium flux. The purpose of this experiment was to investigate the impact of altered calcium flux, by varying the frequency of skeletal muscle contraction, on the activation of contraction-mediated signalling cascades and on the expression of genes of metabolic genes following an acute bout of exercise. We hypothesised that there would be greater activation of the calcium-induced signalling cascades (CaMKII in particular) in response to cycling exercise with a greater frequency of contraction. We also predicted that cycling at a higher contraction frequency would result in greater induction of the metabolic genes regulated by the calcium-activated signalling pathways compared with low contraction frequency.

5.2 Experimental design

A detailed overview of the experimental design was provided in Chapter 3. Briefly eight healthy, sedentary males performed two isocaloric (400 kcal) cycle exercise trials at

identical power outputs, eliciting approximately 50% VO_{2peak}, pedalling at a cadence of either 50 RPM or 80 RPM. Skeletal muscle biopsies from the *m. vastus lateralis* were taken at rest and at +0 h, +3 h and +19 h after exercise.

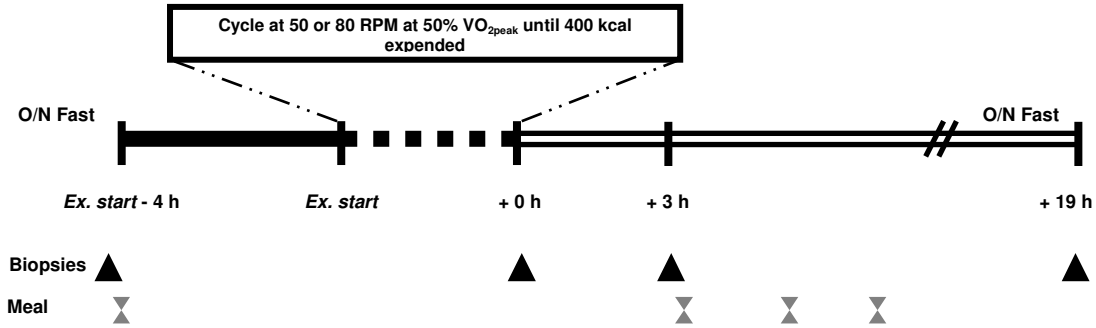

Figure 5.1 Schematic representation of experimental design for experiment II

Table 5.1 Physical characteristics of participants.

	n=8
Age (yr)	21.6±4.3
Height (m)	1.82±0.06
Mass (kg)	81±7.2
BMI ($kg \cdot m^{-2}$)	24.5±2.48
Body fat (%)	13.4±5.6
VO_{2peak} ($mL \cdot kg.min^{-1}$)	37.4±5.9

(Values are mean±SE)

5.3 Results for Experiment II

5.3.1 Whole body energy expenditure and substrate utilisation

Total energy expenditure and the rate of energy expenditure were similar between trials (Table). The low frequency trial lasted approximately 6-mins longer despite working at the same power output in both trials. The non-significant difference in exercise duration was related to a lower relative oxygen consumption during the low frequency trial ($p<0.05$). This difference may also have accounted for the greater rate of carbohydrate oxidation ($p<0.05$) despite a similar respiratory exchange ratio. There was no difference in the fat oxidation rate or total fat utilisation between trials. Plasma lactate concentration was unaltered from baseline at any time point with no variation between contraction frequencies. This was probably due to the low intensity nature of the exercise.

Table 5.2 Energy expenditure and substrate utilization during isocaloric exercise trials at 50 and 80 RPM.

	50 RPM	80 RPM
Total EE (kcal)	409±2	411±2
Rate of EE (kcal·min^{-1})	7.0±0.3	8.1±0.425
Exercise intensity (%VO_{2peak})	47.8±1.1	54.6±1.4**
Exercise time (min)	58.5±3.1	52.6±3.0
RER	0.96±0.01	0.98±0.01
CHO oxidation rate (g•min^{-1})	1.44±0.10	1.74±0.11**
Total carbohydrate oxidized (g)	81.9±2.3	89.8±2.7**
Rate of fat oxidation (g•min^{-1})	0.13±0.01	0.11±0.01
Total fat oxidized (g)	7.61±1.01	5.53±0.65
Plasma lactate at termination (mM)	1.74±0.16	2.02±0.25

Values are mean±SE. ** significantly different compared to 50 RPM trial ($p<0.05$).

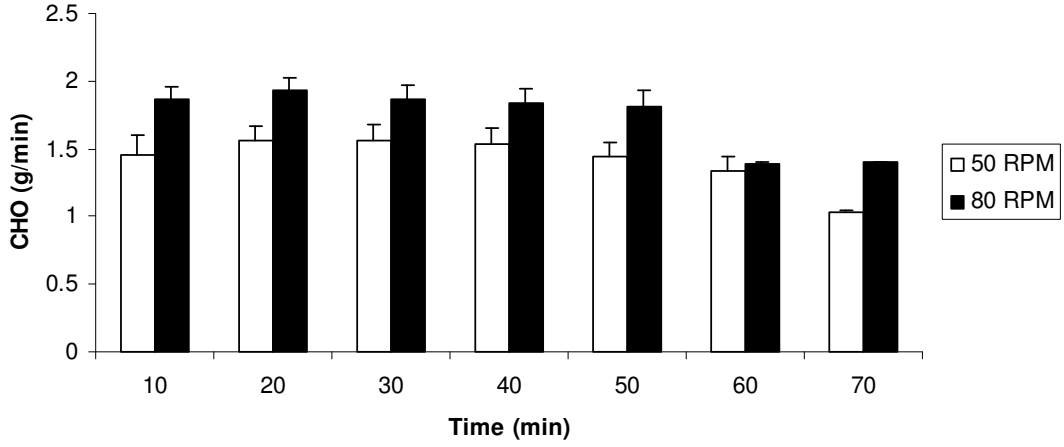

Figure 5.2 Breakdown of the rate of carbohydrate utilisation by time over the course of each exercise protocol. (Values are mean±SE)

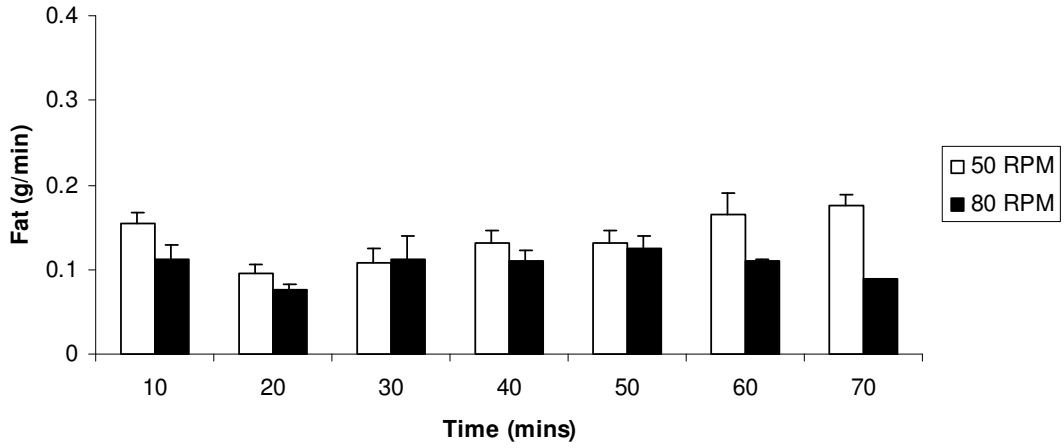

Figure 5.3 Breakdown of the rate of fat utilisation by time over the course of each exercise protocol. (Values are mean±SE)

5.3.2 Contraction-activated signalling cascades

Phosphorylation of AMPK was not significantly different at any time point with no difference observed between exercise trials. ACCβ phosphorylation was increased ($p<0.01$) immediately after exercise compared with baseline, 3 h and 19 h of recovery, while there was no difference between contraction frequencies.

There was a decrease in Total CaMKII phosphorylation 19 h following exercise compared with resting and immediately post exercise, and this change was independent of the frequency of contraction. Phosphorylation of CREB was unchanged by cycling at either 50 or 80 RPM.

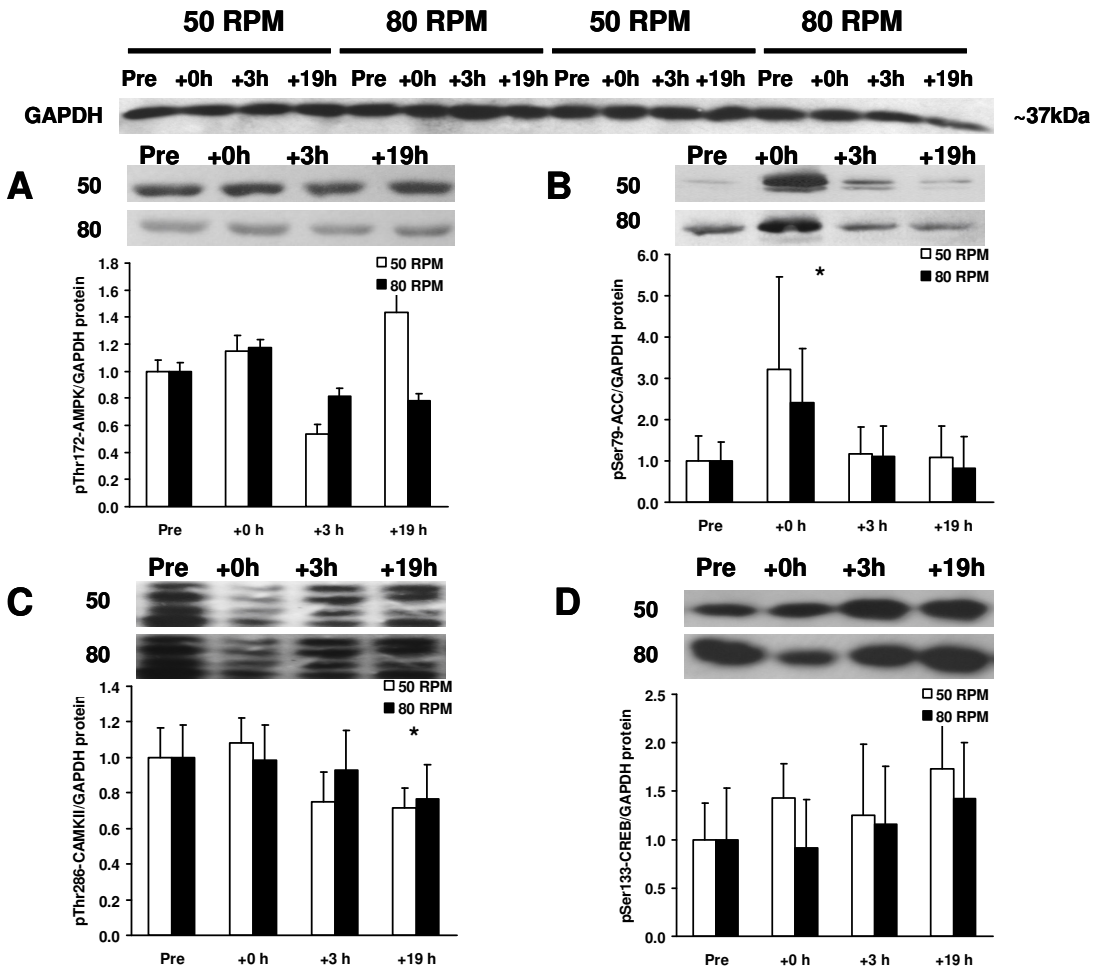

Figure 5.4 Protein phosphorylation of (A) AMPK, (B) ACCβ, (C) CaMKII and (D) CREB, normalised to GAPDH protein content. Representative blots for each protein are included. Values are mean±SE. * significantly different to Pre ($p<0.05$).

5.3.3 Gene expression

In this experiment we focused on the expression of genes that were exercise responsive in experiment I. The intensity of exercise in this experiment was close to the low intensity trial in experiment I. There was a transient change in the expression of some, but not all of the metabolic genes analysed in response to exercise. Despite the fact that AMPK and CaMK phosphorlation were not significantly increased following exercise,

PGC-1α mRNA was significantly greater 3 h after the high frequency trial ($p<0.05$). The magnitude of the increase was similar to the low intensity exercise trial in experiment I. PGC-1α expression was increased approximately 2-fold after the low frequency trial but this did not reach statistical significance. There was also a significant difference between trials 3-hrs after exercise ($p<0.01$).

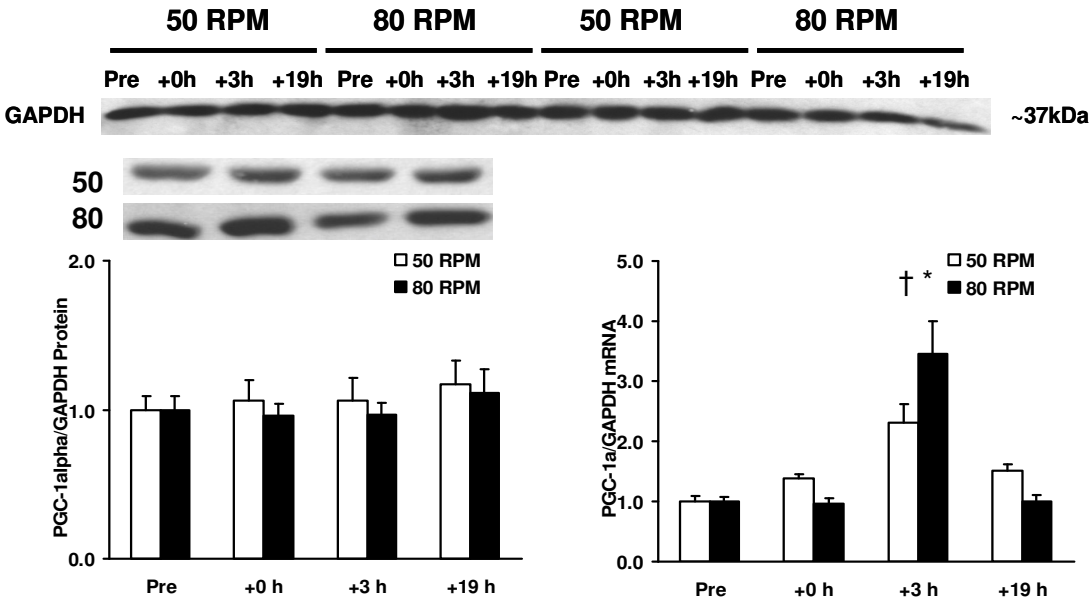

Figure 5.5 PGC-1α protein and mRNA expression. Protein data is relative to GAPDH protein. Representative blots are included. mRNA data is relative to GAPDH mRNA. Values are mean±SE, normalised to GAPDH mRNA. * significantly different to Pre ($p<0.05$); † significant difference between trials ($p<0.05$).

The response of PGC-1α led us to examine some of the transcriptional coactivator's downstream targets. Expression of FOXO1A mRNA was increased immediately and 3 h after exercise ($p<0.05$) with no difference between contraction frequencies. PDK4 mRNA was above baseline at 3 h and 19h post-exercise in both trials ($p<0.05$). In this experiment we decided not to measure PGC-1α acetylation as we were unsure about the specificity of the antibody and the cost of running the experiment was not practical. Instead we determined if there was a change in the expression of SIRT1 mRNA, the NAD^+-dependent deacetylase that acts on PGC-1α. SIRT1 mRNA was upregulated 19 h after exercise ($p<0.05$) compared with all other time points, while there was no difference between frequency of contraction trials. These results suggest that SIRT 1 regulation of PGC-1α acetylation may persist in the hours or days post exercise. CREB mRNA increased 19 h post exercise compared with baseline values ($p<0.05$) with no

difference between exercise trials. Unlike the exercise intensity trial there was no change in the expression of RIP140 or ERRα mRNA at any time point and subsequently RIP140 and ERRα protein expression was similarly unaffected. However, no changes were observed at the protein level in any of the genes we looked at. PGC-1α protein expression was unaltered at any time point with no difference between trials. FOXO1 protein was unchanged after exercise at any time point in either trial.

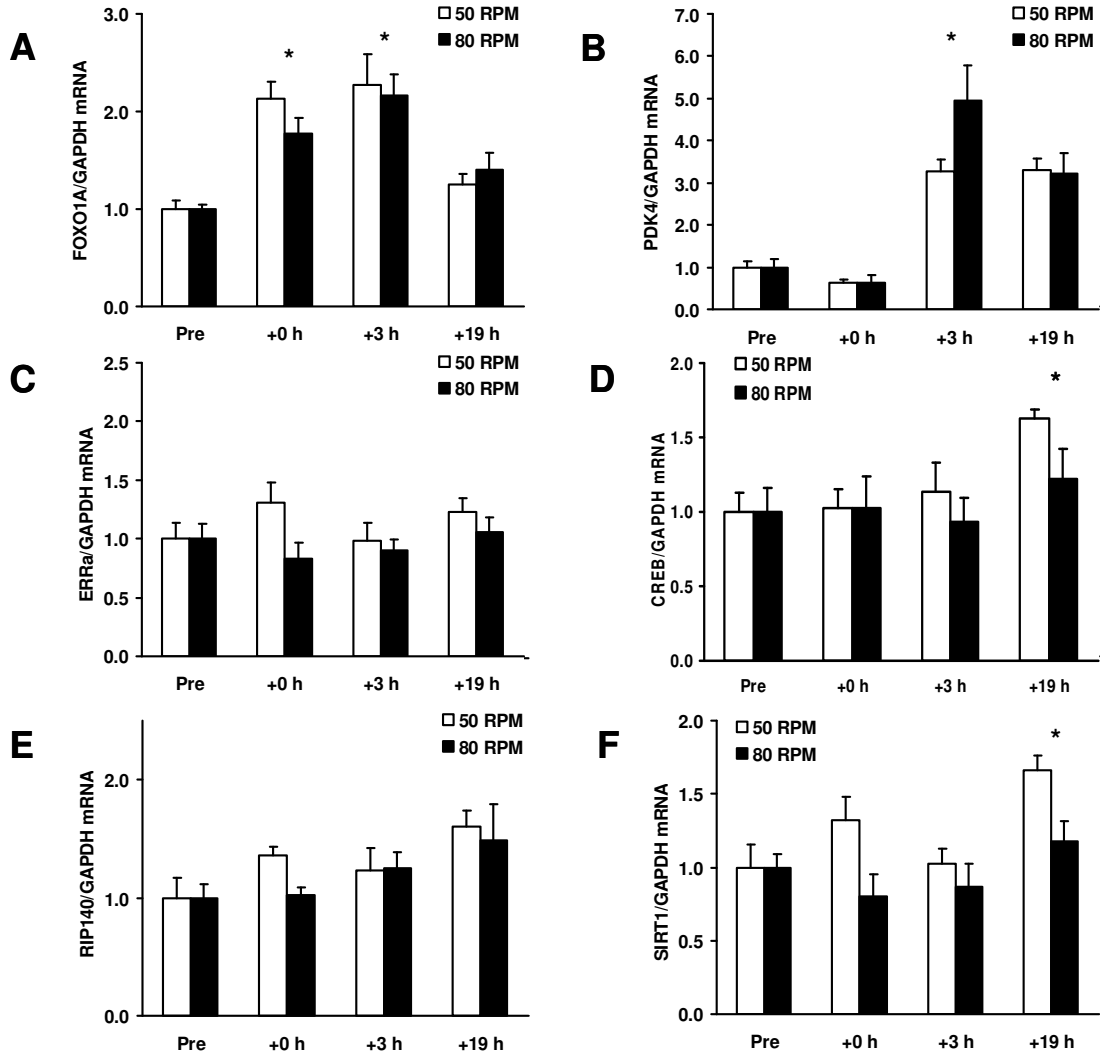

Figure 5.6 mRNA expression of selected transcriptional regulators and metabolic genes, (A) FOXO1A, (B) PDK4, (C) ERRα, (D) CREB, (E) RIP140, (F) SIRT1. Values are mean±SE, normalised to GAPDH mRNA. * significantly different to Pre ($p<0.05$).

5.4 Summary

In summary, I did not see upregulation of either AMPK or CaMKII phosphorylation after exercise at high or low contraction frequency. There was a significant increase in ACCβ phosphorylation immediately after exercise indicating there was some activiation of the cascades regulated by ATP turnover. Interestingly, there was an increase in PGC-1α mRNA in both exercise conditions. However, there was greater upregulation of PGC-1α mRNA with high contraction frequency compared with low contraction frequency. This suggests that the frequency of contraction during exercise regulates PGC-1α mRNA expression. This may have implications for a number of PGC-1α co-activated genes and downstream gene targets. FOXO1A mRNA was increased immediately and 3 h after both exercise conditions. Exercise increased PDK4 mRNA 3 h after exercise after both high and low contraction frequency. These results suggest that exercise regulates substrate utilisation and that the contraction frequency during exercise may play a role in that regulation.

Chapter VI Discussion

6.1 Introduction

The major findings of this thesis are that an acute bout of exercise differentially activates intracellular signalling cascades and the transcription of selected metabolic genes under varying conditions in human skeletal muscle. We found that the intensity of exercise was a more potent stimulator of metabolic gene expression but that contraction frequency was also capable of moderate changes. We demonstrate that both AMPK and calcium flux signalling pathways are modulated by the intensity of exercise. We also show that expression of the transcriptional coactivator PGC-1α is intensity-dependent as well as being regulated differentially by contraction frequency. Genes involved in lipid metabolism, such as FOXO1A and PPARδ are also under the influence of exercise intensity. The finding that RIP140 mRNA was upregulated after exercise is novel as is the exercise-induced increase in the NAD^+-dependent deacetylase, SIRT1 mRNA. In this section I will discuss the impact of an acute bout of exercise on metabolic gene expression in the control of substrate utilisation and how these outcomes describe a sensitive, highly regulated system, in which, there is a exercise specific response to the physiological demands being placed on the muscle.

6.2 Regulation of intracellular signalling cascades by acute exercise

AMPK has been shown to be activated in response to exercise due to an increase in the AMP:ATP ratio (Richter & Ruderman, 2009). In this study we show that AMPK phosphorylation of Thr^{172} occurs in an intensity-dependent manner. AMPK phosphorylation increased immediately following exercise at 80% but not 40% VO_2peak. We found a similar result in Experiment II where AMPK phosphorylation was not increased by exercise at 50% VO_2peak. (Howlett *et al.*, 1998). suggested that low intensity exercise is sufficient to increase ATP turnover, but not necessarily the AMP/ATP ratio, which may explain the response in these two experiments (Howlett *et al.*, 1998). Exercise has been shown to activate AMPK at higher intensities previously (Wojtaszewski et al., 2000), but phosphorylation at low intensity only seems to occur

when the exercise is of a significantly longer duration (Wojtaszewski *et al.*, 2002). Even though AMPK phosphorylation was not increased at low exercise intensities there was an increase in the ACCβ phosphorylation. ACCβ has been shown to be a substrate of AMPK in human skeletal muscle and is tightly linked to AMPK activation (Stephens *et al.*, 2002). This suggests that ACCβ phosphorylation may act as a downstream marker of AMPK activation. We showed a 3.6- and 3.2-fold increase in ACCβ phosphorylation at 40% and 50% VO_2peak respectively. We also found an intensity-dependent increase in ACCβ phosphorylation which supports the evidence that it is tightly coupled with AMPK activity. In this study we used phosporylation as an indicator of the activation of AMPK. This data suggests that AMPK may be transiently activated in response to low intensity exercise but these changes are undetectable following low intensity exercise. Therefore, the direct measurement of AMPK activity, as previously described (Barnes *et al.*, 2005); (Yu *et al.*, 2003) may be a more sensitive indicator of changes following low exercise intensity.

ACCβ phosphorylation decreases protein activity and may play an important role in the AMPK-mediated control of lipid metabolism. Deactivation of ACCβ decreases formation of malonyl-CoA, a potent inhibitor of CPT-I, the rate limiting enzyme in mitochondrial FA uptake (Winder & Hardie, 1996). In addition, AMPK can lower malonyl CoA activity by phosphorylating and activating malonyl CoA decarboxylase, the enzyme responsible for decarboxylating malonyl CoA to acetyl CoA (Hardie & Hawley, 2001). AMPK activation is also associated with FAT/CD36 translocation to the plasma membrane and a parallel increase in fatty acid uptake (Bonen *et al.*, 1999). This suggests that exercise at both high and low intensity acts through ACCβ and AMPK to increase mitochondrial fatty acid oxidation by lowering malonyl CoA and relieving CPT1 inhibition while translocating FAT/CD36 to the plasma membrane. In light of the results shown here the magnitude of this increase in FA uptake may also be intensity-dependent. However, we found a decrease in relative and absolute FA oxidation as the intensity of exercise increases suggesting that other cellular events influence substrate utilisation during exercise but the AMPK-mediated regulation of lipid metabolism may be more important during recovery. This explanation is supported by numerous studies which report a switch to lipid metabolism in the post-exercise period (van Loon *et al.*, 2003); (Mourtzakis *et al.*, 2006); (Kimber *et al.*, 2003).

The results shown here indicate that AMPK is transiently activated as a result of acute exercise and the degree of phosphorylation of AMPK is dependent upon the intensity of the exercise. AMPK activation is likely to be a result of ATP turnover and a change in the ATP/AMP ratio, allowing the muscle to adapt to the demands being placed on it. The results suggest that this adaptation would be greater with high intensity exercise and only moderately influenced by contraction frequency.

Exercise and muscle contraction alter calcium flux which, in turn activates the calcium sensitive protein kinases in the muscle cell (Chin, 2004). In this study we found that the calcium-activated CaMKII is phosphorylated in an intensity-dependent manner. Exercise at 80% VO_2peak increased CaMKII phosphorylation by 42% while there was no change at 40% or 50% VO_2peak. An intensity-dependent increase in CaMKII activity and its downstream target phospholamban has previously been reported (Rose *et al.*, 2006). In contrast to this we did not find an increase in phosphoamban or serum response factor-1, another CaMKII target. (Rose & Hargreaves, 2003) also reported an increase in CaMKII activity after maximal aerobic exercise compared with submaximal exercise. The greater increase in CaMKII activity with high-intensity exercise may be related to the recruitment of more skeletal muscle fibres (Sale, 1987), the recruitment of different fibre types (Baylor & Hollingworth, 2003), and/or greater Ca^{2+}–CaM signalling in recruited fibres. We used phosphorylation of CaMKII as an indicator of its activation in this study whereas Rose et al. measured CaMKII activity (Rose *et al.*, 2006). This may be a limitation of our findings but they are in line with the current research and the CaMK activity assay has not been widely used in published manuscripts.

Calcium flux is both rapid and transient and impossible to measure in human skeletal muscle in real-time. We anticipated that calcium flux would increase CaMKII autophosphorylation and this would be a suitable marker of contraction-activated calcium signalling. Our in vivo experiment was designed to alter the amplitude and frequency of the calcium transient by varying the frequency of contraction, which we thought would produce a differential response in calcium-activated kinases. We hypothesised that a higher contraction frequency would lead to greater calcium flux leading to increased calcium signalling. However, we did not find in difference in CaMKII phosphorylation following exercise at 50% VO_2peak at 50 or 80 RPM. As with AMPK, CaMKII phosphorylation may not be a sensitive indicator of calcium signalling

following low intensity exercise and the challenge of accurately quantified calcium flux in human skeletal muscle remains. In an attempt to further investigate calcium signalling we looked at known downstream targets of CaMK. However, we did not find a change in CREB phosphorylation in response to changes in exercise intensity or the frequency of contraction. This is supported by other studies following exercise at 40%, 70% and 75% VO_2max (Widegren *et al.*, 1998), (Widegren *et al.*, 2000). In my study, CREB phosphorylation tended to increase 19-hrs after exercise at 80% VO_2peak. Interestingly, CREB mRNA was significantly greater 19 h after exercise in Experiment II with no difference between 50 and 80 RPM. CaMKII is known to target the transcription factor CREB by phosphorylation, indicating that CREB is downstream of CaMKII and may therefore explain the time delay in activation of CREB (Sun *et al.*, 1994). Therefore, exercise may have a sustained impact on metabolic function through calcium-mediated transcription of CREB and subsequent regulation of gene expression. This response was not evident following an acute bout of exercise.

Increased calcium concentrations in response to caffeine and electrical stimulation of rat muscle have been shown to increase FA uptake and oxidation. The effect on FA oxidation is completely blocked by incubation with the CaMK inhibitor KN93, and contraction-mediated FA uptake is decreased by 33% (Raney & Turcotte, 2008). This suggests that CaMKII may have a role in the upregulation of FA oxidation during exercise though we see no evidence of it in this study, as fat oxidation was decreased after exercise at 80% VO_2peak, despite an increase in CaMKII phosphorylation. We do not believe that CaMKII is repressing fat oxidation as there are many other factors to consider during high intensity exercise such as the supply of oxygen coupled with the requirement to use the most efficient energy source which is carbohydrate (Jeukendrup & Wallis, 2005). CaMKII may exert its effects on fat metabolism in the post-exercise period, in which case, the intensity of exercise may have implications for FA oxidation. Similarly, as discussed earlier, AMPK has a role to play in FA oxidation in the recovery from exercise and it is likely that both pathways are simultaneously regulating this process. It is unlikely that these pathways act independently of each other. CaMKK has been suggested as a possible regulator of AMPK activity as CaMK kinase (CaMKK) inhibition by STO-609 abolishes AMPK phosphorylation and siRNA against CaMKK reduces AMPK and ACCβ phosphorylation in HeLa cells (Hurley *et al.*, 2005). In electrically stimulated rat muscle the CaMKII inhibitor KN93 decreases contraction-induced α2-AMPK activity suggesting that CaMKII lies upstream of AMPK (Raney &

Turcotte, 2008). Cross-talk between these pathways makes physiological sense as it ensures tight regulation of physiological processes.

6.3 Regulation of PGC-1α by acute exercise

6.3.1 PGC-1α expression

One of the major findings of our studies was that acute exercise upregulated the mRNA expression of the transcriptional coactivator PGC-1α. PGC-1α mRNA was increased in an intensity-dependent manner, with a 3.8- and 10.2-fold increase 3 h after low and high intensity exercise, respectively. Numerous other studies report similar increases PGC-1α mRNA following acute exercise. Mahoney et al. reported a 2.9-fold increase in PGC-1α mRNA 3 h after 75 minutes of high intensity cycling exercise and Cluberton and colleagues found a ~3-fold increase in the mRNA content of PGC-1α following 60 minutes of exercise at ~74% VO_2peak (Mahoney *et al.*, 2005); (Cluberton *et al.*, 2005). The intensity-dependent increase in PGC-1α we found following exercise is supported by one other study that was published at the same time our data was collected (Sriwijitkamol *et al.*, 2007). This study report PGC-1α mRNA increased by approximately 5- and 15-fold following acute exercise at either 50 or 70% VO_2max respectively. This was accompanied by a statistical difference between the divergent intensities. Interestingly, similar to our findings, phosphorylation of AMPK was greater at the higher intensity of exercise.

PGC-1α mRNA was increased in Experiment I in a comparable manner to that of the signalling cascades. Both CaMKII and AMPK phosphorylation were upregulated in an intensity-dependent manner suggesting this may have manifested in the divergent effect seen in PGC-1α mRNA and this hypothesis is supported by the results of (Sriwijitkamol *et al.*, 2007). In light of this it is important to note that AMPK and CaMKII are involved in PGC-1α transcription. Activation of AMPK by βGPA in mice has been shown to increase the expression of PGC-1α (Zong *et al.*, 2002). Six hours of low intensity swimming in rats and electrical stimulation of rat muscle increases AMPK activation and PGC-1α mRNA expression (Terada *et al.*, 2002), (Atherton *et al.*, 2005). In addition, AMPK phosphorylates PGC-1α at Thr^{177} and Ser^{538} stimulating PGC-1α

activation of its own promoter (Jager *et al.*, 2007). Therefore, AMPK activation is associated with increased transcription of PGC-1α and it is not unreasonable to assume PGC-1α expression may reflect the level of AMPK phosphorylation. AMPK has been shown to phosphorylate HDACs, and decrease nuclear localisation, increasing transcriptional activity on the MEF2 binding site of the PGC-1α promoter (McGee & Hargreaves, 2008). In a similar way CaMKII may remove the repression of HDAC4 on MEF2 by excluding it from the nucleus and increase PGC-1α expression (Liu *et al.*, 2005). Again the level of phosphorylation of CaMKII and AMPK may provide an explanation for the intensity-dependent increase in PGC-1α mRNA expression.

Another major finding of Experiment II was the differential expression of PGC-1α mRNA by contraction frequency. PGC-1α mRNA was increased 2.3- and 3.5-fold 3 h following cycling at a cadence of 50 or 80 RPM, respectively. These results were only significant for the high frequency trial and there was a frequency dependent difference also. The study may not have been adequately powered to detect a statistical difference after the low frequency trial. The increase in PGC-1α mRNA was comparable to the low intensity trial in Experiment I where participants cycled at a cadence of 75-80 RPM also. There was no difference between the trials in the phosphorylation of AMPK, ACCβ, CaMKII or CREB and the responses of these signalling pathways all reflect what we observed in Experiment I at low intensity. Therefore, the disparity in PGC-1α expression cannot be explained by the signalling data available to us.

Though we did not find an impact of calcium flux on cellular signalling activation, by varying the frequency of contraction while maintaining the absolute workload, we believed we would have altered the amplitude and duration of the calcium transients. We speculate that this may actually be the case, but CaMKII or CREB phosphorylation may not be sensitive enough to decode this change in calcium flux or the alteration may not be large enough, particularly at such a low intensity of exercise (50% VO_2peak). This phenomenon is not reflected in the expression of any of the other genes we analysed in this study. We can only speculate that there is some change in the signal that may be acting through alternative calcium sensing pathways such as calcineurin or PKC. Neither of these pathways have been shown to directly regulate PGC-1α expression but both have been shown to act on MEF2 (calcineurin through DNA-binding of NFAT on the MEF2 promoter and PKC through nuclear exclusion of HDAC) which can regulate PGC-1α expression (Chin *et al.*, 1998); (Derave *et al.*, 2000); (Wu *et al.*, 2001), (Vega

et al., 2004). It is also possible that the rate of energy expenditure could influence the increased PGC-1α expression observed here. Even though the power output (workload) and total energy expenditure were identical in both trials, at the higher cadence the rate of energy expenditure was significantly greater than the lower contraction frequency. The relative exercise intensity and substrate contribution was also significantly different at 80 RPM compared with 50 RPM. The increased PGC-1α expression following high-frequency contraction could be related to greater recruitment of Type II fibers due to the speed of contraction at 80 RPM or the slightly higher intensity. There may also be greater recruitment of individual skeletal muscle fibres due to the higher intensity or rate of energy expenditure (Sale, 1987).

An increase in mRNA is often referred to as an increase in gene expression in the literature. This is technically incorrect, as increased gene expression and its related functions are not manifested until there is an increase in the abundance of the protein encoded by the gene. Gene expression can be controlled at various points beyond transcription, so while gene transcription is an indicator of protein expression, the extent to which protein content will increase in response to an adaptive stimulus cannot be accurately predicted from the increase in mRNA (Baar *et al.*, 2002). Despite the fairly robust increases in PGC-1α mRNA seen in these experiments this is not manifested as an increase in PGC-1α at the protein level. In this instance acute exercise of varying intensity and duration does not result in a change in PGC-1α protein expression. We were restricted in our timing and number of muscle biopsies in the study design. Considering the half-life of PGC-1α is estimated at ~2.3 h there is a possibility that we may have missed an increase in PGC-1α protein (Puigserver *et al.*, 2001). This is unlikely as PGC-1α protein expression has been observed between 0-5 h after exercise which means, if elevated we would have observed it at the 3 h time point.

Previously it has been noted that acute changes in mRNA expression are not always predictive of changes in protein abundance (Gygi *et al.*, 1999). A number of studies reporting increases in PGC-1α mRNA in humans after an acute bout of exercise do not present data for PGC-1α protein (Sriwijitkamol *et al.*, 2007), (Cluberton *et al.*, 2005), (Pilegaard *et al.*, 2005), (Russell *et al.*, 2005), (Mahoney *et al.*, 2005), (Cartoni *et al.*, 2005), (Vissing *et al.*, 2005), (Norrbom *et al.*, 2004). This may be due to the fact that there have been issues with the commercial antibodies available in the last 2-3 years as I previously highlighted in the methods section. Numerous studies in animal muscle have

demonstrated an exercise-induced increase in PGC-1α protein expression (Baar *et al.*, 2002), (Terada *et al.*, 2002;Terada & Tabata, 2004). An acute bout of swimming results in greater expression of PGC-1α mRNA and an increase in protein of 75% and 95% immediately and 6 h after exercise respectively (Terada *et al.*, 2002;Terada & Tabata, 2004). In the same study, treadmill running increased the PGC-1α protein content by 175% in soleus muscle, but there was no increase in the epitrochlearis muscle suggesting that the response is specific to the muscle being recruited (Terada *et al.*, 2002;Terada & Tabata, 2004). A study in humans comparing resistance and endurance exercise in subjects unaccustomed to one mode of exercise saw increases in PGC-1α mRNA in response to endurance exercise (Coffey *et al.*, 2006). In support of our findings they did not find a change in PGC-1α protein despite an 8-10-fold increase in mRNA. Gibala *et al.* (2009) reported a 2-fold increase in PGC-1α mRNA following 3 h of recovery from an intermittent high intensity bout of cycling (Gibala *et al.*, 2009). Similarly, no change in PGC-1α protein abundance was reported. It seems strange that an increase in protein abundance of PGC-1α is regularly shown in animal models but not in human studies. We speculate that the nature of the exercise bouts in animal studies may be an influencing factor as they are often extreme in duration (4-6 hrs) and stress (swimming with weighted tails). On the other hand human studies tend to submaximal exercise of moderate duration. Take for example the protocol in the papers by (Terada *et al.*, 2002;Terada & Tabata, 2004). The duration of exercise (6 h) coupled with the fact that there was a 45 minute break in between bouts which would allow transcription of mRNA to occur is certainly more likely to produce a response than the exercise protocol used in Experiments I and II.

(Mathai *et al.*, 2008) found that exercise to exhaustion at 65% VO_2max resulted in a ~3- and ~7-fold increase in PGC-1α mRNA immediately and 2 h after exercise. In contrast to our findings, this increase in mRNA was accompanied by a 23% increase of PGC-1α protein immediately and 2 h post exercise which was 16% greater 24 h after exercise ($p<0.05$). The discrepancy seen between these findings and our results may be explained by the duration of the exercise which was greater than 2 h in this case. However, a 23% increase is modest and based on my own experience of the variability of a western blot I imagine a result of this magnitude would be very difficult to reproduce and may be within the margin of error based on the sensitivity of a western blot. A study by (De Filippis *et al.*, 2008) also demonstrated a modest increase in PGC-1α mRNA and protein following a single exercise session in human skeletal muscle.

Participants performed 8 minutes at 70% HRmax and 2 minutes at 90% HRmax followed by 2 minutes rest and repeated this set 4 times. PGC-1α mRNA was increased ~8-fold accompanied by a ~50% increase in PGC-1α protein concentration 5 h after exercise. The high intensity, intermittent nature of this session may account for the discrepancies in PGC-1α protein expression seen between this study and the experiments described here.

A number of recent studies have shown that while acute bouts of exercise transiently increase PGC-1α mRNA, exercise training is required to increase PGC-1α protein content (Burgomaster et al., 2008)(Gibala et al 2009). This is consistent with the hypothesis that physiological adaptations are mediated by the accumulation of translated protein transcribed from pulses of transiently elevated mRNA associated with acute bouts of exercise (Mahoney & Tarnopolsky, 2005); (Fluck, 2006). Further work is required to determine if the marked difference in mRNA during the present protocols would be manifested as divergent increases in PGC-1α protein content after a period of exercise training at the respective intensities.

In summary, these experiments have showed that the mRNA expression of PGC-1α is increased 3 h after exercise in an intensity-dependent manner with a far greater increase at high intensity. PGC-1α mRNA expression is also regulated by the frequency of contraction with a higher contraction frequency resulting in a greater response 3 h after exercise. Importantly, these increments in PGC-1α mRNA are not translated into increases in protein. This leads us to ask the question that if there is not an increase in the abundance of the protein how is PGC-1α exerting control on metabolism in response to exercise?

6.3.2 PGC-1α Activation

The necessity for a change in PGC-1α protein content to alter metabolic gene expression has been brought into question (Wright *et al.*, 2007b);(Leick *et al.*, 2008). Regulation of transcriptional activity occurs via changes in the amount or activity of transcriptional regulators (Spiegelman & Heinrich, 2004). As mentioned earlier the activity of PGC-1α is regulated by a number of posttranslational modifications including phosphorylation,

acetylation and methylation (Wright *et al.*, 2007b;Knutti *et al.*, 2001); (Rodgers *et al.*, 2005); (Teyssier *et al.*, 2005). (Wright *et al.*, 2007b) discovered that the mRNA of a number of PGC-1α mitochondrial targets, was increased in rat skeletal muscle immediately after 6h of swimming, despite the fact PGC-1α protein did not increase until 3 hr after exercise. This suggests that PGC-1α was activated via phosphorylation by p38 MAPK prior to an increase in protein in response to exercise. Furthermore, DNA binding of NRF-1 and NRF-2 to the promoters of cytochrome c and COXIV, respectively, was markedly increased in response to exercise and occurred before an increase in PGC-1α protein (Wright *et al.*, 2007b). Therefore, the activity of the protein may be even more important than its abundance. SIRT1 has been shown to deacetylate PGC-1α and increase the transcriptional activity of the coactivator (Rodgers *et al.*, 2005); (Gerhart-Hines *et al.*, 2007). p38 MAPK phosphorylates PGC-1α on Thr^{262}, Thr^{298} and Ser^{265} in the repressor region of PGC-1α ,where phosphorylation disrupts the association of the p160myb binding protein, a protein that binds to PGC-1α and decreases its transcriptional activity (Fan *et al.*, 2004). This leads to a protein that is more stable and active (Knutti *et al.*, 2001); (Puigserver *et al.*, 2001;Fan *et al.*, 2004). AMPK phosphorylates PGC-1α at Thr^{177} and Ser^{538} stimulating PGC-1α activation of its own promoter (Jager *et al.*, 2007). Akt/PKB phosphorylation of PGC-1α at Ser^{570} leads to a less stable protein with decreased transcriptional activity (Li *et al.*, 2007).

6.3.3 PGC-1 Acetylation

In light of the increase in SIRT1 mRNA and the fact that we had not observed a change in PGC-1α protein , we decided to measure the acetylation status of PGC-1α as a means of quantifying the activation of PGC-1α. PGC-1α acetylation had not been measured in human muscle samples previously and required the development of a novel technique. (Rodgers *et al.*, 2005) had previously published a method to determine PGC-1α acetylation in rat hepatocytes. We used a modified version of this protocol where we firstly, immunoprecipitated PGC-1α protein, followed by a western blot and finally, probed with an antibody for acetyl-lysine (see general methodology). As mentioned earlier in the methods section, there were several issues with the development of this protocol and the technique took quite a lot of refinement. We found that the acetylation of PGC-1α did not change in response to exercise at low or high intensity. In contrast, (Canto *et al.*, 2009) recently found that an exhaustive single bout of treadmill running in

mice transiently activated SIRT1 in an AMPK-dependent manner, deacetylated PGC-1α and increased PGC-1α mediated gene expression, with a maximal effect 3 h after exercise. The disparity between these findings and our own may be explained by the fact that the mice in this study ran to exhaustion which would have depleted glycogen and activated AMPK further. In addition, we only see an increase in SIRT1 mRNA 19h post-exercise, suggesting SIRT1 is not activated by 3 h as in the study above. Therefore, it is possible that deacetylation may occur later than 19 h after exercise in Experiment I although this is unlikely. There may also be methodological issues as the lysis buffers used in the two experiments are different. (Canto *et al.*, 2009) used an extra deacetylase inhibitor (sodium butyrate) that we were unaware of at the time of analysis which may have improved the measurement of acetylation in our samples if we had used it.

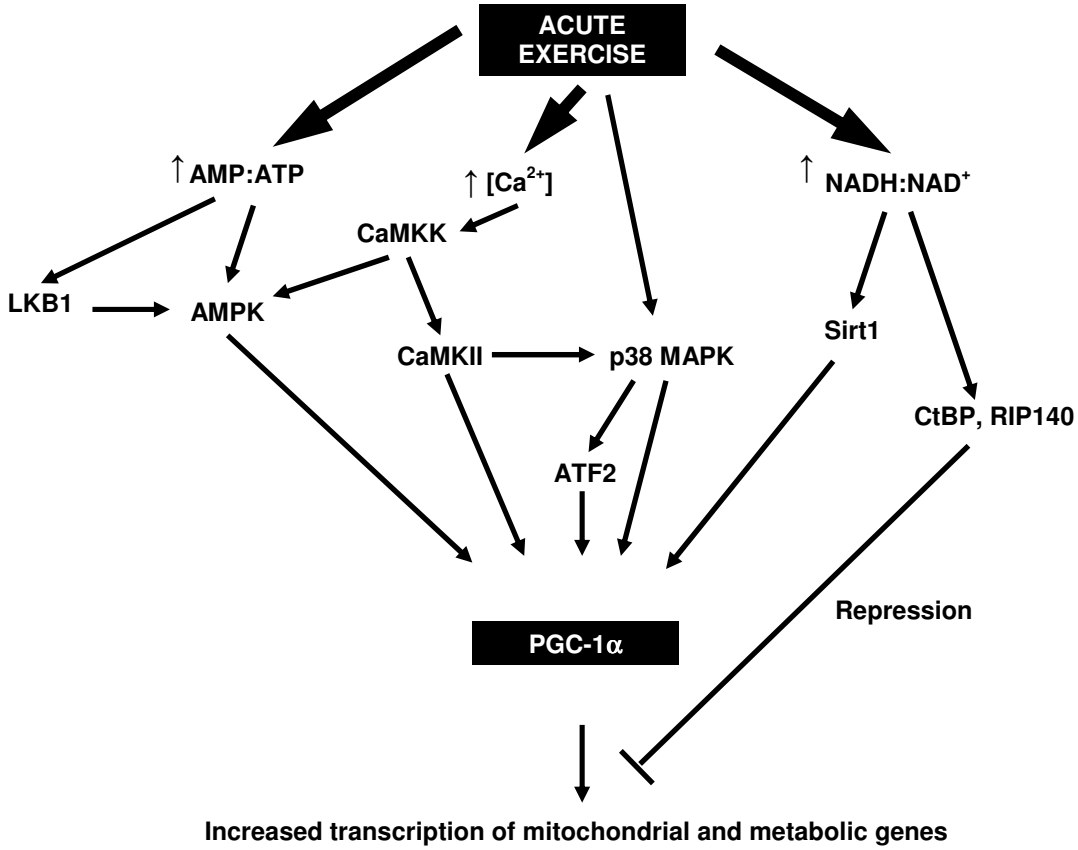

Figure 6.1 Overview of proposed mechanism of transcriptional regulation following an acute bout of exercise.

6.4 PGC-1α-mediated expression of genes involved in substrate use

An important finding of these experiments was the upregulation of several genes that regulate lipid metabolism including FOXO1A, PDK4 and PPARδ. Exercise training leads to a shift in substrate utilisation from relying on carbohydrate to fat metabolism during submaximal exercise (Henriksson, 1995). We report an increase in the expression of FOXO1A mRNA immediately and 3 h after exercise with an intensity dependent effect at 3-hrs post exercise. Contraction frequency had no effect on the expression of FOXO1A as there was a similar increase in mRNA at 0 and 3h in both trials at 50% VO_2peak. We found no change in FOXO1 protein after exercise at 50% VO_2peak at either 50 or 80 RPM despite the increase in FOXO1 mRNA. These findings are not surprising as FOXO1A mRNA has previously been shown to be induced by acute exercise in human muscle (Pilegaard *et al.*, 2005), (Mahoney *et al.*, 2005), (Russell *et al.*, 2005). Similarly, none of these studies report a subsequent increase in FOXO1 protein. However, the finding here that the regulation of FOXO1A is reliant upon exercise intensity is novel.

FOXO1A promotes the expression of genes involved in energy metabolism resulting in the transition from carbohydrate oxidation to lipid oxidation in response to fasting and exercise (Bastie *et al.*, 2005). An inducible form of FOXO1 in C_2C_{12} myotubes positively regulates FA metabolism through a number of mechanisms including FA uptake and oxidation. Activation of FOXO1 increases FA uptake by a 10-fold increase in the plasma membrane content of FAT/CD36, and improves FA oxidation by suppressing ACCβ and the induction of Acetyl CoA oxidase and PPARδ (Bastie *et al.*, 2005). Ectopic expression of FOXO1 also increases the expression of LPL in C_2C_{12} muscle cells (Kamei *et al.*, 2003). The robust increase in FOXO1A mRNA observed in these experiments provides a mechanism for the greater reliance on fat as a fuel source following exercise and the influence of exercise intensity on substrate utilisation. The upregulation of FOXO1A in our experiments suggests a shift from carbohydrate to FA oxidation during the recovery from exercise.

We demonstrate a robust increase in PDK4 mRNA 3 h after exercise independent of intensity. Induction of the PDK4 gene may also control the switch to fat metabolism after exercise by phosphorylation and inactivation of the PDC complex to prevent the

conversion of pyruvate to acetyl CoA, resulting in allosteric inhibition of glycolysis and suppression of glucose oxidation (Pilegaard & Neufer, 2004). . PDK4 may also be part of a transient stress response to exercise that promotes FA oxidation in a bid to conserve carbohydrate stores. These findings are well established as numerous studies support our results that an acute bout of exercise induces PDK4 expression in human skeletal muscle (Cluberton *et al.*, 2005), (Pilegaard *et al.*, 2005), (Mahoney *et al.*, 2005), (Coffey *et al.*, 2006), (Pilegaard *et al.*, 2002), (Civitarese *et al.*, 2005). Similarly, PDK4 transcription was previously shown to be increased by both low and high intensity exercise, with no difference between trials (Hildebrandt *et al.*, 2003). The mechanism controlling the expression of PDK4 may be mediated through FOXO1A. Expression of PDK4, similar to FOXO1, 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.*, 2003b;Tsintzas *et al.*, 2006). Activation of an inducible form of FOXO1 results in greater expression of PDK4 mRNA in C_2C_{12} cells (Bastie *et al.*, 2005). Starvation in mice led to the direct binding of FOXO1 to the promoter and subsequent induction of the PDK4 gene (Furuyama *et al.*, 2003). In our experiments the induction of FOXO1A occurs immediately after exercise, thus preceding an increase in PDK4 mRNA, giving further credence to the idea of FOXO1 regulating PDK4. However, PDK4 may not solely be under the regulation of FOXO1 as the intensity-dependent effect of exercise on FOXO1A expression is not reflected in the induction of PDK4.

The expression of PGC-1α is known to positively regulate lipid metabolism. PGC-1α co-activates the ERRα promoter, an orphan nuclear receptor that regulates mitochondrial biogenesis and fatty acid oxidation. A significant number of nuclear encoded mitochondrial gene promoters that are upregulated by PGC-1α contain binding sites for ERRα (Mootha *et al.*, 2004). PGC-1α overexpression in C_2C_{12} myoblasts induces the expression of ERRα (Mootha *et al.*, 2004). ERRα is involved in the PGC-1α mediated expression of lipid metabolic genes as it has been shown to target MCAD, which mediates the first step in β-oxidation of fatty acids, as well as CPT1, FABP3, CD36 and Acyl CoA oxidase (Huss *et al.*, 2004). PGC-1α has been shown to induce PDK4 mRNA and protein expression, promoting glucose sparing and fatty acid oxidation. This is mediated by a nuclear receptor binding site occupied by ERRα and the effect is lost in ERRα-null mice (Wende *et al.*, 2005). These results suggest that ERRα is involved in the PGC-1α mediated control of substrate selection.

However, we did not find a change in ERRα mRNA following any of the exercise trials, depsite greater PGC-1α and PDK4 mRNA levels. In contrast, (Cartoni *et al.*, 2005) reported an increase in ERRα mRNA 2 h after high intensity cycling exercise. This may be explained by the fact that these subjects were trained as opposed to the untrained males who participated in our study. PGC-1α has been shown to bind and coactivate FOXO1 in hepatic cells (Puigserver *et al.*, 2003). Interestingly, 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). As FOXO1A increases prior to induction of PGC-1α mRNA in these experiments, could FOXO1A possibly be regulating PGC-1α? The fact that both genes were upregulated in an exercise intensity-dependent manner adds strength to this theory. Taken together, this data suggests that a model involving interaction between FOXO1A, PGC-1α, ERRα, PPARδ and PDK4 is controlling the switch from carbohydrate metabolism to a greater reliance on lipid oxidation after exercise. However, PDK4 expression may not solely be under the control of FOXO1 and PGC-1α as the intensity-dependent effect of exercise on FOXO1A and PGC-1α expression was not reflected in the induction of PDK4. The expression of ERRα is regulated by PGC-1α and is necessary for PDK4 expression (Wende *et al.*, 2005;Araki & Motojima, 2006). However, it must be remembered that an increase in mRNA does not represent a change in the abundance of the functional protein. The regulation of PDK4 mRNA in response to exercise has previously been linked to muscle glycogen content (Pilegaard *et al.*, 2005) and the increased rate of glycogen utilization during high intensity exercise would have been expected to elicit a greater response. In light of this, PDK4 mRNA may be controlled by other factors such as energy expenditure which was similar in all trials. A study carried out by Vissing et al. also showed that the response of PDK may be independent of exercise. A control group which didn't undergo an exercise intervention saw similar increases in PDK4 mRNA to those that did exercise, suggesting the response of PDK4 mRNA was due to fasting (Vissing *et al.*, 2005).

Exercise increased PPARδ mRNA expression after 3 h of recovery in experiment I with a greater increase at 80% VO_2peak. An increase in PPARδ mRNA has been demonstrated in human and animal exercise after acute exercise and exercise training (Russell *et al.*, 2005);(Mahoney *et al.*, 2005);(Fritz *et al.*, 2006); (Spangenburg *et al.*, 2009). However, an exercise intensity-dependent effect on PPARδ has not been

described previously. Activation of PPARδ in skeletal muscle cells promotes fatty acid oxidation and utilization (Wang *et al.*, 2003). Activation of an inducible form of FOXO1 also increases the expression of PPARδ mRNA in C_2C_{12} cells (Bastie *et al.*, 2005). This suggests that the model used to describe PDK4 mRNA induction by FOXO1A earlier may also apply to PPARδ. Correspondingly, FOXO1A mRNA was increased prior to the change in PPARδ mRNA. Furthermore, both FOXO1A and PPARδ mRNA are upregulated by exercise in an intensity-dependent manner.

6.5 Regulation of glucose oxidation/transport by intracellular signalling cascades

AMPK is considered to be a 'fuel guage' for the cell, sensing the energy status, switching off anabolic processes and switching on alternative pathways for ATP regeneration (Jessen & Goodyear, 2005). To this end, AMPK is thought to increase glucose transport and uptake by translocating GLUT-4 containing vesicles to the plasma membrane and attenuate glycogen synthesis by phosphorylating glycogen synthase (Koistinen et al., 2003), (Carling & Hardie, 1989;Jorgensen *et al.*, 2004). Koistinen et al. (2003) found an increase in GLUT-4 translocation following AICAR-stimulated AMPK activation. AMPK has been shown to increase GLUT-4 expression by removing HDAC inhibition of MEF2, which is responsible for the regulation of GLUT-4 transcription (Ojuka *et al.*, 2002). AMPK also phosphorylates GEF *in vitro* which increases it's DNA binding activity and GLUT-4 expression (Holmes & Dohm, 2004). However, we did not find a change in GLUT4 or GEF mRNA in response to either high or low intensity exercise despite considerable activation of AMPK. CaMKII is also thought to regulate glucose transport and uptake through GLUT-4. (Smith *et al.*, 2008) found that a 2-fold increase in CaMKII phosphorylation following exercise led to hyperacetylation of the H3 histone around the MEF2 binding site on the promoter of GLUT-4. This increased DNA-binding of MEF2 to the promoter and a subsequent increase in GLUT-4 mRNA and protein expression. Furthermore, these effects were abolished with KN93 (CaMKII inhibitor) supplementation (Smith *et al.*, 2008). Similar results were demonstrated *in vitro* with caffeine as well as a decrease in nuclear localistaion of HDAC5 which inhibits MEF2 binding to the GLUT4 promoter (Mukwevho *et al.*, 2008). These results suggest that phosphorylation of CaMKII is necessary to increase GLUT-4 epxression.

However, we did not find an increase in GLUT4 mRNA despite increased phosphorylation of CaMKII at high intensity and, as mentioned previously, AMPK activation. We did not find a change in MEF2A or MEF2D mRNA at any time point after exercise. (Kraniou *et al.*, 2006) have previously reported an increase in GLUT-4 mRNA expression following low and high intensity exercise comparable to the protocol seen here with no difference between trials in humans.

CaMKII activity may play a role in glucose oxidation in the muscle. Studies in myotubes and rat epitrochlearis muscle have shown that an increase in calcium concentration increased glucose uptake in resting muscle (Holloszy & Narahara, 1967); (Youn *et al.*, 1991) ; (Wright *et al.*, 2004). This effect was blocked by the CaMK inhibitor KN62, which also reduced the contraction-mediated increase in glucose transport by 50% (Wright *et al.*, 2004). The intensity-dependent CaMKII phosphorylation in this study may explain, at least in part, the increase in carbohydrate oxidation during exercise seen at 80% VO_2peak.

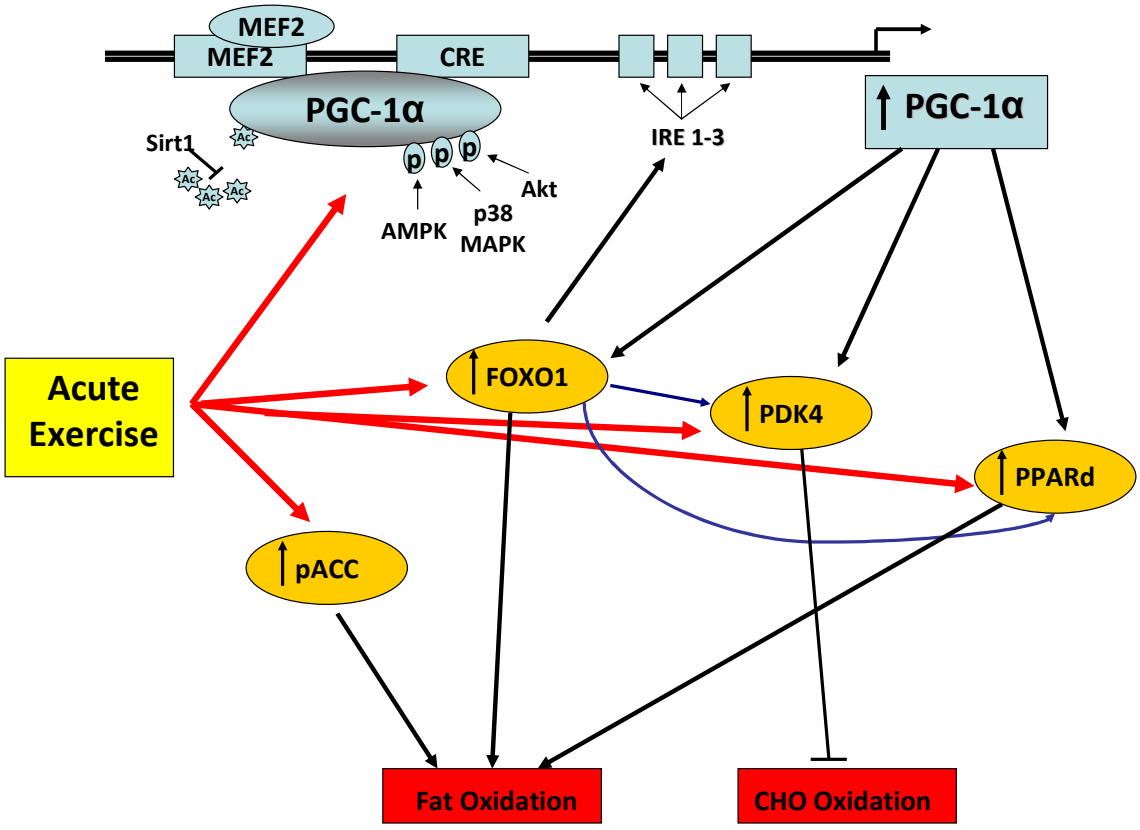

Figure 6.2. Proposed mechanism of the effect of acute exercise on substrate utilisation

6.6 Regulation of SIRT-1 by exercise

One of the major findings of this study was that SIRT1 mRNA was greater 19 h after exercise ($p<0.05$) compared with all other time points, while no effect was observed for the frequency of contraction. This is the first study to the best of our knowledge to report an exercise-induced increase in SIRT1 mRNA in human skeletal muscle. A recent paper described an increase in SIRT1 protein expression 2 h after a single bout of treadmill running in rats (Suwa *et al.*, 2008). This increase in SIRT1 protein was accompanied by an increase in PGC-1α protein 18 h after the bout of acute exercise. SIRT1 is an NAD^+-dependent deacetylase and fluctuations in NAD^+ have been shown to upregulate protein levels and enzyme activity of SIRT1 (Rodgers *et al.*, 2005), (Lagouge *et al.*, 2006). SIRT1 has been shown to deacetylate PGC-1α to increase the transcriptional activity of the coactivator (Rodgers *et al.*, 2005); (Gerhart-Hines *et al.*, 2007). SIRT1 has also been shown to deacetylate FOXO transcription factors to confer target gene specificity (Brunet *et al.*, 2004).

Interestingly, in the study by (Suwa *et al.*, 2008), SIRT1 protein expression preceded PGC-1α expression, suggesting that SIRT1 could deacetylate PGC-1α in the post-exercise period. Recently, a study in mouse muscle found that AMPK enhances SIRT1 activity by increasing cellular NAD^+ levels, resulting in the deacetylation and modulation of the activity of downsteeam SIRT1 targets including PGC-1α (Canto *et al.*, 2009). This may help explain the mechanism behind increased SIRT1 protein expression in the study by (Suwa *et al.*, 2008), whereby AMPK may have been activated by exercise and increased expression of SIRT1. This may also explain the increase in SIRT1 mRNA seen in our study although the time course is very different as we only see upregulation of SIRT1 19 h after exercise. Interestingly, SIRT1 is a NAD^+-sensing enzyme, but the $NADH/NAD^+$ ratio increases during exercise. Therefore, SIRT1 may be inactivated during exercise and would contrast with other findings (Suwa *et al.*, 2008) unless other regulatory systems become more important. However, this may provide an explanation for the delay in SIRT1 expression in our experiment. In the post exercise period NADH is oxidised causing an increase in NAD^+. This increase in NAD^+ during recovery from exercise could activate SIRT1, increase SIRT1 expression and promote PGC-1α deacetylation and increase PGC-1α activity.

Another potential mechanism for our SIRT1 findings can be supported by the findings of (Canto *et al.*, 2009). We have shown that AMPK is phosphorylated immediately after exercise at high intensity. This activated form of AMPK could enhance SIRT1 activity in the post-exercise period resulting in PGC-1α deacetylation and activation. Unfortunately, the increase we see in SIRT1 mRNA is after exercise at 50% VO_2peak where we did not find an increase in AMPK phosphorylation after exercise. However, phosphorylation of ACCβ is increased in response to exercise at 50% VO_2peak implying that AMPK was somewhat activated during this trial. Using this assumption SIRT1 mRNA may have been induced through this theoretical mechanism.

6.7 Regulation of the transcriptional co-repressor RIP140

Corepressors have a significant role to play in the regulation of metabolic transcription in skeletal muscle. In the unliganded state many nuclear receptors are associated with corepressors, such as N-CoR and SMRT, that dissociate from the receptors upon ligand binding allowing recruitment of the coactivator (Jepsen & Rosenfeld, 2002). There are also ligand-dependent corepressors such as RIP140 which associate with nuclear receptors upon the binding of a specific ligand (Christian *et al.*, 2006). RIP140 is of particular interest as several genes that are repressed by RIP140 are also targets of PGC-1α (Christian *et al.*, 2006). In particular, RIP140 has been shown to control ERRα transactivation by binding to it (Castet *et al.*, 2006). Following depletion of RIP140 most of the genes that encode enzymes of the TCA cycle, glycolysis, fatty acid oxidation, oxidative phosphorylation and mitochondrial biogenesis are upregulated (Powelka *et al.*, 2006). RIP140 serves as a scaffold for the docking of additional cofactors and enzymes similarly to PGC-1α, except RIP140 recruits HDACs instead of HATs, remodelling chromatin in such a way that leads to transcriptional repression (Christian *et al.*, 2006). RIP140 contains 4 repression domains that recruit HDACs mediated by the binding of C-terminal Binding Proteins (CtBPs) (Christian *et al.*, 2004). CtBP is a NAD^+ sensitive dehydrogenase indicating that the redox state may affect RIP140 transcriptional repression (Kumar *et al.*, 2002). The interaction between RIP140 and CtBP is modulated by phosphorylation and acetylation. Acetylation prevents the interaction between RIP140 and CtBP (Vo *et al.*, 2001) thus reducing RIP140's ability to recruit HDACs and repress transcription. MAPK-mediated phosphorylation of

RIP140 increases transcriptional repression by increasing recruitment of HDACs (Gupta *et al.*, 2005). Phosphorylation of RIP140 can also lead to relocation to the cytoplasm via interaction with 14-3-3 (Zilliacus *et al.*, 2001).

The interaction of corepressors and coactivators and their subsequent effect on gene transcription is an interesting area. As PGC-1α and RIP140 share similar targets and act in a similar manner, although with opposing consequences, the balance between their activation could be very important for the transcriptional regulation of muscle metabolism.

We report the novel finding that the expression of RIP140 is increased immediately and 3 h after exercise regardless of intensity. This is the first study to show an increase in RIP140 mRNA after exercise in human skeletal muscle. As mentioned previously, RIP140 contains 4 repression domains that recruit HDACs mediated by the binding of CtBPs (Christian *et al.*, 2004). The repressor function of CtBP is increased by decreasing the NAD^+/NADH ratio, similar to the redox response with exercise (Zhang *et al.*, 2002b). This would increase the interaction of RIP140 and CtBPs to enhance their repressor function in the post-exercise period. It s possible that RIP140 may have counteracted the gene expression profile co-activated by PGC-1α and explain why we did not find a change in the expression of ERRα or other metabolic targets. However more evidence is required about the protein content, cellular localisation and co-localisation with PGC-1α before any further conclusions can be made.

6.8 Exercise-mediated mitochondrial biogenesis

One of the major outcomes of exercise training is an increase in mitochondrial content and function, referred to as mitochondrial biogenesis. PGC-1α is thought to be central to exercise-stimulated mitochondrial biogenesis as it activates the transcription factors that modulate genes encoding mitochondrial proteins (NRF-1, NRF-2, ERRα and TFAM) (Lin *et al.*, 2005). As was discussed earlier, PGC-1α mRNA and protein increases in response to a single bout of exercise and this is thought to mediate the increase in mitochondrial biogenesis (Holloszy, 2008;Mathai *et al.*, 2008). However, we did not see an increase in PGC-1α protein after exercise despite a significant increase in mRNA. This suggests that the exercise stimulus was not sufficient to increase mitochondrial

biogenesis and would explain the fact that the expression of NRF-1, NRF-2 and ERRα or their downstream targets COXIV, CPT1 and UCP3 did not increase following exercise. Other studies have reported that CPT1 expression does not change following high intensity running or resistance exercise (Yang *et al.*, 2005) or low or high-intensity exercise (Hildebrandt *et al.*, 2003). (Pilegaard *et al.*, 2005) has reported an increase in the transcriptional activity of CPT1 following bicycle exercise at 75% VO_2peak but there was no change in mRNA.

UCP3 is also shown to be transcriptionally activated by acute exercise in human skeletal muscle (Hildebrandt *et al.*, 2003); (Pilegaard *et al.*, 2005); (Pilegaard *et al.*, 2002) and UCP3 mRNA levels were also increased following 60 minutes of cycling where there was no increase in PGC-1α protein (Cluberton *et al.*, 2005). Similarly, COX IV mRNA has previously been shown to increase 24 hours following a 10km bike trial without an increase in PGC-1α protein suggesting that increased PGC-1α activity and mRNA is sufficient to promote mitochondrial biogensesis (Cartoni *et al.*, 2005). In support of this, a study by (Wright *et al.*, 2007b) found that the mRNA levels of a number of PGC-1α mitochondrial targets, such as cytochrome c and citrate synthase, were increased in rat skeletal muscle immediately after 6h of swimming, despite the fact PGC-1α protein did not increase until 3 hr after exercise. This suggests that PGC-1α activity was increased prior to an increase in protein following exercise. Furthermore, DNA binding of NRF-1 and NRF-2 to the promoters of cytochrome c and COXIV, respectively, was markedly increased in response to exercise and occurred before an increase in PGC-1α protein (Wright *et al.*, 2007b). However, this does not explain the lack of an increase in the expression of either the transcription factors NRF-1, NRF-2 and ERRα, or the nuclear-encoded mitochondrial proteins COXIV, CPT1 and UCP3 observed here. This evidence suggests that the exercise bout was insufficient to increase both PGC-1α protein and PGC-1α functional activity enough to induce mitochondrial biogenesis. Alternatively, RIP140 mRNA expression was increased immediately after exercise and is known to affect the transcriptional activity of ERRα (Castet *et al.*, 2006). RIP140 has also been shown to negatively effect expression of metabolic and mitochondrial genes including CPT-1 and members of the cytochrome c oxidase family (Powelka *et al.*, 2006). It is possible that in this case RIP140 is playing an antagonistic role to that of PGC-1α in the regulation of mitochondrial biogenesis. This, in concert with the absence of an increase in PGC-1α protein expression may go some way to explaining the lack of induction of the mitochondrial proteins observed in this study.

6.9 Limitations

The experiments described within this thesis have a number of limitations. Firstly, carrying out human research carries with it a lot of difficulties. For example, the number of biopsies that could be performed as well as the amount of tissue that could be extracted were limited due to ethical considerations. This limited the analysis in terms of the number and type of laboratory techniques that were carried out. The invasive nature of the study and the time commitment involved made subject recruitment a difficult and lengthy process. This, in turn, made the task of scheduling subjects and the research team (including Doctor qualified to perform biopsies) difficult.

Controlling for variability in a human population provided a tough task. It is important to control for factors such as diet and activity and several measures were taken to ensure this (see Methodologies), however, it is impossible to say conclusively if subjects followed all directions accurately or reported behaviour outside the laboratory honestly. There is also the issue of whether the expression of a number of the genes presented in this thesis were actually regulated by exercise. A study by Vissing et al. showed that PDK4 mRNA is more likely to be regulated by fasting and re-feeding than by exercise and the results of a similar study by Pilegaard et al suggest something similar (Vissing *et al.*, 2005); (Pilegaard *et al.*, 2003a). We endeavoured to control this through dietary control but this has to be put down as a limitation.

There were some issues with the analysis. A considerable period of time was dedicated to developing a technique to analyse PGC-1α acetylation, which had not previously been reported in human muscle samples. Eventually it became apparent that the antibody being used may not actually bind the correct target which could explain, in part at least, the difficulties encountered with setting up the technique. This is discussed in further detail in the methodologies section.

6.9 Future Directions

The next important step to explain the regulation of gene expression following an acute bout of exercise is to gain a better understanding of PGC-1α activation. As mentioned previously, a number of posttranslational modifications control the functional activity of PGC-1α including phosphorylation, acetylation and methylation (Knutti *et al.*, 2001); (Rodgers *et al.*, 2005); (Teyssier *et al.*, 2005). Though there was no change in PGC-1α protein after exercise it is likely that PGC-1α is still regulating transcription through an increase in its functional activity and demonstration of this would help to explain some of our findings. Similar analysis should be carried out on RIP140 to investigate the functional activity of this corepressor. This is of particular interest as it is novel and the potential interplay between RIP140 and PGC-1α in the regulation of their common targets would provide an insight into the complex balance between transcriptional activation and repression and the time-course of these events in response to an exercise bout.

Exercise studies in human metabolism to date have generally focused on descriptive outcomes and do little to further the knowledge of the mechanisms at play. Future research should focus on elucidating the mechanisms in control of transcriptional and translational regulation of genes and how this results in the expression of enzymatically active proteins. This could include investigating the functional activity of transcription factors and their coregulators, their co-localisation, their subcellular localisation as well as their expression to give a more complete picture of muscle plasticity in response to exercise.

The experiments described here focus on the metabolic adaptation to an acute bout of exercise and give an insight into the signalling pathways and main protagonists involved in the regulation of metabolic gene expression and substrate selection. Further investigation into the role of exercise in maintaining metabolic homeostasis and prevention of disease is necessary. The adaptations that occur following an acute bout of exercise are transient, therefore, investigation into repeated bouts of exercise or exercise training would be beneficial. It would be interesting to examine how the exercise intensity- or contraction frequency-dependent effects observed here would manifest themselves after a period of exercise training following the protocols described here.

Novel means of regulating gene expression have recently been discovered. Gene expression can be regulated at a number of stages but the recent findings of post-transcriptional modification of mRNA by microRNA (miRNA) has opened up a whole new regulatory mechanism. miRNAs are short sequences of 19-22 nucleotides that regulate gene expression by post-transcriptional modification of messenger RNA (mRNA) at the 3' untranslated region (3'UTR). The miRNA interaction with mRNA represses protein translation and can destabilise mRNA (Filipowicz et al., 2008; Valencia-Sachez et al., 2006). At least 600-700 human miRNA's have been identified (www.microrna.org) and while the total number is unclear it is thought that they may account for 2-3% of all genes in the human genome (Bethel et al. 2008, Berezikov et al., 2005). miRNA are evolutionary conserved in single and multi-cellular organisms and may target upto one-third of all human genes (Lewis et al., 2005). miRNA can be transcribed in isolation or in parallel with mRNA and may be dependent on the location of encoding DNA (30) but it is generally accepted that primary-miRNA are initially transcribed as double stranded RNA (dsRNA) by RNA polymerase II with a 5' cap and 3' poly(A) tail similar to mRNA (3,34).). The majority of mammalian miRNA fold into double stranded hairpins that imperfectly pair with mRNA and subsequently either (i) block translation initiation/elongation, (ii) initiate the proteolytic degradation of polypeptides or (iii) destabilise mRNA by deadenylating the 3'-poly(A) tail (8,35). The mechanisms of miRNA mediated translation repression are poorly understood. Evidence to date would suggest that the interaction of mRNA with the translation initiation machinery can be impaired but that repression can also occur at a distal step in the process (reviewed in Filipowicz et al., 2008). The majority of evidence from skeletal muscle, to date, suggests that miRNA regulate gene expression during development (8) but there are no reports in the literature of post-transcriptional regulation of skeletal muscle metabolic gene expression by miRNA. There are currently no research publications on the role of miRNA in human skeletal muscle, not least in response to exercise.

6.10 Practical Relevance

The metabolic syndrome is clinically defined as a disorder characterised by at least three of the following: central obesity, hyperglycaemia, hypertension, hypertriglyceridaemia and a decrease in circulating levels of HDL cholesterol (Grundy,

2006). Exercise in addition to preventing obesity, is a means of prevention and treatment of the metabolic syndrome and type II diabetes (Hawley, 2004); (Pedersen, 2006); (Goodyear & Kahn, 1998). The findings of these experiments may provide information that can optimise exercise prescription to prevent and treatment metabolic diseases. Public health guidelines generally recommend 30-60 min of moderate-intensity exercise on all or most days of the week. However, most adults fail to meet even the minimum physical activity guidelines. In experiment I, caloric expenditure was identical; however, the time to completion was almost half in the high intensity trial. In the modern era where time is considered a precious commodity, this information must be considered when designing a training programme. Exercising at a higher intensity may provide a more efficient method in gaining metabolic benefits and expending the necessary calories to maintain health. (Gibala & McGee, 2008) propose high intensity interval training as a time-efficient means of increasing skeletal muscle oxidative capacity and endurance performance and improving metabolic control. In addition to saving time, in this study, high intensity exercise resulted in greater activation of the signalling cascades associated with control of metabolic gene expression than low intensity exercise. This would suggest, that high intensity exercise is more beneficial for health and metabolic control even when caloric expenditure is the same. In terms of contraction frequency, it would seem that a higher contraction frequency during exercise would prove more beneficial as this resulted in greater upregulation of PGC-1α mRNA despite similar caloric expenditure and identical workload. However, anecdotally the higher cadence used in experiment II is likely to be the self-selected cadence by exercising individuals.

6.11 Conclusion

As a result of this research it is clear that an acute bout of exercise results in a transient adaptation in human skeletal muscle and that the nature of the response is specific to the metabolic requirement of the exercise. This adaptation is mediated by the activation of a number of signalling kinases including AMPK, CaMKII, p38 MAPK and NAD^+ that control the activity of transcription factors and transcriptional co-regulators central to muscle metabolism. These transcriptional regulators translate the physiological stimulus of exercise into a transcriptional adaptation in a number of enzymes that regulate

metabolic processes such as substrate utilisation and mitochondrial function. (Desvergne *et al.*, 2006).

The transcriptional co-activator PGC-1α seems to act as a sensitive point of regulation in the control of these metabolic processes in response to varying types of muscle contraction. The fact PGC-1α can be so easily regulated combined with its ability to interact with a number of important metabolic transcription factors make PGC-1α a very interesting research target.

Exercise still represents a useful tool to research the regulation of PGC-1α as well as other metabolic genes. The findings from this research will shed some light on the adaptation of muscle metabolism and substrate utilisation after exercise, however, there is still much to learn about the mechanisms involved in these adaptations. This information may have implications for the prevention and treatment of diseases such as Type 2 Diabetes Mellitus and obesity, therefore, further investigation into the adaptive response of human skeletal muscle to acute bouts of exercise is required.

Bibliography

Reference List

Achten J, Gleeson M, & Jeukendrup AE (2002). Determination of the exercise intensity that elicits maximal fat oxidation. *Med Sci Sports Exerc* **34**, 92-97.

Akimoto T, Pohnert SC, Li P, Zhang M, Gumbs C, Rosenberg PB, Williams RS, & Yan Z (2005). Exercise stimulates Pgc-1alpha transcription in skeletal muscle through activation of the p38 MAPK pathway. *J Biol Chem* **280**, 19587-19593.

Alberini CM (2009). Transcription factors in long-term memory and synaptic plasticity. *Physiol Rev* **89**, 121-145.

Amat R, Planavila A, Chen SL, Iglesias R, Giralt M, & Villarroya F (2009). SIRT1 Controls the Transcription of the Peroxisome Proliferator-activated Receptor-{gamma} Co-activator-1{alpha} (PGC-1{alpha}) Gene in Skeletal Muscle through the PGC-1{alpha} Autoregulatory Loop and Interaction with MyoD. *J Biol Chem* **284**, 21872-21880.

Andersen P & Henriksson J (1977). Capillary supply of the quadriceps femoris muscle of man: adaptive response to exercise. *J Physiol* **270**, 677-690.

Araki M & Motojima K (2006). Identification of ERRalpha as a specific partner of PGC-1alpha for the activation of PDK4 gene expression in muscle. *FEBS J* **273**, 1669-1680.

Aronson D, Boppart MD, Dufresne SD, Fielding RA, & Goodyear LJ (1998). Exercise stimulates c-Jun NH2 kinase activity and c-Jun transcriptional activity in human skeletal muscle. *Biochem Biophys Res Commun* **251**, 106-110.

Aronson D, Dufresne SD, & Goodyear LJ (1997). Contractile activity stimulates the c-Jun NH2-terminal kinase pathway in rat skeletal muscle. *J Biol Chem* **272**, 25636-25640.

Atherton PJ, Babraj J, Smith K, Singh J, Rennie MJ, & Wackerhage H (2005). Selective activation of AMPK-PGC-1alpha or PKB-TSC2-mTOR signaling can explain specific

adaptive responses to endurance or resistance training-like electrical muscle stimulation. *FASEB J* **19**, 786-788.

Baar K, Wende AR, Jones TE, Marison M, Nolte LA, Chen M, Kelly DP, & Holloszy JO (2002). Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. *FASEB J* **16**, 1879-1886.

Bahr R (1992). Excess postexercise oxygen consumption--magnitude, mechanisms and practical implications. *Acta Physiol Scand Suppl* **605**, 1-70.

Bahr R, Hansson P, & Sejersted OM (1990). Triglyceride/fatty acid cycling is increased after exercise. *Metabolism* **39**, 993-999.

Bahr R, Ingnes I, Vaage O, Sejersted OM, & Newsholme EA (1987). Effect of duration of exercise on excess postexercise O2 consumption. *J Appl Physiol* **62**, 485-490.

Bangsbo J, Gollnick PD, Graham TE, Juel C, Kiens B, Mizuno M, & Saltin B (1990). Anaerobic energy production and O2 deficit-debt relationship during exhaustive exercise in humans. *J Physiol* **422**, 539-559.

Barnes BR, Glund S, Long YC, Hjalm G, Andersson L, & Zierath JR (2005). 5'-AMP-activated protein kinase regulates skeletal muscle glycogen content and ergogenics. *FASEB J* **19**, 773-779.

Bassel-Duby R & Olson EN (2006). Signaling pathways in skeletal muscle remodeling. *Annu Rev Biochem* **75**, 19-37.

Bastie CC, Nahle Z, McLoughlin T, Esser K, Zhang W, Unterman T, & Abumrad NA (2005). FoxO1 stimulates fatty acid uptake and oxidation in muscle cells through CD36-dependent and -independent mechanisms. *J Biol Chem* **280**, 14222-14229.

Baylor SM & Hollingworth S (2003). Sarcoplasmic reticulum calcium release compared in slow-twitch and fast-twitch fibres of mouse muscle. *J Physiol* **551**, 125-138.

bdel-Hafiz HA, Heasley LE, Kyriakis JM, Avruch J, Kroll DJ, Johnson GL, & Hoeffler JP (1992). Activating transcription factor-2 DNA-binding activity is stimulated by phosphorylation catalyzed by p42 and p54 microtubule-associated protein kinases. *Mol Endocrinol* **6**, 2079-2089.

Beeson M, Sajan MP, Dizon M, Grebenev D, Gomez-Daspet J, Miura A, Kanoh Y, Powe J, Bandyopadhyay G, Standaert ML, & Farese RV (2003). Activation of protein kinase C-zeta by insulin and phosphatidylinositol-3,4,5-(PO4)3 is defective in muscle in type 2 diabetes and impaired glucose tolerance: amelioration by rosiglitazone and exercise. *Diabetes* **52**, 1926-1934.

Benton CR, Nickerson JG, Lally J, Han XX, Holloway GP, Glatz JF, Luiken JJ, Graham TE, Heikkila JJ, & Bonen A (2008). Modest PGC-1alpha overexpression in muscle in vivo is sufficient to increase insulin sensitivity and palmitate oxidation in subsarcolemmal, not intermyofibrillar, mitochondria. *J Biol Chem* **283**, 4228-4240.

Bessman SP & Carpenter CL (1985). The creatine-creatine phosphate energy shuttle. *Annu Rev Biochem* **54**, 831-862.

Birk JB & Wojtaszewski JF (2006). Predominant alpha2/beta2/gamma3 AMPK activation during exercise in human skeletal muscle. *J Physiol* **577**, 1021-1032.

Bloor CM (2005). Angiogenesis during exercise and training. *Angiogenesis* **8**, 263-271.

Bonen A, Dyck DJ, Ibrahimi A, & Abumrad NA (1999). Muscle contractile activity increases fatty acid metabolism and transport and FAT/CD36. *Am J Physiol* **276**, E642-E649.

Bonen A, Han XX, Habets DD, Febbraio M, Glatz JF, & Luiken JJ (2007). A null mutation in skeletal muscle FAT/CD36 reveals its essential role in insulin- and AICAR-stimulated fatty acid metabolism. *Am J Physiol Endocrinol Metab* **292**, E1740-E1749.

Booth FW, Chakravarthy MV, Gordon SE, & Spangenburg EE (2002a). Waging war on physical inactivity: using modern molecular ammunition against an ancient enemy. *J Appl Physiol* **93**, 3-30.

Booth FW, Chakravarthy MV, & Spangenburg EE (2002b). Exercise and gene expression: physiological regulation of the human genome through physical activity. *J Physiol* **543**, 399-411.

Booth FW, Gordon SE, Carlson CJ, & Hamilton MT (2000). Waging war on modern chronic diseases: primary prevention through exercise biology. *J Appl Physiol* **88**, 774-787.

Boppart MD, Aronson D, Gibson L, Roubenoff R, Abad LW, Bean J, Goodyear LJ, & Fielding RA (1999). Eccentric exercise markedly increases c-Jun NH(2)-terminal kinase activity in human skeletal muscle. *J Appl Physiol* **87**, 1668-1673.

Boppart MD, Asp S, Wojtaszewski JF, Fielding RA, Mohr T, & Goodyear LJ (2000). Marathon running transiently increases c-Jun NH2-terminal kinase and p38 activities in human skeletal muscle. *J Physiol* **526 Pt 3**, 663-669.

Boppart MD, Hirshman MF, Sakamoto K, Fielding RA, & Goodyear LJ (2001). Static stretch increases c-Jun NH2-terminal kinase activity and p38 phosphorylation in rat skeletal muscle. *Am J Physiol Cell Physiol* **280**, C352-C358.

Brechtel K, Niess AM, Machann J, Rett K, Schick F, Claussen CD, Dickhuth HH, Haering HU, & Jacob S (2001). Utilisation of intramyocellular lipids (IMCLs) during exercise as assessed by proton magnetic resonance spectroscopy (1H-MRS). *Horm Metab Res* **33**, 63-66.

Bright NJ, Thornton C, & Carling D (2009). The regulation and function of mammalian AMPK-related kinases. *Acta Physiol (Oxf)* **196**, 15-26.

Brooks GA, Hittelman KJ, Faulkner JA, & Beyer RE (1971). Tissue temperatures and whole-animal oxygen consumption after exercise. *Am J Physiol* **221**, 427-431.

Brooks GA & Mercier J (1994). Balance of carbohydrate and lipid utilization during exercise: the "crossover" concept. *J Appl Physiol* **76**, 2253-2261.

Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, Tran H, Ross SE, Mostoslavsky R, Cohen HY, Hu LS, Cheng HL, Jedrychowski MP, Gygi SP, Sinclair DA, Alt FW, & Greenberg ME (2004). Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science* **303**, 2011-2015.

Calvo JA, Daniels TG, Wang X, Paul A, Lin J, Spiegelman BM, Stevenson SC, & Rangwala SM (2008). Muscle-specific expression of PPAR{gamma} coactivator-1{alpha} improves exercise performance and increases peak oxygen uptake. *J Appl Physiol* **104**, 1304-1312.

Canto C, Gerhart-Hines Z, Feige JN, Lagouge M, Noriega L, Milne JC, Elliott PJ, Puigserver P, & Auwerx J (2009). AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. *Nature* **458**, 1056-1060.

Carling D & Hardie DG (1989). The substrate and sequence specificity of the AMP-activated protein kinase. Phosphorylation of glycogen synthase and phosphorylase kinase. *Biochim Biophys Acta* **1012**, 81-86.

Cartoni R, Leger B, Hock MB, Praz M, Crettenand A, Pich S, Ziltener JL, Luthi F, Deriaz O, Zorzano A, Gobelet C, Kralli A, & Russell AP (2005). Mitofusins 1/2 and ERR{alpha} expression are increased in human skeletal muscle after physical exercise. *J Physiol* **567**, 349-358.

Castet A, Herledan A, Bonnet S, Jalaguier S, Vanacker JM, & Cavailles V (2006). Receptor-interacting protein 140 differentially regulates estrogen receptor-related receptor transactivation depending on target genes. *Mol Endocrinol* **20**, 1035-1047.

Chabowski A, Momken I, Coort SL, Calles-Escandon J, Tandon NN, Glatz JF, Luiken JJ, & Bonen A (2006). Prolonged AMPK activation increases the expression of fatty acid transporters in cardiac myocytes and perfused hearts. *Mol Cell Biochem* **288**, 201-212.

Chad KE & Wenger HA (1988). The effect of exercise duration on the exercise and post-exercise oxygen consumption. *Can J Sport Sci* **13**, 204-207.

Chen HC, Bandyopadhyay G, Sajan MP, Kanoh Y, Standaert M, Farese RV, Jr., & Farese RV (2002). Activation of the ERK pathway and atypical protein kinase C isoforms in exercise- and aminoimidazole-4-carboxamide-1-beta-D-riboside (AICAR)-stimulated glucose transport. *J Biol Chem* **277**, 23554-23562.

Chen ZP, Stephens TJ, Murthy S, Canny BJ, Hargreaves M, Witters LA, Kemp BE, & McConell GK (2003). Effect of exercise intensity on skeletal muscle AMPK signaling in humans. *Diabetes* **52**, 2205-2212.

Chin ER (2004). The role of calcium and calcium/calmodulin-dependent kinases in skeletal muscle plasticity and mitochondrial biogenesis. *Proc Nutr Soc* **63**, 279-286.

Chin ER (2005). Role of Ca2+/calmodulin-dependent kinases in skeletal muscle plasticity. *J Appl Physiol* **99**, 414-423.

Chin ER, Olson EN, Richardson JA, Yang Q, Humphries C, Shelton JM, Wu H, Zhu W, Bassel-Duby R, & Williams RS (1998). A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. *Genes Dev* **12**, 2499-2509.

Choi CS, Befroy DE, Codella R, Kim S, Reznick RM, Hwang YJ, Liu ZX, Lee HY, Distefano A, Samuel VT, Zhang D, Cline GW, Handschin C, Lin J, Petersen KF, Spiegelman BM, & Shulman GI (2008). Paradoxical effects of increased expression of PGC-1alpha on muscle mitochondrial function and insulin-stimulated muscle glucose metabolism. *Proc Natl Acad Sci U S A* **105**, 19926-19931.

Christian M, Tullet JM, & Parker MG (2004). Characterization of four autonomous repression domains in the corepressor receptor interacting protein 140. *J Biol Chem* **279**, 15645-15651.

Christian M, White R, & Parker MG (2006). Metabolic regulation by the nuclear receptor corepressor RIP140. *Trends Endocrinol Metab* **17**, 243-250.

Civitarese AE, Hesselink MK, Russell AP, Ravussin E, & Schrauwen P (2005). Glucose ingestion during exercise blunts exercise-induced gene expression of skeletal muscle fat oxidative genes. *Am J Physiol Endocrinol Metab* **289**, E1023-E1029.

Clark AR & Docherty K (1993). Negative regulation of transcription in eukaryotes. *Biochem J* **296 (Pt 3)**, 521-541.

Cleland PJ, Appleby GJ, Rattigan S, & Clark MG (1989). Exercise-induced translocation of protein kinase C and production of diacylglycerol and phosphatidic acid

in rat skeletal muscle in vivo. Relationship to changes in glucose transport. *J Biol Chem* **264**, 17704-17711.

Cluberton LJ, McGee SL, Murphy RM, & Hargreaves M (2005). Effect of carbohydrate ingestion on exercise-induced alterations in metabolic gene expression. *J Appl Physiol* **99**, 1359-1363.

Coffey VG, Zhong Z, Shield A, Canny BJ, Chibalin AV, Zierath JR, & Hawley JA (2006). Early signaling responses to divergent exercise stimuli in skeletal muscle from well-trained humans. *FASEB J* **20**, 190-192.

CONSOLAZIO CF, MATOUSH LR, NELSON RA, TORRES JB, & ISAAC GJ (1963). Environmental temperature and energy expenditures. *J Appl Physiol* **18**, 65-68.

Cordain L, Gotshall RW, Eaton SB, & Eaton SB, III (1998). Physical activity, energy expenditure and fitness: an evolutionary perspective. *Int J Sports Med* **19**, 328-335.

Creer A, Gallagher P, Slivka D, Jemiolo B, Fink W, & Trappe S (2005). Influence of muscle glycogen availability on ERK1/2 and Akt signaling after resistance exercise in human skeletal muscle. *J Appl Physiol* **99**, 950-956.

Czubryt MP, McAnally J, Fishman GI, & Olson EN (2003). Regulation of peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 alpha) and mitochondrial function by MEF2 and HDAC5. *Proc Natl Acad Sci U S A* **100**, 1711-1716.

Daitoku H, Yamagata K, Matsuzaki H, Hatta M, & Fukamizu A (2003). Regulation of PGC-1 promoter activity by protein kinase B and the forkhead transcription factor FKHR. *Diabetes* **52**, 642-649.

Dawson B, Straton S, & Randall N (1996). Oxygen consumption during recovery from prolonged submaximal cycling below the anaerobic threshold. *J Sports Med Phys Fitness* **36**, 77-84.

De Filippis E, Alvarez G, Berria R, Cusi K, Everman S, Meyer C, & Mandarino LJ (2008). Insulin-resistant muscle is exercise resistant: evidence for reduced response of nuclear-encoded mitochondrial genes to exercise. *Am J Physiol Endocrinol Metab* **294**, E607-E614.

De Koninck P & Schulman H (1998). Sensitivity of CaM kinase II to the frequency of Ca2+ oscillations. *Science* **279**, 227-230.

De Lange P, Farina P, Moreno M, Ragni M, Lombardi A, Silvestri E, Burrone L, Lanni A, & Goglia F (2006). Sequential changes in the signal transduction responses of skeletal muscle following food deprivation. *FASEB J* **20**, 2579-2581.

Deckert J & Struhl K (2001). Histone acetylation at promoters is differentially affected by specific activators and repressors. *Mol Cell Biol* **21**, 2726-2735.

DeFronzo RA, Simonson D, & Ferrannini E (1982). Hepatic and peripheral insulin resistance: a common feature of type 2 (non-insulin-dependent) and type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* **23**, 313-319.

Denis C, Dormois D, Linossier MT, Geyssant A, & Lacour JR (1991). Total muscle NADH during 30-min submaximal exercise: effects of endurance training. *Acta Physiol Scand* **142**, 301-302.

Derave W, Ai H, Ihlemann J, Witters LA, Kristiansen S, Richter EA, & Ploug T (2000). Dissociation of AMP-activated protein kinase activation and glucose transport in contracting slow-twitch muscle. *Diabetes* **49**, 1281-1287.

Desvergne B, Michalik L, & Wahli W (2006). Transcriptional regulation of metabolism. *Physiol Rev* **86**, 465-514.

Donsmark M, Langfort J, Holm C, Ploug T, & Galbo H (2004). Regulation and role of hormone-sensitive lipase in rat skeletal muscle. *Proc Nutr Soc* **63**, 309-314.

Dufour SP, Ponsot E, Zoll J, Doutreleau S, Lonsdorfer-Wolf E, Geny B, Lampert E, Fluck M, Hoppeler H, Billat V, Mettauer B, Richard R, & Lonsdorfer J (2006). Exercise training in normobaric hypoxia in endurance runners. I. Improvement in aerobic performance capacity. *J Appl Physiol* **100**, 1238-1248.

Dufresne SD, Bjorbaek C, El-Haschimi K, Zhao Y, Aschenbach WG, Moller DE, & Goodyear LJ (2001). Altered extracellular signal-regulated kinase signaling and glycogen metabolism in skeletal muscle from p90 ribosomal S6 kinase 2 knockout mice. *Mol Cell Biol* **21**, 81-87.

Durnin JV (1996). Energy requirements: general principles. *Eur J Clin Nutr* **50 Suppl 1**, S2-S9.

Eaton SB, Konner M, & Shostak M (1988). Stone agers in the fast lane: chronic degenerative diseases in evolutionary perspective. *Am J Med* **84**, 739-749.

Edelman AM, Mitchelhill KI, Selbert MA, Anderson KA, Hook SS, Stapleton D, Goldstein EG, Means AR, & Kemp BE (1996). Multiple Ca(2+)-calmodulin-dependent

protein kinase kinases from rat brain. Purification, regulation by Ca(2+)-calmodulin, and partial amino acid sequence. *J Biol Chem* **271**, 10806-10810.

Enslen H, Sun P, Brickey D, Soderling SH, Klamo E, & Soderling TR (1994). Characterization of Ca2+/calmodulin-dependent protein kinase IV. Role in transcriptional regulation. *J Biol Chem* **269**, 15520-15527.

Esterbauer H, Oberkofler H, Krempler F, & Patsch W (1999). Human peroxisome proliferator activated receptor gamma coactivator 1 (PPARGC1) gene: cDNA sequence, genomic organization, chromosomal localization, and tissue expression. *Genomics* **62**, 98-102.

Fan M, Rhee J, St Pierre J, Handschin C, Puigserver P, Lin J, Jaeger S, Erdjument-Bromage H, Tempst P, & Spiegelman BM (2004). Suppression of mitochondrial respiration through recruitment of p160 myb binding protein to PGC-1alpha: modulation by p38 MAPK. *Genes Dev* **18**, 278-289.

Feige JN & Auwerx J (2007). Transcriptional coregulators in the control of energy homeostasis. *Trends Cell Biol* **17**, 292-301.

Fernandez-Real JM & Ricart W (1999). Insulin resistance and inflammation in an evolutionary perspective: the contribution of cytokine genotype/phenotype to thriftiness. *Diabetologia* **42**, 1367-1374.

Fisher RP, Lisowsky T, Parisi MA, & Clayton DA (1992). DNA wrapping and bending by a mitochondrial high mobility group-like transcriptional activator protein. *J Biol Chem* **267**, 3358-3367.

Fluck M (2006). Functional, structural and molecular plasticity of mammalian skeletal muscle in response to exercise stimuli. *J Exp Biol* **209**, 2239-2248.

Fluck M & Hoppeler H (2003). Molecular basis of skeletal muscle plasticity--from gene to form and function. *Rev Physiol Biochem Pharmacol* **146**, 159-216.

Fluck M, Waxham MN, Hamilton MT, & Booth FW (2000). Skeletal muscle Ca(2+)-independent kinase activity increases during either hypertrophy or running. *J Appl Physiol* **88**, 352-358.

Freyssenet D (2007). Energy sensing and regulation of gene expression in skeletal muscle. *J Appl Physiol* **102**, 529-540.

Freyssenet D, Di CM, & Hood DA (1999). Calcium-dependent regulation of cytochrome c gene expression in skeletal muscle cells. Identification of a protein kinase c-dependent pathway. *J Biol Chem* **274**, 9305-9311.

Freyssenet D, Irrcher I, Connor MK, Di Carlo M, & Hood DA (2004). Calcium-regulated changes in mitochondrial phenotype in skeletal muscle cells. *Am J Physiol Cell Physiol* **286**, C1053-C1061.

Friden J, Kjorell U, & Thornell LE (1984). Delayed muscle soreness and cytoskeletal alterations: an immunocytological study in man. *Int J Sports Med* **5**, 15-18.

Friden J, Sjostrom M, & Ekblom B (1981). A morphological study of delayed muscle soreness. *Experientia* **37**, 506-507.

Friden J, Sjostrom M, & Ekblom B (1983). Myofibrillar damage following intense eccentric exercise in man. *Int J Sports Med* **4**, 170-176.

Fritz T, Kramer DK, Karlsson HK, Galuska D, Engfeldt P, Zierath JR, & Krook A (2006). Low-intensity exercise increases skeletal muscle protein expression of PPARdelta and UCP3 in type 2 diabetic patients. *Diabetes Metab Res Rev* **22**, 492-498.

Fujii N, Boppart MD, Dufresne SD, Crowley PF, Jozsi AC, Sakamoto K, Yu H, Aschenbach WG, Kim S, Miyazaki H, Rui L, White MF, Hirshman MF, & Goodyear LJ (2004). Overexpression or ablation of JNK in skeletal muscle has no effect on glycogen synthase activity. *Am J Physiol Cell Physiol* **287**, C200-C208.

Fujii N, Hayashi T, Hirshman MF, Smith JT, Habinowski SA, Kaijser L, Mu J, Ljungqvist O, Birnbaum MJ, Witters LA, Thorell A, & Goodyear LJ (2000). Exercise induces isoform-specific increase in 5'AMP-activated protein kinase activity in human skeletal muscle. *Biochem Biophys Res Commun* **273**, 1150-1155.

Furuyama T, Kitayama K, Yamashita H, & Mori N (2003). Forkhead transcription factor FOXO1 (FKHR)-dependent induction of PDK4 gene expression in skeletal muscle during energy deprivation. *Biochem J* **375**, 365-371.

Gaesser GA & Brooks GA (1984). Metabolic bases of excess post-exercise oxygen consumption: a review. *Med Sci Sports Exerc* **16**, 29-43.

Garesse R & Vallejo CG (2001). Animal mitochondrial biogenesis and function: a regulatory cross-talk between two genomes. *Gene* **263**, 1-16.

Gerhart-Hines Z, Rodgers JT, Bare O, Lerin C, Kim SH, Mostoslavsky R, Alt FW, Wu Z, & Puigserver P (2007). Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1alpha. *EMBO J* **26**, 1913-1923.

Gibala MJ & McGee SL (2008). Metabolic adaptations to short-term high-intensity interval training: a little pain for a lot of gain? *Exerc Sport Sci Rev* **36**, 58-63.

Gibala MJ, McGee SL, Garnham AP, Howlett KF, Snow RJ, & Hargreaves M (2009). Brief intense interval exercise activates AMPK and p38 MAPK signaling and increases the expression of PGC-1alpha in human skeletal muscle. *J Appl Physiol* **106**, 929-934.

Giguere V (2008). Transcriptional control of energy homeostasis by the estrogen-related receptors. *Endocr Rev* **29**, 677-696.

Gladden LB, Stainsby WN, & MacIntosh BR (1982). Norepinephrine increases canine skeletal muscle VO2 during recovery. *Med Sci Sports Exerc* **14**, 471-476.

Gleyzer N, Vercauteren K, & Scarpulla RC (2005). Control of mitochondrial transcription specificity factors (TFB1M and TFB2M) by nuclear respiratory factors (NRF-1 and NRF-2) and PGC-1 family coactivators. *Mol Cell Biol* **25**, 1354-1366.

Goodpaster BH, Katsiaras A, & Kelley DE (2003). Enhanced fat oxidation through physical activity is associated with improvements in insulin sensitivity in obesity. *Diabetes* **52**, 2191-2197.

Goodpaster BH, Theriault R, Watkins SC, & Kelley DE (2000). Intramuscular lipid content is increased in obesity and decreased by weight loss. *Metabolism* **49**, 467-472.

Goodyear LJ, Chang PY, Sherwood DJ, Dufresne SD, & Moller DE (1996). Effects of exercise and insulin on mitogen-activated protein kinase signaling pathways in rat skeletal muscle. *Am J Physiol* **271**, E403-E408.

Goodyear LJ & Kahn BB (1998). Exercise, glucose transport, and insulin sensitivity. *Annu Rev Med* **49**, 235-261.

Graham T, Sjogaard G, Lollgen H, & Saltin B (1978). NAD in muscle of man at rest and during exercise. *Pflugers Arch* **376**, 35-39.

Green HJ, Helyar R, Ball-Burnett M, Kowalchuk N, Symon S, & Farrance B (1992). Metabolic adaptations to training precede changes in muscle mitochondrial capacity. *J Appl Physiol* **72**, 484-491.

Grundy SM (2006). Does a diagnosis of metabolic syndrome have value in clinical practice? *Am J Clin Nutr* **83**, 1248-1251.

Gupta P, Huq MD, Khan SA, Tsai NP, & Wei LN (2005). Regulation of co-repressive activity of and HDAC recruitment to RIP140 by site-specific phosphorylation. *Mol Cell Proteomics* **4**, 1776-1784.

Gurd BJ, Yoshida Y, Lally J, Holloway GP, & Bonen A (2009). The deacetylase enzyme SIRT1 is not associated with oxidative capacity in rat heart and skeletal muscle and its overexpression reduces mitochondrial biogenesis. *J Physiol* **587**, 1817-1828.

Gygi SP, Rochon Y, Franza BR, & Aebersold R (1999). Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol* **19**, 1720-1730.

Hancock CR, Han DH, Chen M, Terada S, Yasuda T, Wright DC, & Holloszy JO (2008). High-fat diets cause insulin resistance despite an increase in muscle mitochondria. *Proc Natl Acad Sci U S A* **105**, 7815-7820.

Handschin C, Rhee J, Lin J, Tarr PT, & Spiegelman BM (2003). An autoregulatory loop controls peroxisome proliferator-activated receptor gamma coactivator 1alpha expression in muscle. *Proc Natl Acad Sci U S A* **100**, 7111-7116.

Hardie DG (2007). Biochemistry. Balancing cellular energy. *Science* **315**, 1671-1672.

Hardie DG & Hawley SA (2001). AMP-activated protein kinase: the energy charge hypothesis revisited. *Bioessays* **23**, 1112-1119.

Hargreaves M (2000). Skeletal muscle metabolism during exercise in humans. *Clin Exp Pharmacol Physiol* **27**, 225-228.

Harris RC, Edwards RH, Hultman E, Nordesjo LO, Nylind B, & Sahlin K (1976). The time course of phosphorylcreatine resynthesis during recovery of the quadriceps muscle in man. *Pflugers Arch* **367**, 137-142.

Hawley JA (2004). Exercise as a therapeutic intervention for the prevention and treatment of insulin resistance. *Diabetes Metab Res Rev* **20**, 383-393.

Hawley JA & Holloszy JO (2009). Exercise: it's the real thing! *Nutr Rev* **67**, 172-178.

Hawley JA & Zierath JR (2004). Integration of metabolic and mitogenic signal transduction in skeletal muscle. *Exerc Sport Sci Rev* **32**, 4-8.

Hawley SA, Boudeau J, Reid JL, Mustard KJ, Udd L, Makela TP, Alessi DR, & Hardie DG (2003). Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. *J Biol* **2**, 28.

Hawley SA, Pan DA, Mustard KJ, Ross L, Bain J, Edelman AM, Frenguelli BG, & Hardie DG (2005). Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. *Cell Metab* **2**, 9-19.

He J, Watkins S, & Kelley DE (2001). Skeletal muscle lipid content and oxidative enzyme activity in relation to muscle fiber type in type 2 diabetes and obesity. *Diabetes* **50**, 817-823.

Henriksson J (1995). Muscle fuel selection: effect of exercise and training. *Proc Nutr Soc* **54**, 125-138.

Hermanson O, Glass CK, & Rosenfeld MG (2002). Nuclear receptor coregulators: multiple modes of modification. *Trends Endocrinol Metab* **13**, 55-60.

Hildebrandt AL, Pilegaard H, & Neufer PD (2003). Differential transcriptional activation of select metabolic genes in response to variations in exercise intensity and duration. *Am J Physiol Endocrinol Metab* **285**, E1021-E1027.

Hofmann S & Pette D (1994). Low-frequency stimulation of rat fast-twitch muscle enhances the expression of hexokinase II and both the translocation and expression of glucose transporter 4 (GLUT-4). *Eur J Biochem* **219**, 307-315.

Holloszy JO (2008). Regulation by exercise of skeletal muscle content of mitochondria and GLUT4. *J Physiol Pharmacol* **59 Suppl 7**, 5-18.

Holloszy JO & Coyle EF (1984). Adaptations of skeletal muscle to endurance exercise and their metabolic consequences. *J Appl Physiol* **56**, 831-838.

Holloszy JO & Narahara HT (1967). Enhanced permeability to sugar associated with muscle contraction. Studies of the role of Ca++. *J Gen Physiol* **50**, 551-562.

Holloway GP, Bezaire V, Heigenhauser GJ, Tandon NN, Glatz JF, Luiken JJ, Bonen A, & Spriet LL (2006). Mitochondrial long chain fatty acid oxidation, fatty acid translocase/CD36 content and carnitine palmitoyltransferase I activity in human skeletal muscle during aerobic exercise. *J Physiol* **571**, 201-210.

Holmes B & Dohm GL (2004). Regulation of GLUT4 gene expression during exercise. *Med Sci Sports Exerc* **36**, 1202-1206.

Holmes BF, Lang DB, Birnbaum MJ, Mu J, & Dohm GL (2004). AMP kinase is not required for the GLUT4 response to exercise and denervation in skeletal muscle. *Am J Physiol Endocrinol Metab* **287**, E739-E743.

Holmes BF, Sparling DP, Olson AL, Winder WW, & Dohm GL (2005). Regulation of muscle GLUT4 enhancer factor and myocyte enhancer factor 2 by AMP-activated protein kinase. *Am J Physiol Endocrinol Metab* **289**, E1071-E1076.

Hood DA, Irrcher I, Ljubicic V, & Joseph AM (2006). Coordination of metabolic plasticity in skeletal muscle. *J Exp Biol* **209**, 2265-2275.

Hood DA & Terjung RL (1990). Amino acid metabolism during exercise and following endurance training. *Sports Med* **9**, 23-35.

Hook SS & Means AR (2001). Ca(2+)/CaM-dependent kinases: from activation to function. *Annu Rev Pharmacol Toxicol* **41**, 471-505.

Hoppeler H & Fluck M (2002). Normal mammalian skeletal muscle and its phenotypic plasticity. *J Exp Biol* **205**, 2143-2152.

Hoppeler H, Howald H, Conley K, Lindstedt SL, Claassen H, Vock P, & Weibel ER (1985). Endurance training in humans: aerobic capacity and structure of skeletal muscle. *J Appl Physiol* **59**, 320-327.

Howald H, Hoppeler H, Claassen H, Mathieu O, & Straub R (1985). Influences of endurance training on the ultrastructural composition of the different muscle fiber types in humans. *Pflugers Arch* **403**, 369-376.

Howlett RA, Parolin ML, Dyck DJ, Hultman E, Jones NL, Heigenhauser GJ, & Spriet LL (1998). Regulation of skeletal muscle glycogen phosphorylase and PDH at varying exercise power outputs. *Am J Physiol* **275**, R418-R425.

Huang B, Wu P, Bowker-Kinley MM, & Harris RA (2002). Regulation of pyruvate dehydrogenase kinase expression by peroxisome proliferator-activated receptor-alpha ligands, glucocorticoids, and insulin. *Diabetes* **51**, 276-283.

Huang CF, Tong J, & Schmidt J (1992). Protein kinase C couples membrane excitation to acetylcholine receptor gene inactivation in chick skeletal muscle. *Neuron* **9**, 671-678.

Huang H, Iida KT, Sone H, & Ajisaka R (2007). The regulation of adiponectin receptors expression by acute exercise in mice. *Exp Clin Endocrinol Diabetes* **115**, 417-422.

Hudmon A & Schulman H (2002). Structure-function of the multifunctional Ca2+/calmodulin-dependent protein kinase II. *Biochem J* **364**, 593-611.

Hudson ER, Pan DA, James J, Lucocq JM, Hawley SA, Green KA, Baba O, Terashima T, & Hardie DG (2003). A novel domain in AMP-activated protein kinase causes glycogen storage bodies similar to those seen in hereditary cardiac arrhythmias. *Curr Biol* **13**, 861-866.

Hultman E (1973). Energy metabolism in human muscle. *J Physiol* **231**, 56P.

Hurley RL, Anderson KA, Franzone JM, Kemp BE, Means AR, & Witters LA (2005). The Ca2+/Calmodulin-dependent Protein Kinase Kinases Are AMP-activated Protein Kinase Kinases. *J Biol Chem* **280**, 29060-29066.

Huss JM, Torra IP, Staels B, Giguere V, & Kelly DP (2004). Estrogen-related receptor alpha directs peroxisome proliferator-activated receptor alpha signaling in the transcriptional control of energy metabolism in cardiac and skeletal muscle. *Mol Cell Biol* **24**, 9079-9091.

Ihlemann J, Ploug T, Hellsten Y, & Galbo H (2000). Effect of stimulation frequency on contraction-induced glucose transport in rat skeletal muscle. *Am J Physiol Endocrinol Metab* **279**, E862-E867.

Imamura H, Shibuya S, Uchida K, Teshima K, Masuda R, & Miyamoto N (2004). Effect of moderate exercise on excess post-exercise oxygen consumption and catecholamines in young women. *J Sports Med Phys Fitness* **44**, 23-29.

Ingjer F (1979). Effects of endurance training on muscle fibre ATP-ase activity, capillary supply and mitochondrial content in man. *J Physiol* **294**, 419-432.

Irrcher I, Adhihetty PJ, Joseph AM, Ljubicic V, & Hood DA (2003a). Regulation of mitochondrial biogenesis in muscle by endurance exercise. *Sports Med* **33**, 783-793.

Irrcher I, Adhihetty PJ, Sheehan T, Joseph AM, & Hood DA (2003b). PPARgamma coactivator-1alpha expression during thyroid hormone- and contractile activity-induced mitochondrial adaptations. *Am J Physiol Cell Physiol* **284**, C1669-C1677.

Irrcher I, Ljubicic V, Kirwan AF, & Hood DA (2008). AMP-activated protein kinase-regulated activation of the PGC-1alpha promoter in skeletal muscle cells. *PLoS ONE* **3**, e3614.

Jager S, Handschin C, St-Pierre J, & Spiegelman BM (2007). AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. *Proc Natl Acad Sci U S A* **104**, 12017-12022.

Jansson E, Esbjornsson M, Holm I, & Jacobs I (1990). Increase in the proportion of fast-twitch muscle fibres by sprint training in males. *Acta Physiol Scand* **140**, 359-363.

Jensen TE, Rose AJ, Jorgensen SB, Brandt N, Schjerling P, Wojtaszewski JF, & Richter EA (2007). Possible CaMKK-dependent regulation of AMPK phosphorylation and glucose uptake at the onset of mild tetanic skeletal muscle contraction. *Am J Physiol Endocrinol Metab* **292**, E1308-E1317.

Jepsen K & Rosenfeld MG (2002). Biological roles and mechanistic actions of co-repressor complexes. *J Cell Sci* **115**, 689-698.

Jessen N & Goodyear LJ (2005). Contraction signaling to glucose transport in skeletal muscle. *J Appl Physiol* **99**, 330-337.

Jeukendrup AE & Wallis GA (2005). Measurement of substrate oxidation during exercise by means of gas exchange measurements. *Int J Sports Med* **26 Suppl 1**, S28-S37.

Ji LL, Gomez-Cabrera MC, & Vina J (2006). Exercise and hormesis: activation of cellular antioxidant signaling pathway. *Ann N Y Acad Sci* **1067**, 425-435.

Jorgensen SB, Treebak JT, Viollet B, Schjerling P, Vaulont S, Wojtaszewski JF, & Richter EA (2007). Role of AMPKalpha2 in basal, training-, and AICAR-induced GLUT4, hexokinase II, and mitochondrial protein expression in mouse muscle. *Am J Physiol Endocrinol Metab* **292**, E331-E339.

Jorgensen SB, Viollet B, Andreelli F, Frosig C, Birk JB, Schjerling P, Vaulont S, Richter EA, & Wojtaszewski JF (2004). Knockout of the alpha2 but not alpha1 5'-AMP-activated protein kinase isoform abolishes 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranosidebut not contraction-induced glucose uptake in skeletal muscle. *J Biol Chem* **279**, 1070-1079.

Jorgensen SB, Wojtaszewski JF, Viollet B, Andreelli F, Birk JB, Hellsten Y, Schjerling P, Vaulont S, Neufer PD, Richter EA, & Pilegaard H (2005). Effects of alpha-AMPK knockout on exercise-induced gene activation in mouse skeletal muscle. *FASEB J* **19**, 1146-1148.

Kahn BB, Alquier T, Carling D, & Hardie DG (2005). AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab* **1**, 15-25.

Kamei Y, Miura S, Suzuki M, Kai Y, Mizukami J, Taniguchi T, Mochida K, Hata T, Matsuda J, Aburatani H, Nishino I, & Ezaki O (2004). Skeletal muscle FOXO1 (FKHR) transgenic mice have less skeletal muscle mass, down-regulated Type I (slow twitch/red muscle) fiber genes, and impaired glycemic control. *J Biol Chem* **279**, 41114-41123.

Kamei Y, Mizukami J, Miura S, Suzuki M, Takahashi N, Kawada T, Taniguchi T, & Ezaki O (2003). A forkhead transcription factor FKHR up-regulates lipoprotein lipase expression in skeletal muscle. *FEBS Lett* **536**, 232-236.

Kang C, O'Moore KM, Dickman JR, & Ji LL (2009). Exercise activation of muscle peroxisome proliferator-activated receptor-gamma coactivator-1alpha signaling is redox sensitive. *Free Radic Biol Med* **47**, 1394-1400.

Karlsson HK, Nilsson PA, Nilsson J, Chibalin AV, Zierath JR, & Blomstrand E (2004). Branched-chain amino acids increase p70S6k phosphorylation in human skeletal muscle after resistance exercise. *Am J Physiol Endocrinol Metab* **287**, E1-E7.

Katz A, Sahlin K, & Broberg S (1991). Regulation of glucose utilization in human skeletal muscle during moderate dynamic exercise. *Am J Physiol* **260**, E411-E415.

Kelley DE, Goodpaster B, Wing RR, & Simoneau JA (1999). Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. *Am J Physiol* **277**, E1130-E1141.

Kennedy JW, Hirshman MF, Gervino EV, Ocel JV, Forse RA, Hoenig SJ, Aronson D, Goodyear LJ, & Horton ES (1999). Acute exercise induces GLUT4 translocation in skeletal muscle of normal human subjects and subjects with type 2 diabetes. *Diabetes* **48**, 1192-1197.

Kiens B (2006). Skeletal muscle lipid metabolism in exercise and insulin resistance. *Physiol Rev* **86**, 205-243.

Kiens B & Lithell H (1989). Lipoprotein metabolism influenced by training-induced changes in human skeletal muscle. *J Clin Invest* **83**, 558-564.

Kiens B, Lithell H, Mikines KJ, & Richter EA (1989). Effects of insulin and exercise on muscle lipoprotein lipase activity in man and its relation to insulin action. *J Clin Invest* **84**, 1124-1129.

Kiens B & Richter EA (1998). Utilization of skeletal muscle triacylglycerol during postexercise recovery in humans. *Am J Physiol* **275**, E332-E337.

Kiens B, Roepstorff C, Glatz JF, Bonen A, Schjerling P, Knudsen J, & Nielsen JN (2004). Lipid-binding proteins and lipoprotein lipase activity in human skeletal muscle: influence of physical activity and gender. *J Appl Physiol* **97**, 1209-1218.

Kimber NE, Heigenhauser GJ, Spriet LL, & Dyck DJ (2003). Skeletal muscle fat and carbohydrate metabolism during recovery from glycogen-depleting exercise in humans. *J Physiol* **548**, 919-927.

Klein DK, Pilegaard H, Treebak JT, Jensen TE, Viollet B, Schjerling P, & Wojtaszewski JF (2007). Lack of AMPKalpha2 enhances pyruvate dehydrogenase activity during exercise. *Am J Physiol Endocrinol Metab* **293**, E1242-E1249.

Klein S, Coyle EF, & Wolfe RR (1994). Fat metabolism during low-intensity exercise in endurance-trained and untrained men. *Am J Physiol* **267**, E934-E940.

Knuttgen HG (1970). Oxygen debt after submaximal physical exercise. *J Appl Physiol* **29**, 651-657.

Knutti D, Kressler D, & Kralli A (2001). Regulation of the transcriptional coactivator PGC-1 via MAPK-sensitive interaction with a repressor. *Proc Natl Acad Sci U S A* **98**, 9713-9718.

Kramer DK, Al-Khalili L, Guigas B, Leng Y, Garcia-Roves PM, & Krook A (2007). Role of AMP kinase and PPARdelta in the regulation of lipid and glucose metabolism in human skeletal muscle. *J Biol Chem* **282**, 19313-19320.

Kramer HF & Goodyear LJ (2007). Exercise, MAPK, and NF-kappaB signaling in skeletal muscle. *J Appl Physiol* **103**, 388-395.

Kraniou GN, Cameron-Smith D, & Hargreaves M (2006). Acute exercise and GLUT4 expression in human skeletal muscle: influence of exercise intensity. *J Appl Physiol* **101**, 934-937.

Kraniou Y, Cameron-Smith D, Misso M, Collier G, & Hargreaves M (2000). Effects of exercise on GLUT-4 and glycogenin gene expression in human skeletal muscle. *J Appl Physiol* **88**, 794-796.

Krssak M, Petersen KF, Bergeron R, Price T, Laurent D, Rothman DL, Roden M, & Shulman GI (2000). Intramuscular glycogen and intramyocellular lipid utilization during prolonged exercise and recovery in man: a 13C and 1H nuclear magnetic resonance spectroscopy study. *J Clin Endocrinol Metab* **85**, 748-754.

Kumar V, Carlson JE, Ohgi KA, Edwards TA, Rose DW, Escalante CR, Rosenfeld MG, & Aggarwal AK (2002). Transcription corepressor CtBP is an NAD(+)-regulated dehydrogenase. *Mol Cell* **10**, 857-869.

Kuo CH, Browning KS, & Ivy JL (1999). Regulation of GLUT4 protein expression and glycogen storage after prolonged exercise. *Acta Physiol Scand* **165**, 193-201.

Kusuhara K, Madsen K, Jensen L, Hellsten Y, & Pilegaard H (2007). Calcium signalling in the regulation of PGC-1alpha, PDK4 and HKII mRNA expression. *Biol Chem* **388**, 481-488.

LaForgia J, Withers RT, Shipp NJ, & Gore CJ (1997). Comparison of energy expenditure elevations after submaximal and supramaximal running. *J Appl Physiol* **82**, 661-666.

Lagouge M, Argmann C, Gerhart-Hines Z, Meziane H, Lerin C, Daussin F, Messadeq N, Milne J, Lambert P, Elliott P, Geny B, Laakso M, Puigserver P, & Auwerx J (2006).

Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. *Cell* **127**, 1109-1122.

Larsson L & Forsberg A (1980). Morphological muscle characteristics in rowers. *Can J Appl Sport Sci* **5**, 239-244.

Larsson NG, Wang J, Wilhelmsson H, Oldfors A, Rustin P, Lewandoski M, Barsh GS, & Clayton DA (1998). Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nat Genet* **18**, 231-236.

Latchman DS (1992). Gene regulation. *BMJ* **304**, 1103-1105.

Lee WJ, Kim M, Park HS, Kim HS, Jeon MJ, Oh KS, Koh EH, Won JC, Kim MS, Oh GT, Yoon M, Lee KU, & Park JY (2006). AMPK activation increases fatty acid oxidation in skeletal muscle by activating PPARalpha and PGC-1. *Biochem Biophys Res Commun* **340**, 291-295.

Leick L, Wojtaszewski JF, Johansen ST, Kiilerich K, Comes G, Hellsten Y, Hidalgo J, & Pilegaard H (2008). PGC-1alpha is not mandatory for exercise- and training-induced adaptive gene responses in mouse skeletal muscle. *Am J Physiol Endocrinol Metab* **294**, E463-E474.

Leone TC, Lehman JJ, Finck BN, Schaeffer PJ, Wende AR, Boudina S, Courtois M, Wozniak DF, Sambandam N, Bernal-Mizrachi C, Chen Z, Holloszy JO, Medeiros DM, Schmidt RE, Saffitz JE, Abel ED, Semenkovich CF, & Kelly DP (2005). PGC-1alpha deficiency causes multi-system energy metabolic derangements: muscle dysfunction, abnormal weight control and hepatic steatosis. *PLoS Biol* **3**, e101.

Lerin C, Rodgers JT, Kalume DE, Kim SH, Pandey A, & Puigserver P (2006). GCN5 acetyltransferase complex controls glucose metabolism through transcriptional repression of PGC-1alpha. *Cell Metab* **3**, 429-438.

Li X, Monks B, Ge Q, & Birnbaum MJ (2007). Akt/PKB regulates hepatic metabolism by directly inhibiting PGC-1alpha transcription coactivator. *Nature* **447**, 1012-1016.

Li Z, Jiang Y, Ulevitch RJ, & Han J (1996). The primary structure of p38 gamma: a new member of p38 group of MAP kinases. *Biochem Biophys Res Commun* **228**, 334-340.

Lin J, Handschin C, & Spiegelman BM (2005). Metabolic control through the PGC-1 family of transcription coactivators. *Cell Metab* **1**, 361-370.

Lin J, Wu H, Tarr PT, Zhang CY, Wu Z, Boss O, Michael LF, Puigserver P, Isotani E, Olson EN, Lowell BB, Bassel-Duby R, & Spiegelman BM (2002). Transcriptional co-

activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. *Nature* **418**, 797-801.

Liu Y, Randall WR, & Schneider MF (2005). Activity-dependent and -independent nuclear fluxes of HDAC4 mediated by different kinases in adult skeletal muscle. *J Cell Biol* **168**, 887-897.

Long YC, Glund S, Garcia-Roves PM, & Zierath JR (2007). Calcineurin regulates skeletal muscle metabolism via coordinated changes in gene expression. *J Biol Chem* **282**, 1607-1614.

Long YC, Widegren U, & Zierath JR (2004). Exercise-induced mitogen-activated protein kinase signalling in skeletal muscle. *Proc Nutr Soc* **63**, 227-232.

Lu J, McKinsey TA, Nicol RL, & Olson EN (2000). Signal-dependent activation of the MEF2 transcription factor by dissociation from histone deacetylases. *Proc Natl Acad Sci U S A* **97**, 4070-4075.

Lund S, Pedersen O, Holman GD, Clark AE, Zierath JR, & Wallberg-Henriksson H (1997). GLUT4 translocation in human muscle strips. *Biochem Soc Trans* **25**, 466S.

Macfarlane WM (2000). Demystified.... Transcription. *Mol Pathol* **53**, 1-7.

MacLean PS & Winder WW (1995). Caffeine decreases malonyl-CoA in isolated perfused skeletal muscle of rats. *J Appl Physiol* **78**, 1496-1501.

Mahoney DJ, Parise G, Melov S, Safdar A, & Tarnopolsky MA (2005). Analysis of global mRNA expression in human skeletal muscle during recovery from endurance exercise. *FASEB J* **19**, 1498-1500.

Mahoney DJ & Tarnopolsky MA (2005). Understanding skeletal muscle adaptation to exercise training in humans: contributions from microarray studies. *Phys Med Rehabil Clin N Am* **16**, 859-73.

Martineau LC & Gardiner PF (2001). Insight into skeletal muscle mechanotransduction: MAPK activation is quantitatively related to tension. *J Appl Physiol* **91**, 693-702.

Mathai AS, Bonen A, Benton CR, Robinson DL, & Graham TE (2008). Rapid exercise-induced changes in PGC-1{alpha} mRNA and protein in human skeletal muscle. *J Appl Physiol* In Press.

Maughan RJ & Gleeson M (2004). *The Biochemical Basis of Sports Performance* Oxford University Press.

McArdle WD, Katch FI, & Katch VI (2006). *Exercise Physiology: Energy, Nutrition, and Human Performance*, 6th Edition ed. Lippincott, Williams and Williams.

McGee SL & Hargreaves M (2008). AMPK and transcriptional regulation. *Front Biosci* **13**, 3022-3033.

McGee SL & Hargreaves M (2006). Exercise and skeletal muscle glucose transporter 4 expression: molecular mechanisms. *Clin Exp Pharmacol Physiol* **33**, 395-399.

McGee SL, Sparling D, Olson AL, & Hargreaves M (2006). Exercise increases MEF2 and GEF DNA-binding activity in human skeletal muscle. *FASEB J* **20**, 348-349.

McKenna NJ & O'Malley BW (2002). Combinatorial control of gene expression by nuclear receptors and coregulators. *Cell* **108**, 465-474.

McKinsey TA, Zhang CL, & Olson EN (2001). Control of muscle development by dueling HATs and HDACs. *Curr Opin Genet Dev* **11**, 497-504.

Medbo JI, Mohn AC, Tabata I, Bahr R, Vaage O, & Sejersted OM (1988). Anaerobic capacity determined by maximal accumulated O2 deficit. *J Appl Physiol* **64**, 50-60.

Merrill GF, Kurth EJ, Hardie DG, & Winder WW (1997). AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. *Am J Physiol* **273**, E1107-E1112.

Michael LF, Wu Z, Cheatham RB, Puigserver P, Adelmant G, Lehman JJ, Kelly DP, & Spiegelman BM (2001). Restoration of insulin-sensitive glucose transporter (GLUT4) gene expression in muscle cells by the transcriptional coactivator PGC-1. *Proc Natl Acad Sci U S A* **98**, 3820-3825.

Monsalve M, Wu Z, Adelmant G, Puigserver P, Fan M, & Spiegelman BM (2000). Direct coupling of transcription and mRNA processing through the thermogenic coactivator PGC-1. *Mol Cell* **6**, 307-316.

Mootha VK, Handschin C, Arlow D, Xie X, St PJ, Sihag S, Yang W, Altshuler D, Puigserver P, Patterson N, Willy PJ, Schulman IG, Heyman RA, Lander ES, & Spiegelman BM (2004). Erralpha and Gabpa/b specify PGC-1alpha-dependent oxidative phosphorylation gene expression that is altered in diabetic muscle. *Proc Natl Acad Sci U S A* **101**, 6570-6575.

Mora S & Pessin JE (2000). The MEF2A isoform is required for striated muscle-specific expression of the insulin-responsive GLUT4 glucose transporter. *J Biol Chem* **275**, 16323-16328.

Mostaqul H, Gupta P, & Wei LN (2008). Post-translational modifications of nuclear co-repressor RIP140: a therapeutic target for metabolic diseases. *Curr Med Chem* **15**, 386-392.

Mourtzakis M, Saltin B, Graham T, & Pilegaard H (2006). Carbohydrate metabolism during prolonged exercise and recovery: interactions between pyruvate dehydrogenase, fatty acids, and amino acids. *J Appl Physiol* **100**, 1822-1830.

Mukwevho E, Kohn TA, Lang D, Nyatia E, Smith J, & Ojuka EO (2008). Caffeine induces hyperacetylation of histones at the MEF2 site on the Glut4 promoter and increases MEF2A binding to the site via a CaMK-dependent mechanism. *Am J Physiol Endocrinol Metab* **294**, E582-E588.

Mulla NA, Simonsen L, & Bulow J (2000). Post-exercise adipose tissue and skeletal muscle lipid metabolism in humans: the effects of exercise intensity. *J Physiol* **524 Pt 3**, 919-928.

Murgia M, Elbenhardt JT, Cusinato M, Garcia M, Richter EA, & Schiaffino S (2009). Multiple signalling pathways redundantly control GLUT4 gene transcription in skeletal muscle. *J Physiol*.

Musi N, Fujii N, Hirshman MF, Ekberg I, Froberg S, Ljungqvist O, Thorell A, & Goodyear LJ (2001). AMP-activated protein kinase (AMPK) is activated in muscle of subjects with type 2 diabetes during exercise. *Diabetes* **50**, 921-927.

Nader GA & Esser KA (2001). Intracellular signaling specificity in skeletal muscle in response to different modes of exercise. *J Appl Physiol* **90**, 1936-1942.

Nemoto S, Fergusson MM, & Finkel T (2005). SIRT1 functionally interacts with the metabolic regulator and transcriptional coactivator PGC-1{alpha}. *J Biol Chem* **280**, 16456-16460.

Neufer PD & Dohm GL (1993). Exercise induces a transient increase in transcription of the GLUT-4 gene in skeletal muscle. *Am J Physiol* **265**, C1597-C1603.

Newton AC (2001). Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. *Chem Rev* **101**, 2353-2364.

Newton AC (2003). Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm. *Biochem J* **370**, 361-371.

Newton AC (1995). Protein kinase C: structure, function, and regulation. *J Biol Chem* **270**, 28495-28498.

Nielsen JN, Frosig C, Sajan MP, Miura A, Standaert ML, Graham DA, Wojtaszewski JF, Farese RV, & Richter EA (2003). Increased atypical PKC activity in endurance-trained human skeletal muscle. *Biochem Biophys Res Commun* **312**, 1147-1153.

Norrbom J, Sundberg CJ, Ameln H, Kraus WE, Jansson E, & Gustafsson T (2004). PGC-1alpha mRNA expression is influenced by metabolic perturbation in exercising human skeletal muscle. *J Appl Physiol* **96**, 189-194.

Nystrom GJ & Lang CH (2008). Sepsis and AMPK Activation by AICAR Differentially Regulate FoxO-1, -3 and -4 mRNA in Striated Muscle. *Int J Clin Exp Med* **1**, 50-63.

Odland LM, Heigenhauser GJ, & Spriet LL (2000). Effects of high fat provision on muscle PDH activation and malonyl-CoA content in moderate exercise. *J Appl Physiol* **89**, 2352-2358.

Odland LM, Howlett RA, Heigenhauser GJ, Hultman E, & Spriet LL (1998). Skeletal muscle malonyl-CoA content at the onset of exercise at varying power outputs in humans. *Am J Physiol* **274**, E1080-E1085.

Ohira M, Miyashita Y, Murano T, Watanabe F, & Shirai K (2009). Metformin promotes induction of lipoprotein lipase in skeletal muscle through activation of adenosine monophosphate-activated protein kinase. *Metabolism*.

Ojuka EO (2004). Role of calcium and AMP kinase in the regulation of mitochondrial biogenesis and GLUT4 levels in muscle. *Proc Nutr Soc* **63**, 275-278.

Ojuka EO, Jones TE, Han DH, Chen M, & Holloszy JO (2003). Raising Ca2+ in L6 myotubes mimics effects of exercise on mitochondrial biogenesis in muscle. *FASEB J* **17**, 675-681.

Ojuka EO, Jones TE, Nolte LA, Chen M, Wamhoff BR, Sturek M, & Holloszy JO (2002). Regulation of GLUT4 biogenesis in muscle: evidence for involvement of AMPK and Ca(2+). *Am J Physiol Endocrinol Metab* **282**, E1008-E1013.

Park SH, Gammon SR, Knippers JD, Paulsen SR, Rubink DS, & Winder WW (2002). Phosphorylation-activity relationships of AMPK and acetyl-CoA carboxylase in muscle. *J Appl Physiol* **92**, 2475-2482.

Pedersen BK (2006). The anti-inflammatory effect of exercise: its role in diabetes and cardiovascular disease control. *Essays Biochem* **42**, 105-117.

Perrini S, Henriksson J, Zierath JR, & Widegren U (2004). Exercise-induced protein kinase C isoform-specific activation in human skeletal muscle. *Diabetes* **53**, 21-24.

Peters SJ, Harris RA, Wu P, Pehleman TL, Heigenhauser GJ, & Spriet LL (2001). Human skeletal muscle PDH kinase activity and isoform expression during a 3-day high-fat/low-carbohydrate diet. *Am J Physiol Endocrinol Metab* **281**, E1151-E1158.

Phelain JF, Reinke E, Harris MA, & Melby CL (1997). Postexercise energy expenditure and substrate oxidation in young women resulting from exercise bouts of different intensity. *J Am Coll Nutr* **16**, 140-146.

Pilegaard H, Keller C, Steensberg A, Helge JW, Pedersen BK, Saltin B, & Neufer PD (2002). Influence of pre-exercise muscle glycogen content on exercise-induced transcriptional regulation of metabolic genes. *J Physiol* **541**, 261-271.

Pilegaard H & Neufer PD (2004). Transcriptional regulation of pyruvate dehydrogenase kinase 4 in skeletal muscle during and after exercise. *Proc Nutr Soc* **63**, 221-226.

Pilegaard H, Ordway GA, Saltin B, & Neufer PD (2000). Transcriptional regulation of gene expression in human skeletal muscle during recovery from exercise. *Am J Physiol Endocrinol Metab* **279**, E806-E814.

Pilegaard H, Osada T, Andersen LT, Helge JW, Saltin B, & Neufer PD (2005). Substrate availability and transcriptional regulation of metabolic genes in human skeletal muscle during recovery from exercise. *Metabolism* **54**, 1048-1055.

Pilegaard H, Saltin B, & Neufer PD (2003a). Effect of short-term fasting and refeeding on transcriptional regulation of metabolic genes in human skeletal muscle. *Diabetes* **52**, 657-662.

Pilegaard H, Saltin B, & Neufer PD (2003b). Exercise induces transient transcriptional activation of the PGC-1alpha gene in human skeletal muscle. *J Physiol* **546**, 851-858.

Polekhina G, Gupta A, Michell BJ, van DB, Murthy S, Feil SC, Jennings IG, Campbell DJ, Witters LA, Parker MW, Kemp BE, & Stapleton D (2003). AMPK beta subunit targets metabolic stress sensing to glycogen. *Curr Biol* **13**, 867-871.

Powelka AM, Seth A, Virbasius JV, Kiskinis E, Nicoloro SM, Guilherme A, Tang X, Straubhaar J, Cherniack AD, Parker MG, & Czech MP (2006). Suppression of oxidative metabolism and mitochondrial biogenesis by the transcriptional corepressor RIP140 in mouse adipocytes. *J Clin Invest* **116**, 125-136.

Puigserver P, Adelmant G, Wu Z, Fan M, Xu J, O'Malley B, & Spiegelman BM (1999). Activation of PPARgamma coactivator-1 through transcription factor docking. *Science* **286**, 1368-1371.

Puigserver P, Rhee J, Donovan J, Walkey CJ, Yoon JC, Oriente F, Kitamura Y, Altomonte J, Dong H, Accili D, & Spiegelman BM (2003). Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction. *Nature* **423**, 550-555.

Puigserver P, Rhee J, Lin J, Wu Z, Yoon JC, Zhang CY, Krauss S, Mootha VK, Lowell BB, & Spiegelman BM (2001). Cytokine stimulation of energy expenditure through p38 MAP kinase activation of PPARgamma coactivator-1. *Mol Cell* **8**, 971-982.

Puigserver P, Wu Z, Park CW, Graves R, Wright M, & Spiegelman BM (1998). A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* **92**, 829-839.

Raney MA & Turcotte LP (2007). Evidence for the regulation of contraction-induced fatty acid oxidation via extracellular signal-regulated kinase 1/2 activation independent of changes in fatty acid uptake. *Metabolism* **56**, 1192-1200.

Raney MA & Turcotte LP (2008). Evidence for the involvement of CaMKII and AMPK in Ca2+-dependent signaling pathways regulating FA uptake and oxidation in contracting rodent muscle. *J Appl Physiol* **104**, 1366-1373.

Raney MA, Yee AJ, Todd MK, & Turcotte LP (2005). AMPK activation is not critical in the regulation of muscle FA uptake and oxidation during low-intensity muscle contraction. *Am J Physiol Endocrinol Metab* **288**, E592-E598.

Rantanen A, Jansson M, Oldfors A, & Larsson NG (2001). Downregulation of Tfam and mtDNA copy number during mammalian spermatogenesis. *Mamm Genome* **12**, 787-792.

Rasmussen BB & Winder WW (1997). Effect of exercise intensity on skeletal muscle malonyl-CoA and acetyl-CoA carboxylase. *J Appl Physiol* **83**, 1104-1109.

Rasmussen BB & Wolfe RR (1999). Regulation of fatty acid oxidation in skeletal muscle. *Annu Rev Nutr* **19**, 463-484.

Ren B & Dynlacht BD (2004). Use of chromatin immunoprecipitation assays in genome-wide location analysis of mammalian transcription factors. *Methods Enzymol* **376**, 304-315.

Richter EA, Cleland PJ, Rattigan S, & Clark MG (1987). Contraction-associated translocation of protein kinase C in rat skeletal muscle. *FEBS Lett* **217**, 232-236.

Richter EA & Ruderman NB (2009). AMPK and the biochemistry of exercise: implications for human health and disease. *Biochem J* **418**, 261-275.

Rodgers JT, Lerin C, Gerhart-Hines Z, & Puigserver P (2008). Metabolic adaptations through the PGC-1 alpha and SIRT1 pathways. *FEBS Lett* **582**, 46-53.

Rodgers JT, Lerin C, Haas W, Gygi SP, Spiegelman BM, & Puigserver P (2005). Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. *Nature* **434**, 113-118.

Roepstorff C, Helge JW, Vistisen B, & Kiens B (2004a). Studies of plasma membrane fatty acid-binding protein and other lipid-binding proteins in human skeletal muscle. *Proc Nutr Soc* **63**, 239-244.

Roepstorff C, Vistisen B, Donsmark M, Nielsen JN, Galbo H, Green KA, Hardie DG, Wojtaszewski JF, Richter EA, & Kiens B (2004b). Regulation of hormone-sensitive lipase activity and Ser563 and Ser565 phosphorylation in human skeletal muscle during exercise. *J Physiol* **560**, 551-562.

Romijn JA, Coyle EF, Sidossis LS, Gastaldelli A, Horowitz JF, Endert E, & Wolfe RR (1993). Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. *Am J Physiol* **265**, E380-E391.

Rose AJ, Alsted TJ, Kobbero JB, & Richter EA (2007). Regulation and function of Ca2+-calmodulin-dependent protein kinase II of fast-twitch rat skeletal muscle. *J Physiol* **580**, 993-1005.

Rose AJ & Hargreaves M (2003). Exercise increases Ca2+-calmodulin-dependent protein kinase II activity in human skeletal muscle. *J Physiol* **553**, 303-309.

Rose AJ, Kiens B, & Richter EA (2006). Ca2+-calmodulin-dependent protein kinase expression and signalling in skeletal muscle during exercise. *J Physiol* **574**, 889-903.

Rose AJ & Richter EA (2005). Skeletal Muscle Glucose Uptake During Exercise: How is it Regulated? *Physiology (Bethesda)* **20**, 260-270.

Rowan BG, Weigel NL, & O'Malley BW (2000). Phosphorylation of steroid receptor coactivator-1. Identification of the phosphorylation sites and phosphorylation through the mitogen-activated protein kinase pathway. *J Biol Chem* **275**, 4475-4483.

Rubink DS & Winder WW (2005). Effect of phosphorylation by AMP-activated protein kinase on palmitoyl-CoA inhibition of skeletal muscle acetyl-CoA carboxylase. *J Appl Physiol* **98**, 1221-1227.

Russ DW & Lovering RM (2006). Influence of activation frequency on cellular signalling pathways during fatiguing contractions in rat skeletal muscle. *Exp Physiol* **91**, 957-966.

Russell AP, Hesselink MK, Lo SK, & Schrauwen P (2005). Regulation of metabolic transcriptional co-activators and transcription factors with acute exercise. *FASEB J* **19**, 986-988.

Ruthenburg AJ, Li H, Patel DJ, & Allis CD (2007). Multivalent engagement of chromatin modifications by linked binding modules. *Nat Rev Mol Cell Biol* **8**, 983-994.

Ryder JW, Bassel-Duby R, Olson EN, & Zierath JR (2003). Skeletal muscle reprogramming by activation of calcineurin improves insulin action on metabolic pathways. *J Biol Chem* **278**, 44298-44304.

Ryder JW, Chibalin AV, & Zierath JR (2001). Intracellular mechanisms underlying increases in glucose uptake in response to insulin or exercise in skeletal muscle. *Acta Physiol Scand* **171**, 249-257.

Sahlin K, Katz A, & Henriksson J (1987). Redox state and lactate accumulation in human skeletal muscle during dynamic exercise. *Biochem J* **245**, 551-556.

Sale DG (1987). Influence of exercise and training on motor unit activation. *Exerc Sport Sci Rev* **15**, 95-151.

Scarpulla RC (1997). Nuclear control of respiratory chain expression in mammalian cells. *J Bioenerg Biomembr* **29**, 109-119.

Schaeffer PJ, Wende AR, Magee CJ, Neilson JR, Leone TC, Chen F, & Kelly DP (2004). Calcineurin and calcium/calmodulin-dependent protein kinase activate distinct metabolic gene regulatory programs in cardiac muscle. *J Biol Chem* **279**, 39593-39603.

Schreiber SN, Emter R, Hock MB, Knutti D, Cardenas J, Podvinec M, Oakeley EJ, & Kralli A (2004). The estrogen-related receptor alpha (ERRalpha) functions in PPARgamma coactivator 1alpha (PGC-1alpha)-induced mitochondrial biogenesis. *Proc Natl Acad Sci U S A* **101**, 6472-6477.

Schreiber SN, Knutti D, Brogli K, Uhlmann T, & Kralli A (2003). The transcriptional coactivator PGC-1 regulates the expression and activity of the orphan nuclear receptor estrogen-related receptor alpha (ERRalpha). *J Biol Chem* **278**, 9013-9018.

Seip RL, Mair K, Cole TG, & Semenkovich CF (1997). Induction of human skeletal muscle lipoprotein lipase gene expression by short-term exercise is transient. *Am J Physiol* **272**, E255-E261.

Selbert MA, Anderson KA, Huang QH, Goldstein EG, Means AR, & Edelman AM (1995). Phosphorylation and activation of Ca(2+)-calmodulin-dependent protein kinase

IV by Ca(2+)-calmodulin-dependent protein kinase Ia kinase. Phosphorylation of threonine 196 is essential for activation. *J Biol Chem* **270**, 17616-17621.

Shahbazian MD & Grunstein M (2007). Functions of site-specific histone acetylation and deacetylation. *Annu Rev Biochem* **76**, 75-100.

Shearer J, Fueger PT, Rottman JN, Bracy DP, Martin PH, & Wasserman DH (2004). AMPK stimulation increases LCFA but not glucose clearance in cardiac muscle in vivo. *Am J Physiol Endocrinol Metab* **287**, E871-E877.

Sheng M, Thompson MA, & Greenberg ME (1991). CREB: a Ca(2+)-regulated transcription factor phosphorylated by calmodulin-dependent kinases. *Science* **252**, 1427-1430.

Sidossis LS, Gastaldelli A, Klein S, & Wolfe RR (1997). Regulation of plasma fatty acid oxidation during low- and high-intensity exercise. *Am J Physiol* **272**, E1065-E1070.

Simoneau JA & Kelley DE (1997). Altered glycolytic and oxidative capacities of skeletal muscle contribute to insulin resistance in NIDDM. *J Appl Physiol* **83**, 166-171.

Simoneau JA, Lortie G, Boulay MR, Marcotte M, Thibault MC, & Bouchard C (1985). Human skeletal muscle fiber type alteration with high-intensity intermittent training. *Eur J Appl Physiol Occup Physiol* **54**, 250-253.

Smith JA, Collins M, Grobler LA, Magee CJ, & Ojuka EO (2007). Exercise and CaMK activation both increase the binding of MEF2A to the Glut4 promoter in skeletal muscle in vivo. *Am J Physiol Endocrinol Metab* **292**, E413-E420.

Smith JA, Kohn TA, Chetty AK, & Ojuka EO (2008). CaMK activation during exercise is required for histone hyperacetylation and MEF2A binding at the MEF2 site on the Glut4 gene. *Am J Physiol Endocrinol Metab* **295**, E698-E704.

Soderling TR, Chang B, & Brickey D (2001). Cellular signaling through multifunctional Ca2+/calmodulin-dependent protein kinase II. *J Biol Chem* **276**, 3719-3722.

Somwar R, Perreault M, Kapur S, Taha C, Sweeney G, Ramlal T, Kim DY, Keen J, Cote CH, Klip A, & Marette A (2000). Activation of p38 mitogen-activated protein kinase alpha and beta by insulin and contraction in rat skeletal muscle: potential role in the stimulation of glucose transport. *Diabetes* **49**, 1794-1800.

Spangenburg EE, Brown DA, Johnson MS, & Moore RL (2009). Alterations in peroxisome proliferator-activated receptor mRNA expression in skeletal muscle after acute and repeated bouts of exercise. *Mol Cell Biochem.*

Spiegelman BM & Heinrich R (2004). Biological control through regulated transcriptional coactivators. *Cell* **119**, 157-167.

Sriwijitkamol A, Coletta DK, Wajcberg E, Balbontin GB, Reyna SM, Barrientes J, Eagan PA, Jenkinson CP, Cersosimo E, DeFronzo RA, Sakamoto K, & Musi N (2007). Effect of acute exercise on AMPK signaling in skeletal muscle of subjects with type 2 diabetes: a time-course and dose-response study. *Diabetes* **56**, 836-848.

Stanley WC & Connett RJ (1991). Regulation of muscle carbohydrate metabolism during exercise. *FASEB J* **5**, 2155-2159.

Stauber WT, Clarkson PM, Fritz VK, & Evans WJ (1990). Extracellular matrix disruption and pain after eccentric muscle action. *J Appl Physiol* **69**, 868-874.

Steffensen CH, Roepstorff C, Madsen M, & Kiens B (2002). Myocellular triacylglycerol breakdown in females but not in males during exercise. *Am J Physiol Endocrinol Metab* **282**, E634-E642.

Steinberg GR, Michell BJ, van Denderen BJ, Watt MJ, Carey AL, Fam BC, Andrikopoulos S, Proietto J, Gorgun CZ, Carling D, Hotamisligil GS, Febbraio MA, Kay TW, & Kemp BE (2006). Tumor necrosis factor alpha-induced skeletal muscle insulin resistance involves suppression of AMP-kinase signaling. *Cell Metab* **4**, 465-474.

Stephens TJ, Chen ZP, Canny BJ, Michell BJ, Kemp BE, & McConell GK (2002). Progressive increase in human skeletal muscle AMPKalpha2 activity and ACC phosphorylation during exercise. *Am J Physiol Endocrinol Metab* **282**, E688-E694.

Stoppani J, Hildebrandt AL, Sakamoto K, Cameron-Smith D, Goodyear LJ, & Neufer PD (2002). AMP-activated protein kinase activates transcription of the UCP3 and HKII genes in rat skeletal muscle. *Am J Physiol Endocrinol Metab* **283**, E1239-E1248.

Sullivan JE, Brocklehurst KJ, Marley AE, Carey F, Carling D, & Beri RK (1994). Inhibition of lipolysis and lipogenesis in isolated rat adipocytes with AICAR, a cell-permeable activator of AMP-activated protein kinase. *FEBS Lett* **353**, 33-36.

Sun P, Enslen H, Myung PS, & Maurer RA (1994). Differential activation of CREB by Ca2+/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity. *Genes Dev* **8**, 2527-2539.

Suwa M, Nakano H, & Kumagai S (2003). Effects of chronic AICAR treatment on fiber composition, enzyme activity, UCP3, and PGC-1 in rat muscles. *J Appl Physiol* **95**, 960-968.

Suwa M, Nakano H, Radak Z, & Kumagai S (2008). Endurance exercise increases the SIRT1 and peroxisome proliferator-activated receptor gamma coactivator-1alpha protein expressions in rat skeletal muscle. *Metabolism* **57**, 986-998.

Taylor EB, Lamb JD, Hurst RW, Chesser DG, Ellingson WJ, Greenwood LJ, Porter BB, Herway ST, & Winder WW (2005). Endurance training increases skeletal muscle LKB1 and PGC-1alpha protein abundance: effects of time and intensity. *Am J Physiol Endocrinol Metab* **289**, E960-E968.

Terada S, Goto M, Kato M, Kawanaka K, Shimokawa T, & Tabata I (2002). Effects of low-intensity prolonged exercise on PGC-1 mRNA expression in rat epitrochlearis muscle. *Biochem Biophys Res Commun* **296**, 350-354.

Terada S, Kawanaka K, Goto M, Shimokawa T, & Tabata I (2005). Effects of high-intensity intermittent swimming on PGC-1alpha protein expression in rat skeletal muscle. *Acta Physiol Scand* **184**, 59-65.

Terada S & Tabata I (2004). Effects of acute bouts of running and swimming exercise on PGC-1alpha protein expression in rat epitrochlearis and soleus muscle. *Am J Physiol Endocrinol Metab* **286**, E208-E216.

Teyssier C, Ma H, Emter R, Kralli A, & Stallcup MR (2005). Activation of nuclear receptor coactivator PGC-1alpha by arginine methylation. *Genes Dev* **19**, 1466-1473.

Thai MV, Guruswamy S, Cao KT, Pessin JE, & Olson AL (1998). Myocyte enhancer factor 2 (MEF2)-binding site is required for GLUT4 gene expression in transgenic mice. Regulation of MEF2 DNA binding activity in insulin-deficient diabetes. *J Biol Chem* **273**, 14285-14292.

Thomson DM, Herway ST, Fillmore N, Kim H, Brown JD, Barrow JR, & Winder WW (2008). AMP-activated protein kinase phosphorylates transcription factors of the CREB family. *J Appl Physiol* **104**, 429-438.

Toyoda T, Tanaka S, Ebihara K, Masuzaki H, Hosoda K, Sato K, Fushiki T, Nakao K, & Hayashi T (2006). Low-intensity contraction activates the {alpha}1-isoform of 5'-AMP-activated protein kinase in rat skeletal muscle. *Am J Physiol Endocrinol Metab* **290**, E583-E590.

Treebak JT, Birk JB, Rose AJ, Kiens B, Richter EA, & Wojtaszewski JF (2007). AS160 phosphorylation is associated with activation of alpha2beta2gamma1- but not alpha2beta2gamma3-AMPK trimeric complex in skeletal muscle during exercise in humans. *Am J Physiol Endocrinol Metab* **292**, E715-E722.

Trost S, Wilcox A, & Gillis D (1997). The effect of substrate utilization, manipulated by nicotinic acid, on excess postexercise oxygen consumption. *Int J Sports Med* **18**, 83-88.

Tsintzas K, Jewell K, Kamran M, Laithwaite D, Boonsong T, Littlewood J, Macdonald I, & Bennett A (2006). Differential regulation of metabolic genes in skeletal muscle during starvation and refeeding in humans. *J Physiol* **575**, 291-303.

Tullson PC & Terjung RL (1991). Adenine nucleotide metabolism in contracting skeletal muscle. *Exerc Sport Sci Rev* **19**, 507-537.

Turcotte LP, Raney MA, & Todd MK (2005). ERK1/2 inhibition prevents contraction-induced increase in plasma membrane FAT/CD36 content and FA uptake in rodent muscle. *Acta Physiol Scand* **184**, 131-139.

van Loon LJ, Greenhaff PL, Constantin-Teodosiu D, Saris WH, & Wagenmakers AJ (2001). The effects of increasing exercise intensity on muscle fuel utilisation in humans. *J Physiol* **536**, 295-304.

van Loon LJ, Koopman R, Stegen JH, Wagenmakers AJ, Keizer HA, & Saris WH (2003). Intramyocellular lipids form an important substrate source during moderate intensity exercise in endurance-trained males in a fasted state. *J Physiol* **553**, 611-625.

Vavvas D, Apazidis A, Saha AK, Gamble J, Patel A, Kemp BE, Witters LA, & Ruderman NB (1997). Contraction-induced changes in acetyl-CoA carboxylase and 5'-AMP-activated kinase in skeletal muscle. *J Biol Chem* **272**, 13255-13261.

Vega RB, Harrison BC, Meadows E, Roberts CR, Papst PJ, Olson EN, & McKinsey TA (2004). Protein kinases C and D mediate agonist-dependent cardiac hypertrophy through nuclear export of histone deacetylase 5. *Mol Cell Biol* **24**, 8374-8385.

Vega RB, Huss JM, & Kelly DP (2000). The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor alpha in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. *Mol Cell Biol* **20**, 1868-1876.

Virbasius JV & Scarpulla RC (1994). Activation of the human mitochondrial transcription factor A gene by nuclear respiratory factors: a potential regulatory link between nuclear and mitochondrial gene expression in organelle biogenesis. *Proc Natl Acad Sci U S A* **91**, 1309-1313.

Vissing K, Andersen JL, & Schjerling P (2005). Are exercise-induced genes induced by exercise? *FASEB J* **19**, 94-96.

Vo N, Fjeld C, & Goodman RH (2001). Acetylation of nuclear hormone receptor-interacting protein RIP140 regulates binding of the transcriptional corepressor CtBP. *Mol Cell Biol* **21**, 6181-6188.

Vollestad NK & Blom PC (1985). Effect of varying exercise intensity on glycogen depletion in human muscle fibres. *Acta Physiol Scand* **125**, 395-405.

Wadley GD, Lee-Young RS, Canny BJ, Wasuntarawat C, Chen ZP, Hargreaves M, Kemp BE, & McConell GK (2006). Effect of exercise intensity and hypoxia on skeletal muscle AMPK signaling and substrate metabolism in humans. *Am J Physiol Endocrinol Metab* **290**, E694-E702.

Wang YX, Lee CH, Tiep S, Yu RT, Ham J, Kang H, & Evans RM (2003). Peroxisome-proliferator-activated receptor delta activates fat metabolism to prevent obesity. *Cell* **113**, 159-170.

Watt MJ, Heigenhauser GJ, Dyck DJ, & Spriet LL (2002). Intramuscular triacylglycerol, glycogen and acetyl group metabolism during 4 h of moderate exercise in man. *J Physiol* **541**, 969-978.

Watt MJ, Heigenhauser GJ, & Spriet LL (2003a). Effects of dynamic exercise intensity on the activation of hormone-sensitive lipase in human skeletal muscle. *J Physiol* **547**, 301-308.

Watt MJ & Spriet LL (2004). Regulation and role of hormone-sensitive lipase activity in human skeletal muscle. *Proc Nutr Soc* **63**, 315-322.

Watt MJ, Steinberg GR, Heigenhauser GJ, Spriet LL, & Dyck DJ (2003b). Hormone-sensitive lipase activity and triacylglycerol hydrolysis are decreased in rat soleus muscle by cyclopiazonic acid. *Am J Physiol Endocrinol Metab* **285**, E412-E419.

Watt MJ, Stellingwerff T, Heigenhauser GJ, & Spriet LL (2003c). Effects of plasma adrenaline on hormone-sensitive lipase at rest and during moderate exercise in human skeletal muscle. *J Physiol* **550**, 325-332.

Wende AR, Huss JM, Schaeffer PJ, Giguere V, & Kelly DP (2005). PGC-1alpha coactivates PDK4 gene expression via the orphan nuclear receptor ERRalpha: a mechanism for transcriptional control of muscle glucose metabolism. *Mol Cell Biol* **25**, 10684-10694.

Wende AR, Schaeffer PJ, Parker GJ, Zechner C, Han DH, Chen MM, Hancock CR, Lehman JJ, Huss JM, McClain DA, Holloszy JO, & Kelly DP (2007). A role for the transcriptional coactivator PGC-1{alpha} in muscle refueling. *J Biol Chem* **282**, 36642-36651.

Wendorf M & Goldfine ID (1991). Archaeology of NIDDM. Excavation of the "thrifty" genotype. *Diabetes* **40**, 161-165.

Wheeler DG, Barrett CF, Groth RD, Safa P, & Tsien RW (2008). CaMKII locally encodes L-type channel activity to signal to nuclear CREB in excitation-transcription coupling. *J Cell Biol* **183**, 849-863.

Widegren U, Jiang XJ, Krook A, Chibalin AV, Bjornholm M, Tally M, Roth RA, Henriksson J, Wallberg-Henriksson H, & Zierath JR (1998). Divergent effects of exercise on metabolic and mitogenic signaling pathways in human skeletal muscle. *FASEB J* **12**, 1379-1389.

Widegren U, Wretman C, Lionikas A, Hedin G, & Henriksson J (2000). Influence of exercise intensity on ERK/MAP kinase signalling in human skeletal muscle. *Pflugers Arch* **441**, 317-322.

Winder WW & Hardie DG (1996). Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. *Am J Physiol* **270**, E299-E304.

Witczak CA, Hirshman MF, Jessen N, Fujii N, Seifert MM, Brandauer J, Hotamisligil GS, & Goodyear LJ (2006). JNK1 deficiency does not enhance muscle glucose metabolism in lean mice. *Biochem Biophys Res Commun* **350**, 1063-1068.

Wojtaszewski JF, Jorgensen SB, Frosig C, MacDonald C, Birk JB, & Richter EA (2003a). Insulin signalling: effects of prior exercise. *Acta Physiol Scand* **178**, 321-328.

Wojtaszewski JF, Laustsen JL, Derave W, & Richter EA (1998). Hypoxia and contractions do not utilize the same signaling mechanism in stimulating skeletal muscle glucose transport. *Biochim Biophys Acta* **1380**, 396-404.

Wojtaszewski JF, Lynge J, Jakobsen AB, Goodyear LJ, & Richter EA (1999). Differential regulation of MAP kinase by contraction and insulin in skeletal muscle: metabolic implications. *Am J Physiol* **277**, E724-E732.

Wojtaszewski JF, MacDonald C, Nielsen JN, Hellsten Y, Hardie DG, Kemp BE, Kiens B, & Richter EA (2003b). Regulation of 5'AMP-activated protein kinase activity and substrate utilization in exercising human skeletal muscle. *Am J Physiol Endocrinol Metab* **284**, E813-E822.

Wojtaszewski JF, Mourtzakis M, Hillig T, Saltin B, & Pilegaard H (2002). Dissociation of AMPK activity and ACCbeta phosphorylation in human muscle during prolonged exercise. *Biochem Biophys Res Commun* **298**, 309-316.

Wojtaszewski JF, Nielsen P, Hansen BF, Richter EA, & Kiens B (2000). Isoform-specific and exercise intensity-dependent activation of 5'-AMP-activated protein kinase in human skeletal muscle. *J Physiol* **528 Pt 1**, 221-226.

Wretman C, Lionikas A, Widegren U, Lannergren J, Westerblad H, & Henriksson J (2001). Effects of concentric and eccentric contractions on phosphorylation of MAPK(erk1/2) and MAPK(p38) in isolated rat skeletal muscle. *J Physiol* **535**, 155-164.

Wright DC, Geiger PC, Han DH, Jones TE, & Holloszy JO (2007a). Calcium induces increases in peroxisome proliferator-activated receptor gamma coactivator-1alpha and mitochondrial biogenesis by a pathway leading to p38 mitogen-activated protein kinase activation. *J Biol Chem* **282**, 18793-18799.

Wright DC, Han DH, Garcia-Roves PM, Geiger PC, Jones TE, & Holloszy JO (2007b). Exercise-induced mitochondrial biogenesis begins before the increase in muscle PGC-1alpha expression. *J Biol Chem* **282**, 194-199.

Wright DC, Hucker KA, Holloszy JO, & Han DH (2004). Ca2+ and AMPK both mediate stimulation of glucose transport by muscle contractions. *Diabetes* **53**, 330-335.

Wu H, Kanatous SB, Thurmond FA, Gallardo T, Isotani E, Bassel-Duby R, & Williams RS (2002). Regulation of mitochondrial biogenesis in skeletal muscle by CaMK. *Science* **296**, 349-352.

Wu H, Rothermel B, Kanatous S, Rosenberg P, Naya FJ, Shelton JM, Hutcheson KA, DiMaio JM, Olson EN, Bassel-Duby R, & Williams RS (2001). Activation of MEF2 by muscle activity is mediated through a calcineurin-dependent pathway. *EMBO J* **20**, 6414-6423.

Wu P, Inskeep K, Bowker-Kinley MM, Popov KM, & Harris RA (1999a). Mechanism responsible for inactivation of skeletal muscle pyruvate dehydrogenase complex in starvation and diabetes. *Diabetes* **48**, 1593-1599.

Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, & Spiegelman BM (1999b). Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* **98**, 115-124.

Yang Y, Creer A, Jemiolo B, & Trappe S (2005). Time course of myogenic and metabolic gene expression in response to acute exercise in human skeletal muscle. *J Appl Physiol* **98**, 1745-1752.

Youn HD, Chatila TA, & Liu JO (2000). Integration of calcineurin and MEF2 signals by the coactivator p300 during T-cell apoptosis. *EMBO J* **19**, 4323-4331.

Youn JH, Gulve EA, & Holloszy JO (1991). Calcium stimulates glucose transport in skeletal muscle by a pathway independent of contraction. *Am J Physiol* **260**, C555-C561.

Yu M, Blomstrand E, Chibalin AV, Krook A, & Zierath JR (2001). Marathon running increases ERK1/2 and p38 MAP kinase signalling to downstream targets in human skeletal muscle. *J Physiol* **536**, 273-282.

Yu M, Stepto NK, Chibalin AV, Fryer LG, Carling D, Krook A, Hawley JA, & Zierath JR (2003). Metabolic and mitogenic signal transduction in human skeletal muscle after intense cycling exercise. *J Physiol* **546**, 327-335.

Zhang CL, McKinsey TA, & Olson EN (2002a). Association of class II histone deacetylases with heterochromatin protein 1: potential role for histone methylation in control of muscle differentiation. *Mol Cell Biol* **22**, 7302-7312.

Zhang Q, Piston DW, & Goodman RH (2002b). Regulation of corepressor function by nuclear NADH. *Science* **295**, 1895-1897.

Zhao M, New L, Kravchenko VV, Kato Y, Gram H, di PF, Olson EN, Ulevitch RJ, & Han J (1999). Regulation of the MEF2 family of transcription factors by p38. *Mol Cell Biol* **19**, 21-30.

Zilliacus J, Holter E, Wakui H, Tazawa H, Treuter E, & Gustafsson JA (2001). Regulation of glucocorticoid receptor activity by 14--3-3-dependent intracellular relocalization of the corepressor RIP140. *Mol Endocrinol* **15**, 501-511.

Zong H, Ren JM, Young LH, Pypaert M, Mu J, Birnbaum MJ, & Shulman GI (2002). AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation. *Proc Natl Acad Sci U S A* **99**, 15983-15987.