Skeletal muscle metabolic flexibility: contribution of genetics and lifestyle.

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

By

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Research Outcomes

The following publications have or will result from the work undertaken in this thesis:

*Peer-reviewed articles:*


*Abstracts:*


Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Donato A. Rivas

July 2009
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<th>Definition</th>
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<tbody>
<tr>
<td>3MG</td>
<td>3-O-methylglucose</td>
</tr>
<tr>
<td>4EBP1</td>
<td>eukaryotic initiation factor 4E binding protein 1</td>
</tr>
<tr>
<td>ACC</td>
<td>acetyl CoA carboxylase</td>
</tr>
<tr>
<td>ACS</td>
<td>acyl CoA synthetase</td>
</tr>
<tr>
<td>ACoA</td>
<td>acetyl-CoA</td>
</tr>
<tr>
<td>AICAR</td>
<td>5’-aminoimidazole-4-carboxamide ribonucleoside</td>
</tr>
<tr>
<td>Akt</td>
<td>v-akt murine thymoma viral oncogene homolog</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AS160</td>
<td>Akt substrate of 160 kilodaltons</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>β2-AR</td>
<td>β2-adrenoceptor</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>ca</td>
<td>consecutively active</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic AMP</td>
</tr>
<tr>
<td>CF</td>
<td>chow-fed</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>CON</td>
<td>control</td>
</tr>
<tr>
<td>COX</td>
<td>cytochrome c oxidase subunit</td>
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</table>
CPT: carnitine palmitoyl transferase
CS: citrate synthase
DAG: diacylglycerol
DW: dry weight
EXT: exercise training
EDL: extensor digitorum longus
FA: fatty acid
FABPpm: plasma membrane-bound fatty acid binding protein
FA-CoA: fatty acyl-CoA
FAT/CD36: fatty acid translocase
FATP: fatty acid transport protein
GLUT: glucose transport protein
G6P: glucose-6-phosphate
HCR: high capacity runner
HF: high-fat diet
HK: hexokinase
HSL: hormone-sensitive lipase
IMTG: intramuscular triacylglycerol
IR: insulin receptor
IRS: insulin receptor substrate
KHB: Krebs-Henseleit buffer
LCR: low capacity runner
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>LKB1</td>
<td>liver kinase b 1</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>mTORC</td>
<td>mTOR complex</td>
</tr>
<tr>
<td>MVD</td>
<td>mitochondrial volume density</td>
</tr>
<tr>
<td>NFDM</td>
<td>non-fat dry milk</td>
</tr>
<tr>
<td>NRF1</td>
<td>nuclear respiratory factor 1</td>
</tr>
<tr>
<td>Nur77</td>
<td>nuclear orphan receptor 4A1</td>
</tr>
<tr>
<td>P</td>
<td>phosphorylation</td>
</tr>
<tr>
<td>Palm</td>
<td>palmitate</td>
</tr>
<tr>
<td>PAS</td>
<td>phoso-Akt substrate</td>
</tr>
<tr>
<td>PDH</td>
<td>pyruvate dehydrogenase</td>
</tr>
<tr>
<td>PFK</td>
<td>phosphofructokinase</td>
</tr>
<tr>
<td>PGC</td>
<td>peroxisome proliferator-activated receptor gamma coactivator</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>Raptor</td>
<td>regulatory associated protein of mTOR</td>
</tr>
<tr>
<td>RG</td>
<td>red gastrocnemius</td>
</tr>
<tr>
<td>Rictor</td>
<td>rapamycin-insensitive companion of mTOR</td>
</tr>
<tr>
<td>S6K1</td>
<td>p70 ribosomal protein S6 kinase 1</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
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</tbody>
</table>
SED: sedentary
Ser: serine
siRNA: silencing ribonucleic acid
Sol: soleus
STZ: Streptozotocin
T2DM: type 2 diabetes mellitus
TAG: triacylglycerol
TCA: tricarboxylic acid
Thr: threonine
TSC: tuberous sclerosis
Y: tyrosine
UCP: uncoupling protein
WG: white gastrocnemius
ZMP: AICAR monophosphate
Abstract

Chronic metabolic diseases such as insulin resistance, obesity and type 2 diabetes develop from the complex interaction of environmental and genetic factors. However, the extent to which each contributes to these disease states is currently unknown. Whole-body metabolic health is associated with the ability of skeletal muscle to transition between the uptake and oxidation of carbohydrate- and lipid-based fuels in response to their availability and the prevailing hormonal milieu, a process described as “metabolic flexibility”. However, in several lifestyle-related diseases such as obesity and type 2 diabetes, there is a loss of skeletal muscle plasticity or “metabolic inflexibility” such that rates of lipid oxidation do not suppress effectively in response to insulin, nor do they effectively increase during the transition to fasting conditions. The primary aim of the experiments undertaken for this thesis was to enhance our understanding of some of the mechanisms by which lifestyle and genetic factors contribute to skeletal muscle metabolic flexibility/inflexibility. The first investigation (Chapter three) was designed to determine the contribution of intrinsic oxidative capacity of skeletal muscle to metabolic flexibility in a rodent model in which aerobic running capacity was artificially manipulated to generate divergent phenotypes for this trait. Hind limb perfusion for the measures of glucose and lipid metabolism revealed that the skeletal muscle of rats selected for high intrinsic running capacity (HCR) were metabolically ‘flexible’ compared to animals selected for low running capacity (LCR). The superior fuel-handling capacity in HCR was, in part, explained by fibre-type specific increases in
insulin-stimulated phosphorylation to components of the insulin signalling cascade, along with a substantial increase in mitochondrial volume density.

The second experimental chapter (described in Chapter four) determined if a six week programme of endurance exercise training could reverse the skeletal muscle metabolic ‘inflexibility’ observed in LCR (Chapter three). Aerobic exercise training-induced improvements in insulin sensitivity were associated with increased fatty acid oxidation, increased phosphorylation of selected components of the insulin signalling pathway and the increased expression of β-adrrenergic components and target proteins.

In order to ascertain a mechanism for skeletal muscle metabolic inflexibility, the final study (described in Chapter five) determined the independent and interactive effects of increased lipid availability and aerobic exercise training on the mammalian target of rapamycin pathway (mTOR) in a model of diet-induced metabolic inflexibility, the high-fat fed rat. A high-fat diet decreased insulin-stimulated glucose transport and was linked to an increase in the association of mTOR with its binding partners, rictor and raptor. These increases were coupled with the increased activation of the downstream substrates Akt1, S6K1 and the inhibitory serine phosphorylation of IRS1. Aerobic exercise training restored skeletal muscle insulin sensitivity possibly through the activation of the AMP-activated protein kinase and thus the inhibition of the mTOR signalling pathway. In summary, the results from the studies undertaken for this thesis provide novel information regarding the mechanisms by which lifestyle (exercise, nutrition) and genetics determine the metabolic flexibility of skeletal muscle.
CHAPTER ONE: Review of Literature
1.1 Metabolic Flexibility

Metabolic flexibility is the ability of peripheral, insulin sensitive tissue (ie. muscle, adipose, and liver) to transition between glucose and lipid oxidation in response to substrate availability and hormonal signals (Fig 1.1) (Kelley & Mandarino, 2000). In healthy lean, insulin-sensitive individuals, during the postabsorptive period (ie. fasting), there is a reliance on lipid oxidation for metabolism. In contrast, during the postprandial condition (ie. a fed state), the body oxidises predominantly glucose and inhibits lipid metabolism. When these processes are functioning normally the health of the individual is maintained. However, in several lifestyle-related diseases such as obesity and type 2 diabetes, there is a loss of skeletal muscle plasticity, a so-called “metabolic inflexibility”. In metabolically inflexible individuals, the rates of lipid oxidation do not suppress effectively in response to insulin, nor do they effectively increase during the transition to fasting conditions (Kelley, 2005; Kelley & Mandarino, 2000). Insulin sensitive tissues like liver and adipose have significant roles in whole body fuel homeostasis. These include the functions of storage and utilisation that regulate the flow of energy-providing substrates (Frayn, 2002; Frayn et al, 1995; Muller, 1995). However, the focus of the following literature review will be on skeletal muscle and its role in fuel selection, energy homeostasis and metabolic health.
Figure 1.1 Metabolic flexibility/inflexibility in lean, aerobically fit and obese aerobically unfit individuals. Reproduced from Kelley 2005.

1.2 Skeletal Muscle Metabolic Flexibility

Overview

Skeletal muscle accounts for approximately 55% of body mass in non-obese individuals and plays a fundamental role in whole-body energy metabolism and substrate turnover (Zierath & Hawley, 2004). In healthy individuals skeletal muscle accounts for approximately 80% of whole body insulin-stimulated glucose uptake but in normal-weight subjects with insulin resistance, total body glucose metabolism is reduced by up to 40% (DeFronzo et al, 1985). These data highlight the importance of the peripheral tissues in the disposal of glucose and indicate that skeletal muscle is the most important site of the insulin resistance observed in states such as obesity and type 2 diabetes.
Under normal physiological conditions skeletal muscle relies on both carbohydrate and lipid-based fuels for oxidative metabolism. The degree to which skeletal muscle utilises one fuel or another is dependent on a multiple of factors (ie. nutrient availability, nutrient-induced hormone secretion, contractile status etc.). Skeletal muscle fuel metabolism is a highly regulated process that ensures that ATP supply is always closely matched to ATP demand. There are two physiological components of skeletal muscle metabolism that have underlying roles in metabolic flexibility. The first is fuel selection during the postabsorptive and post-prandial condition. The second is the oxidation of fuels that are either obtained from a meal or mobilised from energy stores (glycogen or triacylglycerol). Fuel selection and utilisation has been well investigated by many researchers (Burke & Hawley, 2006; Corpeleijn et al, 2009; Hultman, 1995; Kelley & Mandarino, 2000; Randle, 1998; Sugden & Holness, 1990). It was over 40 years ago that Randle and colleagues (1963) proposed the concept of the glucose-fatty acid cycle to explain resting fuel metabolism in cardiac muscle (Fig 1.2) (Kelley & Mandarino, 2000; Randle et al, 1963). Randle and colleagues hypothesised that fuel selection depended primarily on the availability of lipids, and this was the main factor determining the fuel mix to be oxidised (at least during resting conditions).

Biochemically, fat oxidation, through the accumulation of acetyl-COA and citrate, inhibits pyruvate dehydrogenase (PDH) and phosphofructokinase (PFK). This event leads to the accumulation of glucose-6-phosphate (G6P) and the inhibition of hexokinase (HK) activity, the rise in glucose concentrations would inhibit glucose uptake through a negative feedback mechanism (Fig 1.2) (Randle, 1998). More recently, it has been
observed that hyperglycaemia can inhibit fatty acid oxidation, a concept dubbed the reverse Randle Cycle (Corpeleijn et al, 2009). Without doubt, carbohydrate and lipid-based fuels are the most important energy sources for matching ATP supply to ATP demand in skeletal muscle. Therefore, an understanding the processes involved in the respiration of fat and glucose are essential for comprehending the importance of metabolic flexibility in health.

**Figure 1.2** Glucose-fatty acid cycle in skeletal muscle. Abbreviations: UDP, Uridine diphosphate; HK, hexokinase; PFK, phosphofructokinase; PDK, pyruvate dehydrogenase kinase; TCA, tricarboxylic acid; P, phosphorylation.

### 1.3 Substrate utilisation in skeletal muscle

**Introduction**

Lipids, carbohydrates and amino acids are all important fuels for aerobic metabolism. However, in well-fed individuals the contribution of amino acids to resting
energy metabolism is minimal (1-2%). Therefore, this section will focus on the transport and utilisation of lipids and glucose in skeletal muscle, including a discussion of the molecular pathways involved in the regulation of lipid and glucose metabolism in skeletal muscle.

1.3.1 Carbohydrate metabolism in skeletal muscle.

Overview

The transport of glucose into the cell is the rate limiting step in carbohydrate metabolism (its subsequent uptake and oxidation) (Fueger et al, 2004a; Fueger et al, 2004b; Fueger et al, 2004c; Fueger et al, 2005; Koistinen & Zierath, 2002). This is achieved by the facilitated diffusion of glucose through glucose transporters (Glut1-12) during postabsorptive and postprandial conditions (Barnard & Youngren, 1992; James et al, 1988). In skeletal muscle this process is mediated by the translocation of the insulin-sensitive glucose transporter 4 (Glut4) (Barnard & Youngren, 1992; James et al, 1988; Koistinen & Zierath, 2002). This is accomplished by two distinct signalling pathways; one pathway is mediated by insulin (i.e., the insulin-dependent pathway) and the other is activated independently by muscle contraction. Insulin and contraction have a robust effect on the uptake, oxidation and storage of glucose in skeletal muscle.

Insulin-dependent glucose metabolism

The effect of insulin on whole-body carbohydrate metabolism was first observed in the 1920’s with its discovery and isolation by Banting, Best, Collip and Macleod (Banting, 1937). Some three decades later it was demonstrated that insulin changed the permeability of muscle to glucose (Park & Johnson, 1955). Subsequently, using
highly purified plasma membranes prepared from rat liver, it was discovered that cell membranes contained specific binding sites for insulin (Freychet et al, 1971). Using in vitro skeletal muscle preparations, Beatty et al. (1960) observed that insulin increased the uptake, oxidation and storage of glucose (Beatty et al, 1960). This occurred to a similar extent in rats made diabetic by the removal of 95% of their pancreas (Beatty et al, 1960). In 1980, Cushman (Cushman & Wardzala, 1980) and Suzuki (Suzuki & Kono, 1980) independently reported that insulin induced the translocation of a glucose transport protein (Glut4) from an intracellular store to the plasma membrane. It is now understood that insulin acting through its receptor and signalling cascade initiates the transport of the insulin-sensitive glucose transporter, Glut4 (James et al, 1988; Taniguchi et al, 2006).

The postprandial uptake of glucose into muscle cells is initiated by the binding of insulin to the extramyocellular α–subunit of the insulin receptor (IR), which results in IR β–subunit autophosphorylation and the subsequent tyrosine phosphorylation of the insulin receptor substrate (IRS). Phosphorylated IRS then binds and activates phosphoinositide 3-kinase (PI3K) leading to the activation and phosphorylation of Akt and protein kinase C (PKC). The activated Akt [most likely mediated through the Akt2 isoform (Brozinick et al, 2003)] subsequently phosphorylates and inhibits its downstream substrate the Akt substrate of 160 kDa (AS160). The phosphorylation of AS160 allows its binding to the 14-3-3 protein and the untethering from the Glut4 vesicle (Howlett et al, 2008). These initial signalling events converge on several downstream effectors that promote the metabolic actions of insulin, including the
translocation of Glut4 to the myocellular membrane allowing for the disposal of plasma glucose. (Fig 1.3)

**Figure 1.3** Simplified schematic of insulin-dependent and independent glucose transport in skeletal muscle. Bars represent inhibition, arrows represent activation. Abbreviations: IRS, insulin receptor substrate; PI3-K, Phosphoinositide 3-kinase; PKC, protein kinase C; AS160, Akt substrate of 160 kDa; HK, hexokinase; G-6-P, glucose-6-phosphate; AICAR, 5’-aminoimidazole carboxamide ribonucleotide; ZMP, AICAR monophosphate; AMP, adenosine monophosphate; LKB1, liver kinase B1; AMPK, 5’AMP-activated protein kinase; Y, tyrosine phosphorylation; P, phosphorylation.

**Contraction induced glucose metabolism**

Investigations using aerobic exercise or electrically-stimulated muscle contraction have revealed that glucose disposal is activated to a similar extent as with insulin (Ploug et al, 1984; Richter et al, 1982; Richter et al, 1985). Ploug et al. (1984) demonstrated that *in situ* contraction, in the absence of insulin, increased the uptake and oxidation of glucose in the perfused intact hindlimb (Ploug et al, 1984). This result was observed, in an effect similar to insulin, in rats made diabetic with streptozotocin (STZ; a compound that induces β-cell destruction) (Ploug et al, 1984). Winder and
Hardie (1996) first observed that exercise/contraction increased the activation of the 5′–AMP-activated protein kinase (AMPK) (Winder & Hardie, 1996). Subsequently, when using the AMP mimetic 5′-aminoimidazole-4-carboxamide ribonucleoside (AICAR), these researchers proposed a role for AMPK activation in increased glucose uptake into skeletal muscle (Merrill et al, 1997). Since these studies, the role of AMPK in contraction-stimulated glucose metabolism has been well researched (Jessen & Goodyear, 2005; Winder & Hardie, 1999; Wojtaszewski et al, 2003). However as noted earlier, AMPK is not the only contributor to glucose metabolism in this tissue. Indeed, muscle contraction induces the activation of several signalling mechanisms that have been associated with increased GLUT4 translocation including Ca2+/calmodulin, nitric oxide, PKC and IL-6 [For review see (Cortwright & Dohm, 1997; Farese, 2002; Febbraio & Pedersen, 2005; Jessen & Goodyear, 2005)]. The following section will focus on the roles of AMPK signalling pathway in skeletal muscle insulin-independent glucose metabolism.

AMPK is a cellular energy sensor that restrains energy consuming processes and concurrently increases energy producing processes, like β–oxidation. AMPK is a heterotrimeric complex that is composed of a catalytic subunit, that is encoded by two distinct genes (α1, α2) and two regulatory subunits, that is also encoded by two distinct genes (β1, β2) and the γ subunit that is encoded by 3 genes (γ1, γ2, γ3) (Fig 1.3). It is currently unclear what role the different AMPK catalytic isoforms (α1, α2) have on the substrates downstream of AMPK. AMPK is highly sensitive to, and allosterically activated by 5′–AMP. This activation is inhibited by ATP, which makes the AMP/ATP ratio a good indicator of cellular energy status and AMPK activation (Fujii et al, 2006; Karagounis &
Hawley, 2009; Winder & Hardie, 1999). Any cellular event that decreases ATP synthesis (i.e. hypoxia, heat stress) or increases ATP consumption (i.e. muscle contraction) activates the AMPK signalling pathway. Although AMP alone can allosterically activate AMPK, activation can also occur covalently through phosphorylation of its Thr172 residue by an upstream kinase that was recently identified as LKB1 (Hawley et al, 2003). Since LKB1 itself is allosterically activated by AMP, and the binding of AMP to AMPK makes it a better substrate for LKB1, the result is a highly amplified activation cascade sensitive to small changes in AMP.

The precise mechanism by which AMPK stimulates the transport of glucose is currently unknown, but studies that have utilised AMPK isoform-specific knockout models indicate that the α1 and α2 subunits play distinct roles in the regulation of whole-body glucose homeostasis (Viollet et al, 2003). It is believed that activation of AMPK results in the phosphorylation of AS160, which then leads to the translocation of GLUT4 protein to the plasma membrane (Cartee & Wojtaszewski, 2007; Fujii et al, 2006; Treebak et al, 2007; Winder & Hardie, 1999). Evidence to support this hypothesis comes from studies using the pharmacological activator of AMPK, AICAR. AICAR is transported across the cell membrane and is then converted to the AMP analogue, ZMP, inside the intracellular compartment. ZMP has the ability to activate both LKB1 and AMPK in the same manner as AMP, making it a useful tool to study AMPK action without inducing the multiple confounding factors involved with increasing cellular AMP levels.

An early study reported that in perfused skeletal muscle, glucose uptake across the rat hindlimb was increased two-fold in response to AICAR (Merrill et al, 1997).
Further work demonstrated that AICAR-stimulated muscle glucose uptake was independent of insulin, and was not inhibited by the PI3K inhibitor wortmannin (Hayashi et al, 1998). Investigations using fluorescent labelling techniques revealed that AICAR-induced glucose transport was indeed associated with the translocation of Glut4 protein to the sarcolemma (Russell et al, 1999). Recently, Treebak et al. (2006) using genetically manipulated models of AMPK revealed the skeletal muscle of AMPK α2 knockout, γ3 knockout, and α2 kinase–dead mice had impaired phosphorylation of AS160 in response to AICAR treatment (Long & Zierath, 2006; Treebak et al, 2006). Taken together these studies provide proof of a relationship between AMPK activation and skeletal muscle glucose uptake.

Although the precise mechanisms underlying the role of AMPK in contraction-stimulated glucose uptake is not currently known, the ability of AMPK to stimulate glucose metabolism makes it an important target for the treatment of metabolic diseases (ie. the metabolic syndrome, type 2 diabetes) (Musi & Goodyear, 2002; Winder, 2000). In addition to its role in insulin-independent glucose uptake, chronic AMPK activation has the ability to increase insulin-stimulated Glut4 translocation in muscle, possibly due to an increase in Glut4 protein expression (Buhl et al, 2001). The expression of AMPK and its activation in response to exercise appears to be normal in the muscle of individuals with type 2 diabetes (Musi et al, 2001). Taken collectively, the results from these studies identify AMPK activation as a potential therapeutic target for the enhancement of glucose uptake by both insulin-dependent and -independent means.
1.3.2 Fatty-acid metabolism in skeletal muscle

In humans during times of low energy status (ie. fasting, strenuous muscle contraction) insulin levels decrease considerably and the concentration of free-fatty acids (FA) increase 5-10 fold above resting levels. Most of the resting energy requirements are obtained from lipolysis in adipose tissue (Costill et al, 1977; Jeukendrup, 2002; Vukovich et al, 1993). This liberation of FA is accomplished by the release of catecholamines that induce the activation of the β-adrenergic pathway (Jeukendrup, 2002; Wolfe et al, 1990). This subsequently leads to the formation of cyclic AMP (cAMP) through the adenylylcyclase system. cAMP activates the cAMP-associated protein kinase A (PKA) and leads to the phosphorylation of hormone sensitive lipase (HSL), the key rate-limiting enzyme in lipolysis (Langfort et al, 1999; Stich & Berlan, 2004). At rest the amount of FA released from adipose exceeds the amount oxidised and a large portion are reesterified back into triacylglycerol by the liver (Horowitz & Klein, 2000). During aerobic exercise the amount of triacylglycerol reesterified decreases by about 50% (Horowitz & Klein, 2000). Wolfe et al. (1990) observed that exercising humans increased rates of lipolysis by threefold compared to at rest (Wolfe et al, 1990). The authors believed this was mainly due to increased β-adrenergic activation (Wolfe et al, 1990). In a low energy state (ie. fasting, exercise) circulating FA are the predominant fuel and are readily taken up by skeletal muscle.

The precise regulatory mechanisms of lipid uptake by skeletal muscle are not completely understood. During transport of FA from blood to muscle, there are several potential rate-limiting areas that might slow the eventual FA uptake. These are the
membranes of the vascular endothelial cells, the interstitial space between endothelium and muscle cell, and finally the muscle cell membrane (Kiens, 1998). The transport of FA from the blood circulation to the cytosol of skeletal muscle cells is likely the combined result of passive diffusion across the myocellular membrane and protein-mediated membrane transport (Kiens, 2006). There are at least three FA transport proteins that have been identified in skeletal muscle: 1) FAT/CD36, 2) plasma membrane FABPpm, and 3) FATP1-6 (Bonen et al, 2000; Luiken et al, 2001). Of these the FA transporter, FAT/CD36 has been the most well characterised with regard to its role in skeletal muscle lipid metabolism (Bonen et al, 2000). Muscle-specific overexpression of FAT/CD36 in mice results in reduced plasma lipids as well as increased palmitate oxidation (Ibrahimi et al, 1999). Aerobic exercise training and a high-fat diet up-regulate FA transport and oxidation and are accompanied by an increase in the expression of FAT/CD36 (Cameron-Smith et al, 2003; Holloway et al, 2006). Furthermore, it was observed in the skeletal muscle of obese women that there was an enhancement in triacylglycerol storage and FA transport that corresponded with an increased translocation of FAT/CD36 to the plasma membrane (Bonen et al, 2004). Taken together, these observations indicate that FA transport into skeletal muscle is a highly regulated process that may influence whole-body lipid metabolism (Fig 1.4).

FAT/CD36, along with carnitine palmitoyl transferase I (CPTI), regulates the transport and oxidation of lipid in the skeletal muscle. When FA are translocated into the cytosol, they are activated in a reaction catalysed by acyl CoA synthetase (ACS), resulting in the formation of fatty acyl-CoA (FA-CoA) (Large et al, 2004). These FA-CoA
esters can act as substrates for β-oxidation, be incorporated into intermediate lipid signalling molecules such as ceramide and diacylglycerol (DAG), or be stored as triacylglycerol (TAG). FA-CoA that is targeted for FA oxidation must first cross the mitochondrial membrane in a rate-controlling step that is regulated by CPTI. CPTI transfers FA-CoA into the mitochondria a process that is allosterically inhibited by malonyl-CoA, an enzyme which is synthesized by acetyl-CoA carboxylase (ACC). The activity of ACC is regulated by reversible phosphorylation and both glucose and insulin increase ACC activity while abundant FA-CoA inhibits ACC activation. During times of low energy status (eg. fasting, prolonged exercise), malonyl-CoA levels decline and the reliance on FA oxidation increases. When FA enters the mitochondria they proceed through a repetitive biochemical process called β–oxidation. During this process the FA is broken down with the release of two carbons at a time, as acetyl-CoA. Acetyl-CoA is then disposed by way of the tricarboxylic acid (TCA) cycle. Citrate Synthase (CS) is the first step in the TCA cycle for the production of CO₂ from acetyl-CoA and is used as a marker of mitochondrial activity and content. β–oxidation and the TCA cycle produce energy rich donors (NADH, succinate) for the synthesis of adenosine triphosphate (ATP) in the electron transport chain (Fig 1.4).
Figure 1.4 Simplified overview of lipid metabolism in skeletal muscle. Abbreviations: FA, fatty acids, FAT/CD36, fatty acid translocase CD36, CPTI, Carnitine palmitoyltransferase I; NADH, nicotinamide adenine dinucleotide; TCA, tricarboxylic acid; ETC, electron transport chain; ATP, adenosine triphosphate.

1.4 ‘Metabolic inflexibility’ in skeletal muscle

Overview

The composition and biochemistry of skeletal muscle are altered in obesity and type 2 diabetes compared to healthy individuals (Kelley, 2002). Rates of fatty acid (FA) oxidation do not suppress effectively in response to insulin, nor do they effectively increase during the transition to fasting conditions. In addition, glucose uptake and oxidation are relatively unresponsive to an insulin stimulus. Almost a decade ago, Kelley & Mandarino (2000) coined the term “metabolic inflexibility” to describe the metabolic
disturbances of skeletal muscle fuel handling (Kelley & Mandarino, 2000). Decreases in the rates of fat oxidation that are observed in individuals with metabolic inflexibility have been proposed to be a predictor of whole-body insulin resistance (McGarry, 2002). Indeed, Kelley et al. (1999) have observed that in obese humans, an inhibition in FA oxidation (but not FA uptake) is responsible for the increased storage of lipids in the muscle of these patients (Kelley et al, 1999). Thus, a direct consequence of metabolic inflexibility is an increased accretion of lipid within muscle that mirrors fat storage in other insulin-sensitive tissues and bears a negative relationship to whole-body insulin sensitivity (McGarry, 2002).

![Metabolic Inflexibility](image)

**Figure 1.5** Metabolic flexibility/inflexibility in lean, obese and diabetic individuals. Determined by indirect calorimetry in lean control subjects, obese subjects without diabetes, and subjects with type 2 diabetes after an overnight fast or at the end of a hyperinsulinemic glucose clamp (insulin-stimulated). Leg RQ of the control subjects during fasting of ~0.8 indicated a predominant use of lipid, which switched to carbohydrate during insulin infusion. In contrast, fasting leg RQs in the obese and diabetic subjects were elevated, indicating a predominance of carbohydrate as fuel, and remained unaltered by insulin infusion. Reproduced from Kelley and Mandarino 2000.

Obesity and elevated levels of circulating nutrients (ie. glucose, AA, FFA) are strongly associated with the development of insulin resistance and type 2 diabetes (Dresner et al, 1999; Kahn & Flier, 2000; McGarry, 2002). These conditions are
exacerbated by excess nutrient intake and a sedentary lifestyle (Hawley & Lessard, 2008). Increased dietary fat consumption has long been associated with the metabolic abnormalities that are associated with over-nutrition (Hawley & Lessard, 2008). However, increases in the intake of other dietary nutrients, such as protein and carbohydrates, can also have negative effects on whole body insulin action (Daly, 2003; Um et al, 2006). The so-called “western diet” is characterised by the consumption of large quantities of processed meats, refined sugars and fats and is closely linked with obesity and insulin resistance (McCullough et al, 2002; van Dam et al, 2002). In addition to nutrient-induced impairments as a consequence of increased lipid availability, hyperglycaemia is also known to impair whole-body glucose disposal and glycogen synthesis (Krebs & Roden, 2004). Exposure to chronically high glucose levels (‘glucose toxicity’) also plays a role in the insulin resistance of skeletal muscle in part by the inhibition of glucose transport/phosphorylation (Cline et al, 1997; Robertson et al, 1994). However, the treatment of hyperglycaemia in patients with type 2 diabetes only partially improves insulin sensitivity strongly suggesting that insulin resistance might be a cause rather than a consequence of hyperglycaemia (Krebs & Roden, 2004). Because skeletal muscle is responsible for the majority of insulin-mediated glucose disposal and the muscle’s sensitivity to insulin is highly responsive to changes in circulating nutrients significant scientific effort has been devoted to elucidating the mechanisms by which over-nutrition leads to impaired insulin signal transduction in skeletal muscle.
1.4.1 Models to investigate ‘metabolic inflexibility’

The low (LCR) and high (HCR) capacity running rat model

The LCR/HCR model of divergent intrinsic aerobic capacity was developed specifically to study mechanisms associated with the metabolic syndrome and cardiovascular disease in a system in which there is a degree of control over both environmental and genetic variables. This model was engineered because traditional animal models failed to accurately mirror human disease (Koch & Britton, 2008). For example, chemical or surgical procedures used to mimic disease, such as using streptozotocin to induce diabetes, more closely resemble the response to the injury rather than the progression of the disease. Furthermore, using single or multiple genetic approaches to studying a disease (ie. gene knockout model) are problematic because complex diseases result from genetic variation to multiple genes that are sensitive to the environment (Koch & Britton, 2008).

Through two-way artificial selection, Britton & Koch (2001) generated an animal model of low or high intrinsic aerobic exercise capacity from a population of genetically heterogeneous rats (N:NIH stock) (Koch & Britton, 2001). Founder animals were obtained from a colony maintained at the National Institutes of Health that originated from the intentional crossbreeding of eight inbred strains (ACI, BN, BUF, F344, M520, MR, WKY and WN). To broaden the initial genetic variance, each rat in the founder population was of different parentage, so that selection was not among brothers and sisters. The crossbreeding produced a population that has wide genetic heterogeneity, providing an ideal starting population for artificial selection. Divergent (two-way)
artificial selection for a complex trait (e.g., superior aerobic phenotype) produces an excellent genetic model to study gene-exercise interactions because contrasting allelic variation is concentrated at the extremes from one generation to the next (Koch & Britton, 2001). Eleven generations of two-way selection produced rats that differed markedly in aerobic exercise capacity (374% difference between LCR and HCR) (Wisloff et al, 2005). Selection for low and high aerobic exercise capacity simultaneously generated animals that differed markedly for metabolic and cardiovascular disease risk factors. LCR rats were insulin-resistant compared with HCR, as verified by higher fasting blood glucose and insulin concentration and impaired glucose tolerance (Wisloff et al, 2005).

Figure 1.6 Divergent running distance to exhaustion in the LCR/HCR model from Generation 1 to Generation 11. On average, the LCR rats decreased 16 m per generation and the HCR rats gained 42 m per generation in distance run to exhaustion. Reproduced from Wisloff et al. 2005.

While the precise molecular mechanisms of metabolic inflexibility remain unknown, this disorder shares two hallmark features with several other chronic diseases: insulin resistance and reduced skeletal muscle oxidative capacity. A direct
consequence of these altered metabolic states is a breakdown in lipid dynamics reflected by elevated levels of circulating FA and TGs, culminating in the buildup of lipids in skeletal muscle and other tissues. In the animal model of divergent intrinsic aerobic exercise capacity, LCR display greater visceral adiposity (63%), higher plasma triglycerides (168%), elevated plasma FFA concentrations (94%) and greater weight gain (32%) compared with HCR (Wisloff et al, 2005). Furthermore, it has recently been reported that HCR animals are resistant to the development of high-fat diet-induced obesity and insulin resistance (Noland et al, 2007). Upon exposure to a chronic (12 wk) high-fat diet, LCR rats gained more weight, fat mass, and their insulin-resistant condition was exacerbated despite a similar energy intake to HCR rats (Noland et al, 2007). The divergent responses to a high-fat diet in this model could be a result of differences intrinsic properties within skeletal muscle (i.e. increased oxidative capacity). Indeed, Noland et al. (2007) observed that HCR had enhanced rates of palmitate oxidation and citrate synthase activity on a normal (15% fat/kCal) diet and were increased after a high-fat feeding (60% fat/kCal) compared to LCR (Noland et al, 2007). Furthermore, mitochondrial proteins, such as Cytochrome oxidase IA (COX IA) and uncoupling protein 3 (UCP3) were additionally increased in HCR compared to LCR (Noland et al, 2007). Taken collectively, these data demonstrate that LCR and HCR rats provide a valid model to address genetic factors that protect or predispose individuals to the metabolic syndrome.
**The high-fat fed rat model of over-nutrition.**

The initial observation that skeletal muscle glucose metabolism was sensitive to changes in lipid availability was made by Randle and co-workers (Randle et al, 1963). The “Randle cycle” describes how an increase in the availability of lipids results in the up-regulation of fatty acid metabolism with the concomitant down-regulation of glucose metabolism and glucose uptake into skeletal muscle (see section 1.2 and Fig 1.2). The observations of Randle et al. (1963) combined with the finding that skeletal muscle acts as the main pool for insulin-stimulated glucose disposal makes this tissue an ideal target for the study of insulin resistance due to over-nutrition (Hawley & Lessard, 2008; Randle et al, 1963). Furthermore, models that mimic the dysfunctional effects of lipotoxicity in skeletal muscle are needed to better understand these impairments in humans.

The most frequently used animal model to examine nutrient-induced impairments to skeletal muscle insulin signal transduction is the high-fat diet (45-60% kcal from fat). After several weeks of a high-fat diet, there is an increase in the storage of lipid species such as triacylglycerol, diacylglycerol and ceramides in muscle, along with impairments to the insulin signalling cascade and the glucose transport system through multiple mechanisms (Hawley & Lessard, 2008). Studies on lipid-induced insulin resistance have demonstrated decreases in the phosphorylation, activation and expression of insulin signalling components such as the IR, IRS, PI3K, Akt, and Glut4 in skeletal muscle (these will be discussed in further detail subsequently, see section 1.5.2) (Bjornholm & Zierath, 2005; Hawley & Lessard, 2008; Lessard et al, 2007). The consumption of a high-fat diet is strongly related to obesity and the onset of obesity-
related health problems such as type 2 diabetes (Lichtenstein et al., 1998). In humans, consumption of a high-fat diet is associated with persistently elevated blood lipids, as well as abnormal glucose homeostasis and insulin resistance (Lombardo & Chicco, 2006). Similarly, mice and rats fed diets high in saturated, monounsaturated or polyunsaturated FA develop glucose intolerance, hyperinsulinaemia and obesity (Lombardo & Chicco, 2006). Although genetic components certainly play a role in the aetiology of insulin resistance and type 2 diabetes, the rapidly increasing prevalence of this disease and its strong link to obesity suggest that lifestyle factors make an important contribution to its onset (Beck-Nielsen et al., 2003). Thus, the induction of insulin-resistance via high-fat feeding is a well-utilised model to investigate possible causes of lipid-induced insulin resistance in the absence of genetic modification.

1.4.2 Molecular mechanism associated with ‘metabolic inflexibility’

Impaired insulin signalling

In patients with type 2 diabetes, impaired insulin-stimulated glucose uptake in skeletal muscle is associated with inadequate recruitment of GLUT4 to the plasma membrane (Bjornholm & Zierath, 2005). Reduced membrane GLUT4 content can occur despite normal expression of GLUT4 protein (Zierath et al., 1997; Zierath et al., 2000), indicating that signalling events that lead to GLUT4 translocation may be causally related to muscle insulin resistance. Defective GLUT4 translocation in skeletal muscle may result from a down-regulation of one or more components of the insulin signal transduction pathway, and/or from disruption of mechanical events that allow for the
translocation/insertion of GLUT4 in the plasma membrane. It is possible that the redistribution of GLUT4 to inaccessible intracellular compartments inhibits GLUT4 trafficking and translocation to the plasma membrane in individuals with type 2 diabetes (Garvey et al, 1998). However, GLUT4 translocation in response to contraction and hypoxia is normal in insulin-resistant rats (Zierath et al, 1997), suggesting that impaired insulin signalling is at least partially responsible for skeletal muscle insulin resistance.

The role of the insulin receptor in skeletal muscle insulin resistance is unclear, as some studies have reported a decrease (Nolan et al, 1994) or no change (Krook et al, 2000) in its expression and insulin-stimulated phosphorylation in the skeletal muscle of insulin-resistant subjects. However, a number of defects to the insulin signalling cascade have been identified in skeletal muscle of insulin resistant animals and humans. Insulin-stimulated activation of the signalling cascade components directly downstream of the insulin receptor, IRS-1 and PI3-kinase, are impaired in the muscle of patients with type 2 diabetes (Bjornholm et al, 1997; Bjornholm & Zierath, 2005; Krook et al, 2000). Aberrant signalling at the level of IRS-1 and PI3-kinase appears to be due to loss of function rather than reduced protein expression (Bjornholm & Zierath, 2005). Further downstream, Akt activation in response to physiological insulin concentrations is normal in individuals with type 2 diabetes (Kim et al, 1999; Krook et al, 1998), but appears to be blunted at supraphysiological (60 nM) insulin concentrations (Krook et al, 1998). Reduced insulin-stimulated Akt activity in skeletal muscle from individuals with type 2 diabetes was recently attributed to decreased phosphorylation of this enzyme at Thr308, and was associated with decreased Akt-mediated AS160 phosphorylation (Karlsson et al, 2005).
It is apparent that defects have been found at virtually every known step of the signalling pathway that ultimately leads to GLUT4 translocation in insulin resistant muscle. However, the extent to which these alterations in signalling are causative in the overall development of insulin resistance is currently unknown. Although there is undoubtedly a genetic contribution to the aetiology of insulin resistance and type 2 diabetes, single-gene mutations that result in impaired insulin signalling are very rare and account for a minority of all cases (So et al, 2000). Rather, it is likely that the onset of insulin resistance is a result of complex interactions between genetic susceptibility and a number of environmental factors (Beck-Nielsen et al, 2003; So et al, 2000). In fact, environmental factors are believed to outweigh those from genetic causes in predisposing to type 2 diabetes, with the strongest environmental predictor of this disease state being obesity (Beck-Nielsen et al, 2003). The robust association between the increasing world-wide prevalence of both obesity and diabetes (Golay & Ybarra, 2005), has prompted some to refer to this co-existence of conditions as “diabesity” (Shafrir, 1996).

**Reduced mitochondrial respiratory metabolism and metabolic inflexibility**

Chronic inactivity, or disuse, results in decreased mitochondrial number, impairment in lipid handling, insulin resistance and metabolic inflexibility [For review: (Hawley & Lessard, 2007)]. Defects in mitochondria have been linked to both metabolic inflexibility and insulin resistance: low mitochondrial oxidative capacity results in impaired rates of fat oxidation and to the accumulation of muscle lipid intermediates
that directly contribute to the pathogenesis of metabolic syndrome (Hawley & Lessard, 2007).

Kelley et al. (1996) first reported an impairment of insulin-stimulated glucose phosphorylation in skeletal muscle from patients with T2DM (Kelley et al, 1996). Subsequently it was suggested that a mismatch between mitochondrial oxidative capacity and the capacity for glycolysis may be an important factor in the development of insulin resistance (Simoneau & Kelley, 1997). Additional work by Kelley and colleagues (2002) demonstrated that the overall capacity of complex I of the electron transport chain was reduced in skeletal muscle from patients with T2DM compared to healthy volunteers and that mitochondria from diabetic patients were ~35% smaller than those from healthy subjects (Kelley et al, 2002b). Of note was the observation that mitochondrial size was significantly associated with whole-body glucose disposal (Kelley et al, 2002b). In follow-up work, others have observed reduced mitochondrial density (Morino et al, 2005), lower rates of mitochondrial phosphorylation (Petersen et al, 2004) and decreased insulin-stimulated ATP synthesis and phosphate transport in muscle (Petersen et al, 2005) from insulin resistant individuals and/or patients with T2DM (Fig 1.7). Some groups have hypothesised that these abnormalities are attributable to defects in mitochondria (Lowell & Shulman, 2005). Finally, there have been several recent reports of coordinated changes in genes involved in oxidative phosphorylation (OXPHOS) in obese individuals and patients with T2DM (Mootha et al, 2003; Patti et al, 2003).
During the past few years researchers have proposed the notion that mitochondrial dysfunction and/or impaired oxidative phosphorylation/metabolism contribute to skeletal muscle insulin resistance (Kelley, 2002; Kelley et al, 2002b; Morino et al, 2005; Petersen et al, 2003; Petersen et al, 2004; Petersen et al, 2005). While mitochondria are undoubtedly a central locus of altered metabolic regulation and are associated with the pathogenic processes that underpin insulin resistance, the precise mechanism by which mitochondrial dysfunction contributes to the insulin resistant state has not been elucidated. Indeed, some evidence suggests that mitochondrial dysfunction may not be the primary etiological event causing insulin resistance or T2DM, and that mitochondrial function can be uncoupled from insulin sensitivity, lipid accretion, or both (Boushel et al, 2007; Goodpaster et al, 2001; Helge & Dela, 2003; Ortenblad et al, 2005; Perdomo et al, 2004; Schrauwen-Hinderling et al, 2007; Wredenberg et al, 2006). In support of this contention, Boushel et al. (2007) observed normal mitochondrial function in the skeletal muscle of type 2 diabetic subjects, although mitochondrial respiration was indeed lower in patients with type 2 diabetes than in the healthy control group (Boushel et al, 2007). The results of Boushel and colleagues (2007) have provided strong evidence to show that the missing link connecting muscle mitochondrial function with metabolic disease risk factors is likely to be the level of habitual contractile activity (Hawley & Lessard, 2007).

Mitochondrial dysfunction and decreased mitochondrial density have been linked to a sedentary lifestyle, nutritional overconsumption and metabolic inflexibility (Kelley et al, 2002b; Morino et al, 2005; Petersen et al, 2003; Petersen et al, 2004). In
contrast, recent studies have observed lipid-induced increases of mitochondrial content and fatty acid oxidation in skeletal muscle (Garcia-Roves et al., 2007; Hancock et al., 2008; Kraegen et al., 2008; Turner et al., 2007). Studies that have utilised animals fed a high-fat diet (Turner et al., 2007) or have artificially induced elevated concentrations of circulating FFA (Garcia-Roves et al., 2007), have reported increases in lipid oxidation and the expression of mitochondrial proteins (i.e., PGC1, PPARδ, citrate synthase). Despite increases in mitochondrial content and fatty-acid oxidation induced by a high-fat diet, Hancock (2008) still observed insulin resistance in skeletal muscle as measured by 2-deoxyglucose uptake (H Hancock et al., 2008). The authors concluded that although a high-fat diet induced increases in the capacity of mitochondria to oxidise fat, this occurred during the period in which insulin resistance developed (H Hancock et al., 2008). Therefore, the high-fat diet-induced insulin resistance in skeletal muscle is possibly not due to mitochondrial deficiency (H Hancock et al., 2008). A sedentary lifestyle and disease states such as Type 2 diabetes and cardiovascular disease are associated with decreases in proteins involved in mitochondrial biogenesis (i.e., PGC1, PPARδ) (Bonen, 2009; Patti et al., 2003; Timmons et al., 2006; Wisloff et al., 2005). Patti et al. (2003) observed decreases in the mRNA expression of the transcriptional coactivator peroxisome proliferator-activated receptor γ coactivator-1 (PGC1)α/β in type 2 diabetics and in high-risk nondiabetic subjects with a family history of diabetes. PGC1 has a known role in the transcription of genes involved in both glucose (i.e., Glut4, HKII) and lipid (i.e., CytoC, COX VB) metabolism and mitochondrial biogenesis [see section 1.5.3 (Choi et al., 2008; Handschin et al., 2007; Leick et al., 2008)]. Aberrant control of lipid and carbohydrate
homeostasis (i.e. metabolic inflexibility) and reduced mitochondrial respiratory metabolism (i.e. mitochondrial dysfunction) contribute to the pathogenesis of skeletal muscle insulin resistance (Fig 1.7).

Figure 1.7 Whole-body insulin-stimulated glucose uptake (A) skeletal muscle lipid content (B) mitochondrial capacity, as determined by ATP synthesis, (C) and mitochondrial density (D) in healthy and insulin resistant individuals. Adapted from Petersen et al. 2004 and Morino et al. 2005

While it seems reasonable to suggest that these conditions are mechanistically linked, it could be argued that disruptions to fuel homeostasis and impaired mitochondrial function and their tight association with diminished insulin sensitivity are simply coincidental characteristics of a cohort of individuals genetically programmed to develop all conditions in parallel, but independently to varying degrees (McGarry, 2002). However, it is not presently known whether the metabolic inflexibility observed in
insulin resistant states is an intrinsic defect or is based on extra muscular mechanisms (i.e. the inability to vary extra-cellular fatty acid levels during insulin stimulation).

**The nutrient sensor mammalian target of rapamycin (mTOR)**

The results from recent studies have implicated a role for high-protein intake in the inhibition of insulin signalling (Patti et al, 1998; Tremblay et al, 2005; Um et al, 2006). Almost 40 years ago it was known that obese, insulin resistant subjects had high circulating levels of plasma amino acids and that the infusion of amino acids inhibited muscle glucose uptake as measured across the forearm muscle (Felig et al, 1969; Pozefsky et al, 1969). While it is becoming clear that nutrient excess results in skeletal muscle insulin resistance, there are several “missing links” in establishing causality between nutrient excess and the subsequent down-regulation of skeletal muscle glucose metabolism. It is thought that molecular sensors identify the nutrient excess and attenuate insulin signalling. Moreover, there have been recent implications that over-nutrition directly inhibits insulin signalling at the level of IRS1 through the “hyperactivation” of the mammalian target of rapamycin pathway (Patti et al, 1998; Tremblay & Marette, 2001).

mTOR regulates the activity and function of multiple cellular targets in skeletal muscle via two putative protein complex formations (Bhaskar & Hay, 2007). The rapamycin sensitive mTOR complex 1 (mTORC1) is mTOR bound with GβL (G protein β–subunit Like protein, also called mLST8), regulatory associated protein of mTOR (Raptor), and proline rich Akt substrate of 40 kDa (PRAS40) [Fig 1.8; (Hara et al, 2002; Hay & Sonenberg, 2004; Kim et al, 2002; Yang & Guan, 2007)]. A primary
phosphorylation target of mTORC1 is the threonine (Thr) 389 site of ribosomal protein p70 S6 Kinase (S6K1) (Hornberger et al., 2007; Jastrzebski et al., 2007). Given the ability of S6K1 to both repress IRS1 activity and enhance translation efficiency, mTORC1 may ultimately orchestrate insulin signalling and translational processes in skeletal muscle (Tremblay & Marette, 2001). In contrast, mTOR complex 2 (mTORC2) is rapamycin-insensitive and comprised of mTOR bound to GβL, rapamycin insensitive companion of mTOR (Rictor), and mammalian stress activated protein kinase [SAPK]-interacting protein 1 (mSIN1) [Fig 1.8; (Bhaskar & Hay, 2007; Frias et al., 2006; Jacinto et al., 2006; Sarbassov et al., 2004)].

Figure 1.8 Schematic representation of the molecular components that form each of the dual mTOR complexes and putative factors regulating mTOR complex activity and function. Bars represent inhibition, arrows represent activation. Abbreviations: mTORC, mammalian target of rapamycin complex; Raptor, regulatory associated protein of mTOR; Rictor, rapamycin insensitive companion of mTOR; GβL, G protein β subunit Like protein; PRAS40, proline rich Akt substrate of 40 kDa; mSIN1, mammalian stress activated protein kinase [SAPK]-interacting protein 1; S6K1, ribosomal protein p70 S6 Kinase; 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1.
The mTORC2 complex has been implicated in Akt regulation via phosphorylation at serine (Ser) 473 (Hresko & Mueckler, 2005; Sarbassov et al, 2005). Akt has been described as a critical node of the insulin signalling pathway and its putative metabolic roles include glucose transport and hypertrophy (Taniguchi et al, 2006). Thus, mTORC2 appears to have a crucial role in regulation of insulin stimulated glucose uptake in skeletal muscle. Paradoxically, mTORC2 seems to be a positive regulator of insulin signalling transduction, while mTORC1 has the capacity to negatively regulate insulin action via its inhibitory actions on IRS1.

Mechanistic understanding of processes by which skeletal muscle cells “sense” nutrient status/availability and subsequently conveys molecular responses in the cell has received intense scientific scrutiny in recent years. In this regard, mTOR is emerging as a primary intra-cellular nutrient sensor involved in regulating energy homeostasis, fuel metabolism and cell growth (Krebs et al, 2007; Tzatsos & Kandror, 2006; Zinzalla & Hall, 2008). Moreover, in addition to its pivotal role in insulin-mediated glucose uptake mTOR has also been shown to be a nutrient sensor for amino acids and lipids (Powers, 2008; Um et al, 2006). The mechanism(s) by which mTOR responds to amino acids has yet to be clearly established but recent evidence indicates modulation of the extra- and/or intra-cellular amino acid pools initiate Rag GTPase protein and Ca2+/Camodulin signalling that translocate and activate mTORC1 (Gulati et al, 2008; Hundal & Taylor, 2009; Sancak et al, 2008). This cascade of events may promote the activity of an additional putative intermediary, the class 3 PI3K vacuolar protein sorting mutant 34 (Vps34), which is activated by amino acids and has been associated with prolonged
mTORC1 activation (MacKenzie et al, 2009; Nobukuni et al, 2005). As noted previously, the pathway through which lipids may modify mTORC signalling is currently undefined but a recent study by Portsmann and colleagues (2008) revealed mTORC1-mediated control of sterol-responsive binding protein activity and regulation of lipogenesis (Porstmann et al, 2008). Regardless, adequate nutrient supply is required to stimulate mTORC1 and mTORC2 activity for their essential roles in cell growth and glucose homeostasis in skeletal muscle. Conversely, with nutrient excess the contrasting functions of each mTOR complex appear incompatible and ultimately result in skeletal muscle insulin resistance.

*mTORC1: a negative regulator of insulin signal transduction with over-nutrition*

In line with its proposed role as a nutrient sensor, results from previous studies demonstrate that the complex formation and activation of mTORC1 is a process sensitive to growth factors (e.g. insulin, insulin-like growth factors), amino acids (AA), glucose, FA, and ATP levels (Crozier et al, 2003; Dennis et al, 2001; Fang et al, 2001; Kim et al, 2002; Lang, 2006; Mordier & Iynedjian, 2007; Sun et al, 2008; Yeshao et al, 2005). Increases in AA availability have been shown to promote the interaction between raptor and mTOR (mTORC1), thereby enhancing the ability of mTORC1 to phosphorylate its well characterised downstream substrates, S6K1 and eukaryotic translation initiation factor 4E-binding protein 1 [4EBP1; (Hay & Sonenberg, 2004; Kim et al, 2002; Um et al, 2006)]. Moreover, recent studies indicate that mTORC1 activation may also alter insulin action in response to increased FA and AA availability [Fig 1.9; (Crozier et al, 2003; Khamzina et al, 2005; Korsheninnikova et al, 2006; Mordier & Iynedjian, 2007; Tremblay
et al, 2007; Um et al, 2004). There is evidence that mTORC1 activation may induce insulin resistance in skeletal muscle and that inhibition of mTOR or its downstream targets may improve insulin action (Tremblay & Marette, 2001). In support of this contention, mTORC1 activation is elevated in the skeletal muscle of high-fat fed, insulin resistant rats (Khamzina et al, 2005). In response to high-fat feeding Khamzina et al. (2005) observed increased mTORC1 activation associated with elevated phosphorylation of mTOR’s downstream target, S6K1 and consequently, elevated inhibitory Ser636/639 phosphorylation of IRS1 in skeletal muscle (Khamzina et al, 2005).

The notion that mTORC1 activation is involved in FA-induced insulin resistance is also supported by the observation that transgenic S6K1 knockout mice (S6K/-) are protected against high-fat diet induced insulin resistance (Um et al, 2004). Enhanced insulin sensitivity in S6K/- mice is accompanied by reduced Ser636/639 phosphorylation of IRS1 in skeletal muscle (Um et al, 2004). In addition, the results of studies using rapamycin treatment demonstrate S6K1 phosphorylation and its inhibition of insulin signal transduction was decreased even in the presence of increased nutrient availability (Berg et al, 2002; Krebs et al, 2007; Tremblay & Marette, 2001). Moreover, the down-regulation of mTORC1 activation with acute rapamycin treatment prevented insulin resistance induced by chronically high insulin levels (Berg et al, 2002) and AA exposure in L6 myotubes (Tremblay & Marette, 2001). The maintenance of insulin sensitivity in rapamycin treated cells has been attributed to an increased IRS1-associated PI3-kinase activation (Tremblay & Marette, 2001). Such an observation is of potential clinical significance because attenuated PI3-kinase activity is associated with impairments in
skeletal muscle from type 2 diabetic and obese insulin resistant rodents (Lessard et al, 2007) and humans (Bjornholm & Zierath, 2005), implicating mTORC1 as a pivotal regulator of insulin action.

**mTORC2: a regulator of insulin signal transduction via Akt**

It is apparent that mTORC1 has the ability to negatively regulate insulin action via its control of inhibitory actions on IRS1. The precise effect that mTORC2 may have on insulin signal transduction is, however, not completely understood. Studies utilising a loss of function approach to inhibit mTOR activity by suppressing the activity of mTOR and Raptor (mTORC1), but not Rictor (mTORC2), prevented the activation of the mTORC1 substrate S6K1 (Thoreen & Sabatini, 2009). These results demonstrate that mTORC1 and mTORC2 are distinct complexes that have diverse cellular functions. An established role for mTORC2 is as an effector of actin cytoskeleton organisation through a downstream substrate PKCα (Jacinto et al, 2004; Sarbassov et al, 2004). In addition, another role for mTORC2 is its modulation of insulin action. Sarbassov and colleagues (2005) were the first to demonstrate that mTORC2 is the kinase that phosphorylates and activates Akt on Ser473 (Sarbassov et al, 2005). In support of this finding, Hresko and Mueckler (2005) utilised siRNA-induced knockdown of Rictor in 3T3-L1 adipocytes and revealed that mTORC2 is responsible for the insulin-induced Akt Ser473 phosphorylation (Hresko & Mueckler, 2005). Furthermore, when Rictor was deleted in skeletal muscle there was a decrease in insulin-stimulated muscle glucose uptake, most likely due to decreases in Ser473 phosphorylation and subsequent activation of Akt and its downstream substrate AS160 (Kumar et al, 2008). Thus, mTORC2–Akt interactions are
critical to our current understanding of glucose homeostasis and are an influential component of insulin action. Akt is part of the AGC protein kinase family expressed in two isoforms in skeletal muscle. It has been proposed that Akt2 (in conjunction with upstream regulation by IRS1) plays a predominant role in the regulation of insulin-stimulated glucose uptake, while Akt1 is involved in the regulation of muscle hypertrophy and subsequent protein synthesis/degradation (Cleasby et al, 2007; Kim et al, 2000). In a study examining isoform specific activation of Akt, Cleasby and colleagues (2007) electroporated constitutively active-Akt (ca-Akt) isoforms into skeletal muscle (Cleasby et al, 2007). These workers found a 200% increase in the Thr389 phosphorylation of S6K1 in the ca-Akt1 electroporated rats versus 53% in ca-Akt2 skeletal muscle (Cleasby et al, 2007). Other studies have also shown that Akt1, via upstream signalling events initiated by IRS2, is also an important regulator of lipid metabolism in skeletal muscle (Bouzakri et al, 2006). Specifically, Bouzakri et al. (2006) identified a role for the IRS2/Akt1 pathway in the regulation of basal palmitate uptake and the insulin-induced suppression of β-oxidation (Bouzakri et al, 2006). Taken collectively, these results suggest that mTORC2 may mediate effects on fatty acid metabolism and glucose transport in skeletal muscle via its ability to activate Akt1 and Akt2, independently. In this regard, a dual role for mTOR in the regulation of both FA and glucose metabolism was proposed by Sipula et al. (2006), who found that high-dose (24-72 h) treatment of L6 myotubes with rapamycin [which inhibits both mTORC1 and mTORC2 (Sarbassov et al, 2006)] decreased insulin-stimulated glucose uptake, and increased fatty acid oxidation (Sipula et al, 2006). These workers suggested that
excessive rapamycin treatment attenuated the ability of insulin to increase glucose uptake and suppress fatty acid oxidation by inducing an inappropriate “fasting” metabolic phenotype in these cells (Sipula et al, 2006). However, the effects of rapamycin-mediated mTOR inhibition on Akt activation were not examined and it is currently unknown whether mTORC2 differentially regulates activation of the Akt1 and Akt2 isoforms in order to mediate its effects on fatty acid and glucose metabolism, respectively.

**Figure 1.9** Simplified putative mTOR signalling pathways in response to over-nutrition which promote negative effects on skeletal muscle insulin sensitivity and metabolic adaptation. Bars represent inhibition, arrows represent activation. Chronic nutrient excess stimulates persistent mTORC1-S6K activity and subsequent attenuation of IRS activity, inhibiting mTORC2-mediated insulin-dependent glucose uptake. Abbreviations: IR, insulin receptor; IRS, insulin receptor substrate; PI3K, phosphatidylinositol 3-kinase; PDK1, phosphoinositide-dependent kinase 1; AS160, AKT substrate of 160 kDa; Glut4, glucose transporter; TSC2, tuberous sclerosis complex-2; mTORC, mammalian target of rapamycin complex; S6K, p70 ribosomal protein S6 kinase.
1.5 Exercise as a treatment of skeletal muscle ‘metabolic inflexibility’.

Overview

The case for a causal link between the rise in physical inactivity during the past century and the increase in a cluster of prevalent metabolic disease states is compelling (Mootha et al, 2003; Myers et al, 2002; Zimmet et al, 2001). Epidemiological studies of subjects with and without coronary artery disease have demonstrated that low aerobic exercise capacity is a strong independent predictor of mortality (Kavanagh et al, 2003). Bruce et al. (2003) observed, in individuals with type 2 diabetes, that whole body insulin sensitivity is best predicted by skeletal muscle oxidative capacity, rather than measures of lipid or carbohydrate status (Bruce et al, 2003). In insulin-resistant elders, there is a 40% reduction in mitochondrial oxidative and phosphorylation activity, largely attributable to impaired skeletal muscle glucose metabolism, while defects in insulin-stimulated mitochondrial density and adenosine trisphosphate (ATP) flux have been observed in skeletal muscle from insulin-resistant offspring of parents with type 2 diabetes (Hawley & Lessard, 2007; Petersen et al, 2005). As noted previously, in patients with type 2 diabetes, a low aerobic capacity is associated with a coordinated reduction in the expression of a subset of genes involved in oxidative phosphorylation (Mootha et al, 2003).

Exercise is one of the most effective therapeutic interventions for enhancing skeletal muscle insulin sensitivity (Hawley, 2004; Hawley & Lessard, 2008; Pedersen & Saltin, 2006). A single bout of aerobic exercise increases insulin stimulated glucose
uptake for up to 48 hr, predominantly in the specific muscle groups recruited during exercise (Wojtaszewski & Richter, 2006). Musi et al. (2001) observed ‘normal’ aerobic exercise-induced glycogen utilisation in type 2 subjects versus healthy controls and a large decrease in blood glucose levels in response to a single bout of aerobic exercise (45 min) (Musi et al, 2001). An important mechanism by which exercise training improves whole-body glucose uptake is through improved insulin action in skeletal muscle (Hawley & Lessard, 2008; Wojtaszewski & Richter, 2006). The exercise-induced enhancements of insulin action in skeletal muscle may occur through multiple mechanisms including improvements in the activation and expression of components of the insulin signalling cascade, increased glucose transporter expression and translocation to the plasma membrane, and increased mitochondrial biogenesis (Hawley et al, 2006; Hawley & Holloszy, 2009; Hawley & Lessard, 2007; Hawley & Lessard, 2008; Wojtaszewski & Richter, 2006).

1.5.1 Exercise induced insulin sensitivity

The observation that endurance exercise training increases both insulin-dependent and independent glucose transport in skeletal muscle is well-established (Garetto et al, 1984; Holloszy, 2005; Holloszy & Narahara, 1965; Ivy et al, 1983; Richter et al, 1982; Richter et al, 1984; Wallberg-Henriksson et al, 1988). Similar observations regarding endurance exercise-induced improvements in skeletal muscle glucose transport have been made in insulin-resistant rats (Etgen et al, 1997), diabetic rats (Wallberg-Henriksson & Holloszy, 1984) and in humans with insulin resistance and type 2 diabetes (Kirwan et al, 2009).
The acute increase in skeletal muscle glucose transport in response to a bout of endurance exercise or muscle contraction may be mediated by a variety of intramyocellular signalling events including AMPK activation, Akt phosphorylation, nitric oxide production, IL6 and calcium-mediated mechanisms involving Ca2+/ CaMK and PKC (Febbraio & Pedersen, 2005; Hawley & Lessard, 2008; Jessen & Goodyear, 2005; Sakamoto & Goodyear, 2002; Sakamoto et al, 2002). However, the insulin-sensitising effects of an acute aerobic exercise bout are short-lived, and may only persist for up to 48 hr if another bout of aerobic exercise is not undertaken (Etgen et al, 1993; Ivy et al, 1983). In contrast, chronic exercise training may produce metabolic adaptations that result in sustained improvements in whole-body and muscle insulin sensitivity (Hawley, 2004; Hawley & Lessard, 2008; Pedersen & Saltin, 2006; Wojtaszewski & Richter, 2006).

In this regard, Houmard et al. (2004) demonstrated a “dose-response” relationship between volume of aerobic exercise training (min/week) and insulin sensitivity in sedentary, obese and overweight individuals that were randomly assigned to endurance exercise programmes of varying volumes and intensities for 6 months (Houmard et al, 2004). The investigators concluded that an aerobic exercise prescription of 170 min of walking or jogging per week was more effective than 115 min for improving whole-body insulin sensitivity, regardless of exercise intensity, although both volumes of exercise improved insulin sensitivity compared to sedentary controls (Houmard et al, 2004). Furthermore, Hansen et al. (2009) recently compared the benefits of six months of aerobic-based, low- to moderate-intensity aerobic exercise training with those of a moderate- to high intensity programme of the same duration (Hansen et al, 2009).
the low- to moderate-intensity and moderate- to high intensity endurance exercise groups had equal improvements in markers of whole-body metabolic health (ie. HbA1c, LDL-cholesterol, BMI etc.), although the authors concluded that high-intensity aerobic exercise training programmes may yield more favourable results than programmes employing low- to moderate-intensity exercise (Hansen et al, 2009).

Exercise-induced increases in muscle insulin sensitivity may be attributed to increased expression and/or activity of signalling proteins involved in the regulation of skeletal muscle glucose uptake (Bjornholm & Zierath, 2005; Hawley & Lessard, 2008). Perhaps the most consistently observed effect of exercise training in healthy and insulin resistant skeletal muscle is increased expression of GLUT4 protein (Hawley & Lessard, 2008; Holloszy, 2005; Wojtaszewski & Richter, 2006). In addition, insulin sensitivity following aerobic exercise training in previously sedentary men (Houmard et al, 1999), sedentary rats (Bernard et al, 2005) and insulin resistant rats (Lessard et al, 2007) was associated with increased insulin-stimulated PI-3 kinase activity. However, Houmard et al. (1999) observed no known mechanism for this increase, as it was not accompanied by increased protein levels of PI-3 kinase or upstream components of the insulin-signalling cascade (see Fig 1.3).

1.5.2 Exercise induced AMPK activation

Upregulation of the AMPK pathway is one potential mechanism by which exercise training can improve the metabolic inflexibility of skeletal muscle. In addition to acute activation of AMPK due to muscle contraction (or AICAR treatment), aerobic exercise training may result in an increase in AMPK protein levels. In healthy individuals,
three weeks of endurance training increased the skeletal muscle protein content of the AMPK α1, β2 and γ1 subunits (Frosig et al, 2004). Similarly, seven weeks of aerobic exercise training (treadmill running) in obese Zucker rats resulted in a 1.5-fold increase in AMPKα1 protein expression and restored impaired AMPK activation to the level of lean controls (Sriwijitkamol et al, 2006). Pold et al (2005) observed that eight weeks of treadmill running in ZDF rats produced similar improvements in insulin sensitivity as daily AICAR administration (Pold et al, 2005). However, unlike leptin-deficient (ob/ob mouse) and leptin receptor-deficient (fa/fa Zucker rat) rodent models of diabetes (Yu et al, 2004), humans with type 2 diabetes do not exhibit decreased AMPK subunit expression or activation compared to healthy controls (Wojtaszewski et al, 2005). Even so, Wojtaszewski et al. (2005) observed a comparable increase in the expression of the α1, β2 and γ3 subunits of AMPK in response to 6 weeks of resistance training in type 2 diabetic individuals and healthy controls (Wojtaszewski et al, 2005).

It has been established that endurance exercise training induces a chronic upregulation of AMPK (Frosig et al, 2004; Lessard et al, 2007; Sriwijitkamol et al, 2006; Wojtaszewski et al, 2005). Furthermore, it has been previously described that chronic activation of AMPK through AICAR results in improved skeletal muscle insulin sensitivity (Hayashi et al, 1998). Thus, it is plausible that the insulin-sensitising effects of exercise training are, in part, attributable to the increased skeletal muscle AMPK protein levels and subsequent activation. Chronic AMPK activation, as seen with exercise training, may act to improve insulin sensitivity by regulating the expression of specific genes involved in glucose and lipid homeostasis (Jorgensen et al, 2006). AMPK activation through daily
AICAR injections (1 mg/g) for 4 weeks was associated with increased expression of GLUT4, hexokinase II (HKII) and mitochondrial proteins (i.e. citrate synthase, cytochrome C) in rodent skeletal muscle (Winder, 2000; Zheng et al, 2001). It is also possible that exercise-induced upregulation of AMPK mediates its effects through distal components of the insulin-signalling cascade. Recently, AICAR was shown to induce AS160 phosphorylation in mouse skeletal muscle, by a mechanism that was independent of insulin, but at least partly attributable to the AMPKα2 isoform (Bruss et al, 2005; Kramer et al, 2006a; Treebak et al, 2006). However, AICAR does not mimic every biochemical adaptation to acute and chronic endurance exercise. Booth & Laye (2009), in a recent review, denoted that many important biochemical processes altered with exercise either were unchanged with AICAR treatment or had the converse result (Booth & Laye, 2009). Interestingly, it was also observed that muscle contraction stimulated AS160 phosphorylation by an unknown mechanism that did not involve AMPK or Akt activation (Bruss et al, 2005; Kramer et al, 2006a).

1.5.3 Effects of exercise on increased skeletal muscle oxidative capacity

Overview

Endurance exercise training results in an increase in the oxidative capacity of skeletal muscle by increasing the expression of proteins involved in mitochondrial biogenesis such as PGC1, PPARα and nuclear respiratory factor 1 (NRF1) (Gollnick & Saltin, 1982; Irrcher et al, 2003a). Oxidative enzyme capacity is low in individuals with insulin resistance, which is thought contribute to a “metabolic inflexibility” (Kelley,
This inflexibility, in turn is thought to contribute to the aberrant skeletal muscle glucose and lipid metabolism that is associated with insulin resistance and type 2 diabetes. Furthermore, the maximal activities of several skeletal muscle oxidative enzymes (i.e. citrate synthase) are good predictors of whole-body insulin sensitivity, suggesting that treatments that increase oxidative capacity may also improve insulin sensitivity (Bruce et al, 2003). In support of this contention, Goodpaster et al. (2003) demonstrated that the strongest predictor of insulin sensitivity following endurance training in obese individuals was enhanced lipid oxidation (Goodpaster et al, 2003). Increased oxidative capacity following aerobic exercise training was recently associated with increased CPT1 activity and decreased ceramide and DAG content in the muscle of obese individuals (Bruce et al, 2007). The findings by Bruce et al. (2006) suggest that aerobic exercise training may improve muscle insulin sensitivity by increasing the proportion of lipids targeted for oxidation, thereby reducing the accumulation of lipid species that are known to inhibit insulin signal transduction (Bruce et al, 2007).

Although there appears to be a strong association between improved muscle oxidative capacity and whole-body insulin sensitivity, this relationship may be age-dependent. A cross-sectional study examining individuals aged between 22-87 years, found that although oxidative capacity increased to a similar degree following 16 weeks of aerobic training in all age groups, this was not associated with an increase in insulin sensitivity in individuals over 60 years of age (Short et al, 2003). Similar results were obtained by others who observed an increase in oxidative capacity following 12 weeks of aerobic exercise training, but only a trend (P=0.14) toward increased insulin
sensitivity in older individuals (67.3 ± 0.7 years) (Pruchnic et al, 2004). The observations of Short et al. (2003) and Pruchnic et al. (2004) suggest that insulin resistance associated with obesity and type 2 diabetes may involve different mechanisms than age-induced insulin resistance (Pruchnic et al, 2004).

**Increased mitochondrial biogenesis and fibre type switching**

Endurance exercise training highly induces increases in the size, number and oxidative capacity of mitochondria (Fig 1.10) (Holloszy, 1967; Holloszy, 2008). This mitochondrial biogenesis is a well established muscle adaptation that can be easily observed with the use of electron microscopy (Lumini et al, 2008). A single bout of exercise is a sufficient stimulus to induce mitochondrial biogenesis and this mitochondrial proliferation can be maintained by repeated bouts of exercise (Baar et al, 2002; Hawley & Holloszy, 2009). Baar et al. (2002) observed that after a single bout of aerobic exercise there was an increase in the mRNA and protein expression of the transcriptional coactivator PGC1 (Baar et al, 2002). A crucial role, of the transcriptional coactivator PGC1, in the induction of mitochondrial biogenesis was demonstrated by overexpressing PGC1 in muscle cells and transgenic mice (Joseph et al, 2006). Wu et al. (1999) observed that overexpression of PGC1 in myotubes induced mitochondrial biogenesis and increased the expression of genes involved in oxidative phosphorylation (eg. COXI and IV) (Wu et al, 1999). Furthermore, the overexpressed PGC1 transgenic mice were observed to have a transition in skeletal muscle with their white muscle having a more red muscle phenotype (Table 1.1) (Joseph et al, 2006). Both a single bout and chronic aerobic training are known to increase gene and protein expression of PGC1
in the skeletal muscle of both humans and rats (Bonen, 2009; Irrcher et al, 2003b; Russell et al, 2003; Terada et al, 2002; Terada et al, 2005; Terada & Tabata, 2004). However recent studies have questioned if PGC1 is required for exercise-induced adaptive responses in skeletal muscle (Bonen, 2009; Leick et al, 2008). Leick et al. (2008), using a transgenic mouse model of PGC1α knockout, observed a decreased pre-exercise expression of mitochondrial proteins (ie. CytoC, COX1) but aerobic exercise training increased the fold-change expression of mitochondrial proteins to the same level as wild-type mice (Leick et al, 2008). Although there are some questions about the extent of PGC1’s role in exercise training-induced adaptations of skeletal muscle the importance of this protein for the determination of fibre type composition is of known importance (Bonen, 2009; Handschin et al, 2007; Lin et al, 2002; Wu et al, 1999). Additionally, exercise induces the conversion of muscle fibers from the more glycolytic type 2x (humans/rodents) and/or 2b (rodents) (white, glycolytic, fast twitch) to the more oxidative type 2a (white, oxidative, medium twitch), that has a more type 1 phenotype (red, oxidative, slow twitch) (Table 1.1) (Garcia-Roves et al, 2006; Puigserver, 2005; Richter et al, 2008; Saltin et al, 1977). Type 1 fibers are characterised by an increased mitochondrial number and oxidative potential as well as a specific set of contractile proteins such as troponin I slow and myoglobin (Table 1.1) (Puigserver, 2005; Saltin et al, 1977). The ability to “shift” skeletal muscle fibres from a more glycolytic fibre to a more oxidative fibre could have a profound effect on metabolic health.
Recent reports have shown that obese and insulin resistant individual have a lower ratio of Type 1 (red muscle) to Type 2 (white muscle) skeletal muscle fibres (He et al, 2001). In contrast, highly trained endurance athletes have a higher ratio of Type 1 to Type 2 skeletal muscle fibre (Zierath & Hawley, 2004). Differences in the physiological properties and function of red and white skeletal muscle have been well characterised [Table 1.1 (Hickson et al, 1976; Maltin et al, 1989; Vandenberghe et al, 1999)]. Red muscle has a higher oxidative capacity, partly because of higher concentrations of mitochondria, and is more insulin sensitive than white muscle (James et al, 1986; Pande, 1971). Additionally, in response to aerobic exercise training several kinases have been
found to be differentially activated in red and white skeletal muscle (Ljubicic & Hood, 2008; Nader & Esser, 2001). For example, Ljubicic & Hood (2008) demonstrated that *in situ* contraction-induced oxygen consumption stimulated Akt activation in white, but not red muscle (Ljubicic & Hood, 2008). The authors concluded in this study that the oxidative capacity of the skeletal muscle determines the activation of intracellular signalling as muscle oxygen consumption increases (Ljubicic & Hood, 2008). Of interest, the most evident change in the increase of postexercise mitochondrial content is observed in white muscle, which usually has only between 1-3% of the total cell volume (Hood et al, 2006).

Skeletal muscle displays remarkable changes in mitochondrial oxidative capacity as an adaptive response to exercise and contractile activity. Interestingly, it was recently observed in obese, sedentary individuals that aerobic exercise and weight loss, but not weight loss alone, increased mitochondrial capacity despite decreases in intramyocellular lipids in both groups (Toledo et al, 2008). Taken together, this data provides evidence that exercise is an important therapy for increases in mitochondrial size, number and function and in the treatment metabolic disease (see Fig 1.10 for overview of exercise induced mitochondrial biogenesis).
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Table 1.1 Characteristics of Skeletal Muscle Fibre Types. Adapted from Saltin et al. 1977. Abbreviations: FT, fast-twitch; ST, slow-twitch; FG, fast, glycolytic; FOG, fast, oxidative, glycolytic; SO, slow, oxidative; FF, fast-contracting, fast-fatigue; FR, fast-contracting, fatigue-resistant; S, slowly contracting.
1.5.4 Effect of exercise on β-Adrenergic signalling

The sympathoadrenal system is hypothesised to have a significant role in fuel mobilisation and subsequent utilisation in skeletal muscle during and after exercise (Borsheim et al, 1998; Hawley et al, 2006). This effect is likely mediated by the release of catecholamines (eg. norepinephrine and epinephrine) into the circulation. In this regard, Galbo (1983) observed that plasma epinephrine levels increased linearly with exercise duration (Galbo, 1983). It was first shown in 1930 that the infusion of even low doses of epinephrine highly stimulated fuel metabolism in humans (Borsheim et al, 1998). Epinephrine (also known as Adrenaline) is a key stimulator of lypolysis and glycogenolysis through β-adrenergic signalling (Borsheim et al, 1998). Catecholamines stimulate the β-adrenergic receptor (β-AR) which leads to the activation cAMP and PKA. PKA subsequently serine phosphorylates HSL on the Ser 563/659/660 residues, for the induction of lypolysis, or glycogen phosphorylase (GPh), to stimulate glycogenolysis [Fig 1.11 (Hawley et al, 2006)].

The stimulation of β-adrenergic signalling in muscle via catecholamine release during exercise is responsible for mediating many chronic training-induced adaptations (Kjaer, 1998; Kraus et al, 1989; Zouhal et al, 2008) and plays an important role in the regulation of muscle metabolism. Specifically, β-adrenergic stimulation regulates lipid turnover in muscle via its activation of HSL, which has been linked to skeletal muscle insulin sensitivity (Mulder et al, 2003). Additionally, β-adrenergic stimulation in soleus muscle while incubated in the presence of epinephrine or with electrically-stimulated contraction induces GPh activity (Langfort et al, 1998). Kraus et al. (1989) observed that
continuous contractile activity of skeletal muscle results in elevated levels of β-AR in skeletal muscle, which coincides with increased expression of genes that encode proteins important for oxidative metabolism (Kraus et al, 1989).

The independent activation of the AMPK and β-adrenergic signalling pathways are also responsible for promoting the transcription of several genes that are important in the regulation of skeletal muscle glucose and lipid metabolism, including the glucose transporter (GLUT4), uncoupling protein (UCP3) and fatty acid transporter (FAT/CD36) (Chabowski et al, 2006; Lessard et al, 2009; Maxwell et al, 2005). Recently, the expression of the NR4A orphan nuclear receptors subfamily, a downstream substrate of β-adrenergic signalling, has been shown to be highly upregulated by exercise and AICAR (Kanzleiter et al, 2009; Kawasaki et al, 2009b; Lessard et al, 2009). Although the exact roles of NR4A receptors in skeletal muscle metabolism are as yet understood, a particular member, Nur77 (NR4A1), has recently been revealed to have an important function in the expression of genes associated with glucose and fatty acid utilisation (e.g. Glut4, FAT/CD36, PGC1, lipin1) (Chao et al, 2007; Lessard et al, 2009; Maxwell et al, 2005). Kanzleiter et al. (2009) observed a reduction in skeletal muscle Nur77 mRNA expression after high-fat feeding and a reversal of this effect with exercise training (Kanzleiter et al, 2009). Furthermore, in the low- and high-running capacity rat model (described in section 1.4.1) there was an observed reduction in Nur77 mRNA and protein expression that was coupled with the decreases of its target genes (FAT/CD36, UCP3, AMPK γ3) in LCR when compared to HCR (Lessard et al, 2009). These data suggest that there is likely to be an important role in exercise induced muscle adaptation
through the β-adrenergic signalling pathways and that NR4A receptors that have a potential utility in the treatment of diseases associated with the metabolic syndrome [Fig 1.11 (ie. insulin resistance, metabolic inflexibility, obesity etc.)].

Figure 1.11 β–adrenergic induced lipolysis, glycogenolysis, and transcription in adipose and skeletal muscle in response to exercise and fasting. Abbreviations: TG, triacylglycerol; FA, fatty-acid; HSL, hormone sensitive lipase; PKA, protein kinase A; cAMP; cyclic AMP; β–AR, β–adrenergic receptor; Nur77, nuclear orphan receptor 77; Glut4, glucose transporter 4; FAT/CD36, fatty acid translocase; UCP3, uncoupling protein 3; GPh, glycogen phosphorylase; P, phosphorylation.

1.5.5 Modulation of mTOR signalling with exercise

Endurance- and resistance-type exercise has been shown to enhance insulin sensitivity and training-specific adaptation in skeletal muscle (Coffey & Hawley, 2007; Hawley & Lessard, 2008; Holten et al, 2004; Houmard et al, 2004; Ishii et al, 1998; Wojtaszewski & Richter, 2006; Yaspelkis, 2006). Similarly, acute increases in mTOR phosphorylation are observed in the hours following bouts of diverse contractile activity.
associated with both aerobic endurance and heavy resistance exercise (Bolster et al, 2003; Dreyer et al, 2006; Mascher et al, 2007; Mascher et al, 2008). Indeed, Coffey et al. (2009) observed similar responses in mTOR phosphorylation during the post exercise recovery period following bouts of endurance and resistance exercise (Coffey et al, 2009). While there is a paucity of data regarding changes in mTOR activity and protein content with chronic exercise available evidence indicates that mTOR phosphorylation is also increased following prolonged training (Benziane et al, 2008; Leger et al, 2006). Moreover, endurance and resistance exercise appear equally capable of enhancing mTOR activity and the diverse contractile stimuli may initiate both reciprocal and independent pathways to promote specific adaptation in skeletal muscle.

Modulation of energy and fuel status with regular bouts of exercise generates elevated glucose transporter protein content and activity in skeletal muscle (Rockl et al, 2008). Moreover, a principal contraction-induced pathway important for glucose transport that is enhanced following exercise is altered Glut4 activity via activation by AS160 [Fig 1.12; (Arias et al, 2007; Kramer et al, 2006b)]. In addition, Akt interactions with adaptor protein containing PH domain, PTB domain, and leucine zipper motif 1 (APPL1) may also regulate Glut4 in skeletal muscle (Saito et al, 2007).
Figure 1.12 Simplified putative mTOR signalling pathways in response to exercise, which promote positive effects on skeletal muscle insulin sensitivity and metabolic adaptation. Bars represent inhibition, arrows represent activation. Exercise promotes mTOR activation: question marks (?) denote our current lack of knowledge regarding the specific exercise-induced complex activation for enhanced translation (mTORC1) and glucose uptake (mTORC2), respectively. Abbreviations: IR, insulin receptor; IRS, insulin receptor substrate; PI3K, phosphatidylinositol 3-kinase; PDK1, phosphoinositide-dependent kinase 1; AS160, AKT substrate of 160 kDa; Glut4, glucose transporter; TSC2, tuberous sclerosis complex-2; mTORC, mammalian target of rapamycin complex; S6K, p70 ribosomal protein S6 kinase; 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1.

Notably, despite the critical role of mTORC2 in regulating Akt activity a causative link with subsequent Akt-AS160 or -APPL mediated glucose transport following exercise has not been fully elucidated. However, given that Rictor deletion impairs glucose transport (Kumar et al, 2008) and that the mTOR pathway may also regulate glucose transporter 1 mediated glucose uptake (Buller et al, 2008), mTOR represents an important putative mediator of enhanced insulin action in response to exercise. Indeed, resistance exercise shown to increase acute leg glucose uptake is associated with elevated mTOR Akt-AS160
phosphorylation (Dreyer et al, 2008; Dreyer et al, 2006). Likewise, changes in Akt-AS160 signal transduction following acute and chronic endurance exercise closely correlate with enhanced glucose transport and uptake (Arias et al, 2007; Frosig et al, 2007; Howlett et al, 2008; Lessard et al, 2007). Thus, increased mTOR activity with resistance and endurance exercise likely promotes enhanced glucose kinetics and metabolic status.

In summary, there are several mechanisms by which exercise training may improve skeletal muscle glucose uptake. These include chronic activation of AMPK, facilitation of insulin signal transduction and Glut4 expression, upregulation of the β-Adrenergic pathway, regulation of mTOR signalling, as well as increases in the expression of several proteins involved in glucose and lipid utilisation and turnover. Furthermore, exercise training has the potential to ameliorate several other conditions associated with the metabolic syndrome, including obesity, hypertension and cardiovascular disease, and is not associated with adverse metabolic side-effects (Hawley & Holloszy, 2009; Pedersen & Saltin, 2006). Therefore, exercise training is an effective therapy for the treatment of insulin-resistance and metabolic inflexibility in skeletal muscle.
CHAPTER TWO: Aims of Thesis
2.1 Aims of the Thesis

The review of the literature (Chapter one) documents that both lifestyle (nutrition and physical activity) and genetic factors may contribute to the regulation/dysregulation of substrate metabolism in skeletal muscle. It has been shown that a key component to impaired skeletal muscle substrate metabolism is the inability to transition between fuels in response to hormonal signals (metabolic inflexibility). However, the contributions of nutrition, physical activity and genetics and how they are interconnected with metabolic inflexibility/flexibility in skeletal muscle is unknown. Specifically, the exact mechanisms that cause or improve skeletal muscle metabolic inflexibility are unidentified. Accordingly, the primary aim of the investigations undertaken for this thesis was to enhance our understanding of the mechanisms of nutrition, genetics and exercise in skeletal muscle flexibility/inflexibility.

The first study (Chapter three), using a genetic model of high and low oxidative capacity, investigated if intrinsic aerobic capacity conferred metabolic inflexibility/flexibility in skeletal muscle. Furthermore, the possible contributing molecular factors (eg. mitochondrial density, insulin signalling etc.) leading to these states were determined. The second study (Chapter four) examined if exercise training could improve the metabolic inflexibility/flexibility in the skeletal muscle of these divergent animal models. In addition, the molecular mechanism(s) (eg. insulin signalling, β–adrenergic signalling etc.) associated with exercise training and metabolic health were examined to determine their contributions to improved substrate metabolism.
Finally, in the third experimental chapter (Chapter five), the mTOR signalling cascade was then investigated to ascertain its contribution to skeletal muscle metabolic inflexibility/flexibility in response to over-nutrition and exercise training. Using *in vivo* and *in vitro* experiments, the aim of this investigation was to characterise the role of two key molecular nutrient sensors, mTOR and AMPK, in skeletal muscle glucose metabolism and insulin resistance.
CHAPTER THREE: Artificial selection for high aerobic running capacity is associated with enhanced insulin sensitivity and increased substrate oxidation in skeletal muscle.

3.1 Introduction

The prevalence of the metabolic syndrome has reached epidemic proportions and is linked with the global epidemic of obesity and diabetes (Eckel et al, 2005). The metabolic syndrome is characterised by metabolic abnormalities including, glucose intolerance, insulin resistance, obesity, and dyslipidaemia (Eckel et al, 2005). The rising incidence of the metabolic syndrome and therefore the elevated risk for diabetes and cardiovascular disease can, in part, be attributed to changes in environmental factors such as human behaviour and lifestyle (Zimmet et al, 2001). However, studies of healthy or insulin resistant first degree relatives of patients with type 2 diabetes have shown there is also a strong genetic component to this disease (Petersen & Shulman, 2006; Ukropcova et al, 2005; Ukropcova et al, 2007). The importance of a genetic contribution to abnormal metabolism is highlighted by the observation that decreased insulin sensitivity, impaired mitochondrial activity and increased fasting plasma fatty-acid concentrations have all been found among the offspring of type 2 diabetics (Perseghin et al, 1997; Petersen et al, 2004).

Skeletal muscle makes an important contribution to whole-body energy metabolism, comprising ~45% of the body mass and accounting for ~80% of a postprandial glucose load (DeFronzo et al, 1985; Zierath & Hawley, 2004). Whole-body metabolic health is associated with the ability of skeletal muscle to adapt fuel oxidation to fuel availability, a process described as “metabolic flexibility” (Galgani et al, 2008). In several lifestyle-related diseases such as obesity and type 2 diabetes, there is a loss of
skeletal muscle plasticity or “metabolic inflexibility” such that rates of substrate oxidation do not increase effectively in response to fuel availability (Galgani et al, 2008; Kelley, 2005; Kelley & Mandarino, 2000).

Muscle oxidative capacity coupled with an enhanced ability of muscle to oxidise lipids are strong predictors of insulin sensitivity (Bruce et al, 2003; Schrauwen-Hinderling et al, 2007). In support of this premise, several groups have reported that skeletal muscle mitochondrial content, mitochondrial function and/or oxidative capacity are reduced in individuals that are insulin resistant or have type 2 diabetes (T2DM) (Holloway et al, 2007; Kelley et al, 2002a; Schrauwen-Hinderling et al, 2007). Taken collectively, the results from these studies suggest that the lower oxidative capacity observed in individuals with obesity, T2DM, or both, may play a significant functional role in the development of insulin resistance. However, it is not known to what extent low oxidative capacity in muscle and the concomitant metabolic inflexibility are a result of genetically predetermined or environmental factors.

Recently, a genetic component to metabolic flexibility has been observed in muscle homogenates and skeletal muscle cells of humans with a family history of type 2 diabetes (T2DM) (Ukropcova et al, 2005; Ukropcova et al, 2007). Furthermore, Morino et al. (2005) reported a lower mitochondrial density in insulin-resistant muscle in offspring of people with T2DM (Morino et al, 2005), suggesting that reduced oxidative capacity may represent an early stage in the progression of insulin resistance, and that low oxidative capacity may be an inherited defect in such people. Indeed rates of lipid
oxidation at rest have been observed to be persistently low even in formerly obese people who subsequently lose weight (Ranneries et al, 1998; Thyfault et al, 2004).

In order to identify the genetic contribution to oxidative capacity, we have developed a novel rat model through two-way artificial selection for either low (LCR) or high (HCR) aerobic treadmill running capacity (Koch & Britton, 2001). After 11 generations of selective breeding, there was a 374% difference in running capacity between phenotypes that was associated with an increase in risk factors for cardiovascular disease, such as hyperinsulinaemia, hyperlipidaemia and increased adiposity in LCR compared to HCR (Noland et al, 2007; Wisloff et al, 2005). HCR rats are also resistant to the development of high-fat diet-induced obesity and insulin resistance (Noland et al, 2007).

The aim of the present study was to test the hypothesis that high intrinsic aerobic running capacity is associated with enhanced skeletal muscle oxidative capacity and metabolic flexibility. This was accomplished by mimicking a “fasted” and “fed” environment in the skeletal muscle of the LCR and HCR and determining rates of insulin-stimulated glucose uptake and oxidation and basal lipid uptake and oxidation using hind limb perfusion techniques. We have attempted to elucidate potential mechanisms that impart the improved metabolic health associated with a high intrinsic running capacity. Therefore, we have measured substrate storage, insulin signal transduction and mitochondrial activity and density in both oxidative and glycolytic muscle, from these divergent phenotypes.
3.2 Material and Methods

Experimental Animals

Forty-eight female LCR/HCR rats (~20 wk old) from generation 20 and 22 (G20 and G22) were used for analysis in the present study. Rats models for high and low aerobic capacity were derived from genetically heterogeneous N:NIH stock rats by artificial selection for low and high treadmill running capacity as previously described (Koch & Britton, 2001). Animals were phenotyped for intrinsic running capacity at 11 wk of age using an incremental treadmill running test with the treadmill constantly at an uphill incline of 15° (Koch & Britton, 2001). Rats were housed two per cage in a temperature-controlled animal room (21°C) maintained on a 12-hour light-dark cycle. Animals were provided with standard chow diets and water ad libitum. All animal experimentation procedures were carried out with the approval of animal ethics committees from California State University, Northridge; RMIT University; and the University of Michigan.

Blood Measures

Fasting blood values were taken after a 5 hr fast and glucose concentrations were determined with the MediSense2 Blood Glucose Testing system (MediSense Australia; Melbourne, Australia). Fasting serum non-esterified fatty acid measures were obtained using an enzymatic colorimetric method (NEFA C; Wako Pure Chemicals, Osaka, Japan).
**Glucose Tolerance Test**

Animals were fasted for 5 hr before receiving an intraperitoneal injection of D-glucose (1 g / kg body mass). Blood glucose concentrations were measured at 0, 15, 30, 45, 60 , 90 and 120 min following the glucose dose and the area under the blood glucose curve (AUC; mM*min) was calculated for each animal.

**Hind limb Perfusions**

Animals were fasted for 5-7 hr before undergoing hind limb perfusion for the measurement of insulin-stimulated D-[\(^{14}\)C(U)]-glucose uptake/oxidation (n=8/ group) or [\(^{14}\)C]-palmitate uptake/oxidation (n=8/group). Rats were anesthetized and surgically prepared for hind limb perfusion as previously described (Lessard et al, 2007; Yaspelkis et al, 2004). Just prior to cannulation, portions of the red (RG) and white (WG) gastrocnemius were excised from the non-perfused left leg, freeze clamped in liquid N\(_2\), and stored at -80\(^\circ\)C until later analysis. In the non-perfused RG and WG, citrate synthase activity, intramuscular triacylglycerol, glycogen content, PPAR\(_\gamma\) coactivator 1 (PGC1) and carnitine palmitoyltransferase I (CPTI) protein content were assessed. The basic perfusate medium consisted of 30% washed time-expired human erythrocytes (Ogden Medical Center, Ogden, UT, USA), 4% dialysed bovine serum albumin (Equitech-Bio Inc., Kerrville, TX USA) and Krebs–Heinseleit Buffer (KHB) [pH 7.4]. The perfusate was continuously gassed with a mixture of 95% O\(_2\)/5% CO\(_2\) and warmed to 37\(^\circ\)C.

**D-[\(^{14}\)C(U)]-glucose uptake/oxidation rates**

Immediately after cannulation, the rats were sacrificed via an intracardiac injection of pentobarbital while the hind limbs were washed out with 20 ml of
heparinised (10 U/ml) KHB. The catheters were then placed in line with a non-recirculating perfusion system, and the hind limb was allowed to stabilise during a 5 min washout period. The perfusate flow rate was set at 5 ml/min during the 5 min stabilization period and subsequent perfusion. Perfusions were performed in the presence of insulin (1 mU/ml) for all experimental groups. Following the stabilization period, the perfusate was changed to one containing 8 mM glucose (0.25 μCi/ml D-[\(^{14}\)C(U)] glucose, PerkinElmer Life Sciences, MA, USA). At the completion of the 30 min perfusion, portions of the RG and WG were removed, blotted on gauze dampened with cold KHB, clamp frozen in liquid N\(_2\) and stored at -80°C until later analysis. For determination of glucose uptake, perfusate samples were taken from the arterial perfusate and well-mixed venous effluent, deproteinised in 10% TCA, centrifuged and quantified using liquid scintillation counting. Muscle glucose uptake was calculated from the arteriovenous difference, the perfusate flow rate and the weight of the muscle perfused.

Arterial and venous samples for the analysis of \(^{14}\)CO\(_2\) were taken immediately at the end of a 5 min equilibration period and at the end of the perfusion (30 min). The liberation and collection of \(^{14}\)CO\(_2\) from perfusate samples was performed by injecting 2 ml of anaerobically collected perfusate into a sealed flask containing an equal volume of 1 mol/l acetic acid. The released \(^{14}\)CO\(_2\) was trapped by an insert containing a strip of filter paper saturated with 500 μl benzethonium hydroxide and quantified using liquid scintillation counting. The rate of glucose oxidation was determined from the arteriovenous difference in \(^{14}\)CO\(_2\) and flow rate.
Lactate concentration was determined from samples obtained from the arterial reservoir and venous effluent, which was collected on ice during the 25 min perfusion. Lactate was determined as previously described (Yaspelkis et al, 2004). Lactate accumulation was calculated from the arteriovenous difference, perfusate flow rate and the weight of the muscle perfused.

$[^{14}C]$ palmitate uptake/oxidation rates

Skeletal muscle fatty acid metabolism was assessed as previously described (Lessard et al, 2007). In brief, immediately after cannulation, the rats were sacrificed via an intracardiac injection of pentobarbital while the hind limbs were washed out with 20 ml of heparinised (10 U/ml) KHB. The catheters were then placed in line with a recirculating perfusion system, and the hind limb was allowed to equilibrate during a 20 min period. The perfusate flow rate was set at 7.5 ml/min during the 50 min perfusion (20 min equilibration / 30 min perfusion) with perfusate containing 4% fatty acid-free BSA (Equitech-Bio Inc.), 500 μmol/l albumin-bound palmitate (Sigma Aldrich; MO, USA), and 5 μCi albumin-bound $[^{14}C]$ palmitate (PerkinElmer; MA, USA). For determination of palmitate uptake, perfusate samples were anaerobically taken from the arterial perfusate and venous lines, centrifuged and quantified using liquid scintillation counting. Muscle palmitate uptake was calculated from the arteriovenous difference, the perfusate flow rate and the weight of the muscle perfused.

Arterial and venous samples for the analysis of $^{14}$CO$_2$ were taken immediately after a 20 min equilibration period and at 50 min. The liberation and collection of $^{14}$CO$_2$ from perfusate samples was performed by injecting 2 ml anaerobically collected
perfusate into a sealed flask containing an equal volume of 1 mol/l acetic acid. The released $^{14}$CO$_2$ was trapped by an insert containing a strip of filter paper saturated with 500 μl benzethonium hydroxide and quantified using liquid scintillation counting.

**Analysis of intramuscular substrate storage and citrate synthase activity**

Portions of RG and WG muscle were freeze dried, powdered and analysed for the content of glycogen, glycerol, and maximal citrate synthase activity as previously described (Lessard et al, 2007; Yeo et al, 2008). Briefly, freeze-dried muscle was powdered and cleaned of all visible connective tissue and blood under magnification. Portions (4-5 mg) of freeze dried, powdered RG (8-10/group) and WG (8-10/group) were used to fluorometrically determine skeletal muscle triacylglycerol (total glycerol) content, following Folch lipid extraction and saponification. Portions (3-4 mg) of freeze dried, powdered RG (8-10/group) and WG (8-10/group) were used to enzymatically analyse glycogen content by fluorometric detection after extraction with 2M HCl and neutralization with 0.67M NaOH. Portions (3-4 mg) of freeze dried, powdered RG (8-10/group) and WG (8-10/group) were homogenised in 100 mM potassium phosphate buffer (pH 7.3, 1:400 dilution), and citrate synthase was assayed spectrophotometrically at 25°C by the reduction of DNTB.

**Western blotting analysis**

Portions of muscle were cut from the RG and WG, weighed frozen and homogenised in an ice-cold homogenization buffer (1:8 wt/vol) containing 50 mM Tris-HCl (pH 7.5), 5 mM Na-pyrophosphate, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 10% glycerol (v/v), 1% Triton-X, 1 mM DTT, 1 mM benzamidine, 1 mM PMSF, 10 μg/ml
trypsin inhibitor, and 2 µg/ml aprotinin. Following centrifugation (21,000 x g, 4° C) for 15 min the supernatant was collected and assayed for protein content. RG muscle lysates (60 µg) were solubilised in Laemmli buffer, separated by SDS-PAGE, and transferred to PVDF membranes. The membranes were then blocked (5% NFDM), and incubated overnight at 4°C with primary antibodies specific for either phospho-Akt Ser473, phospho-Akt Thr308, Akt1, Akt2, phospho-Akt substrate (1:1000; Cell Signaling, MA, USA), phospho-IRS1 Tyr632, CPTI (1:200-500; Santa Cruz Biotechnology, CA, USA), or AS160 (TBCD14) which was produced as previously described (Larance et al, 2005) using a region of human AS160 from amino acids 621-766 fused with GST (1:1000, a gift from Prof. David James, Garvan Institute, Sydney). The measure of PGC1 (1:1000; Millipore, MA, USA) expression was acquired by using the ~92 kDa band that is specified as PGC1 according to the manufacturer. The immunoreactive proteins were detected with enhanced chemiluminescence (Amersham Biosciences) and quantified by densitometry.

**Transmission electron microscopy for determination of mitochondrial density**

Soleus (SOL) [4/group] and extensor digitorum longus (EDL) [4/group] skeletal muscle from a separate cohort of animals (G22) were excised, cut into small longitudinal strips (1x1x2 mm) fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) and postfixed in 2% osmium tetroxide solution. After dehydrating in graded acetone, tissue was embedded in araldite/epon resin. Thick sections (0.5 µm) were cut using Ultracut S ultramicrotome (Leica, North Ryde, New South Wales, Australia) and then stained with 1% methylene blue. Thin sections (90 nm) were cut using the same microtome mounted
on copper/ palladium 200 mesh grids and then stained with 3% aqueous solution of uranyl acetate and lead citrate (Reynold’s stain). Randomly sampled transverse sections of muscle fibres were obtained followed by micrographs acquired with an electron microscope at 60V (Siemens Elmiskop 102 electron microscope) on a final magnification of 10,000x. In order to obtain a valid representation of the whole muscle, two micrographs (1 from the subsarcolemmal region and 1 from the adjacent interfibrillar region) in three separate muscle fibers for each muscle (SOL, EDL) in each group were acquired for a total of 6 micrographs/muscle/animal. Mitochondrial volume density (MVD) was determined using the point-counting stereological analysis methodology with Image J software (Image J 1.41, National Institutes of Health, USA). Each micrograph was counted and then recounted in a double-blind fashion.

3.2.1 Statistical Analysis

Differences between LCR and HCR were identified using a two-tailed t-test with GraphPad Prism version 4.04 for Windows (GraphPad Software, CA, USA, www.graphpad.com). Results are expressed as mean ± SEM and statistical significance was accepted at P<0.05.

3.3 Results

Markers of metabolic health are divergent in HCR and LCR.

Intrinsic treadmill running capacity was 530% greater in HCR compared to LCR (Generation 20, P=0.0001, Table 3.1). HCR had lower body mass and fasting serum glucose levels (P<0.0002 vs. LCR; Table 3.1), and tended to have lower levels of
circulating free-fatty acids (FFA) compared to LCR (P=0.2 vs. LCR; Table 3.1). HCR were more glucose tolerant than LCR, as measured from the AUC after an intraperitoneal glucose tolerance test (IPGTT) following a 5 hr fast (P=0.01; Table 3.1).

<table>
<thead>
<tr>
<th></th>
<th>LCR</th>
<th>HCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Running Capacity (m)</td>
<td>282.4 ± 11.92</td>
<td>1776 ± 35.07 *</td>
</tr>
<tr>
<td>Non-Esterified Fatty Acids (mM)</td>
<td>0.5817 ± 0.09</td>
<td>0.3843 ± 0.02</td>
</tr>
<tr>
<td>Fasting Glucose (mmol/l)</td>
<td>6.23± 0.147</td>
<td>5.18± 0.185 *</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>227.7 ± 2.53</td>
<td>166.3 ± 2.02 *</td>
</tr>
<tr>
<td>Glucose Tolerance Test (mM*min)</td>
<td>916.9 ± 25.56</td>
<td>828.7 ± 25.55 *</td>
</tr>
</tbody>
</table>

Table 3.1 Whole-body metabolic health measures and initial running capacity Significant differences between groups (*= P<0.05 vs. LCR) are indicated by the symbol listed on the table (n=10/group).

**HCR have superior substrate handling in skeletal muscle.**

To determine if intrinsic differences in aerobic capacity were associated with alterations in glucose and lipid handling, “fasted” and “fed” states were mimicked during hind limb perfusions. HCR had superior insulin-stimulated glucose metabolism as demonstrated by a 30% higher rate of glucose uptake (P=0.04 vs. LCR; Figure 3.1A) and a 50% elevation in the rate of glucose oxidation (P=0.04 vs. LCR; Figure 3.1B). HCR also had superior lipid metabolism, with a higher rate of palmitate oxidation (P=0.02 vs. LCR; Figure 3.1D), and a trend for a higher palmitate uptake (P=0.10 vs. LCR; Figure 3.1C). Lactate accumulation during the 30 min perfusion was similar for both phenotypes (4.464 ± 0.46 μmol/g/h vs. 4.052 ± 0.24 μmol/g/h).
Figure 3.1 Skeletal muscle glucose and lipid metabolism were assessed in generation 20 LCR and HCR after a 5 hour fast. Average $[^{14}\text{C}]$ glucose uptake (A) and $[^{14}\text{C}]{\text{CO}}_2$ production due to $[^{14}\text{C}]$ glucose oxidation (B) were calculated using differences in arterial and venous perfusate concentrations during a 30 min hindlimb perfusion with 8 mM glucose and 1 mU/mL insulin. Average $[^{14}\text{C}]$ palmitate uptake (C) and $[^{14}\text{C}]{\text{CO}}_2$ production due to $[^{14}\text{C}]$ palmitate oxidation (D) were calculated using differences in arterial and venous perfusate concentrations during a 30 min hindlimb perfusion with 1.8 mmol/l palmitate. Significant differences between groups (P<0.05) are indicated by the P value listed on the figure (n=7-8/group).

**Substrate storage in skeletal muscle was greater in HCR.**

The intramuscular storage of glycogen and lipid was determined in basal (unstimulated) tissue. There were no differences in glycogen storage in the RG between LCR and HCR, but there was a 21% increase in glycogen content in the WG of HCR (P=0.042 vs. LCR; Figure 3.2A). IMTG concentration was 18% higher in the RG of HCR (P=0.04 vs. LCR; Figure 3.2B).
Figure 3.2 Intramuscular glucose and lipid storage in LCR/HCR. Muscle glycogen (A) and triacylglycerol (B) content were determined in red and white gastrocnemius on separate aliquots of freeze dried/powdered muscle and expressed per mg of dry weight [DW]. Significant differences between groups (P<0.05) are indicated by the P value listed on the figure (n=8-10/group).

**Specific enhancement of insulin signalling in WG muscle of HCR.**

In order to determine insulin-stimulated cell signalling responses in white and red muscle, the phosphorylation of proximal and distal components of the insulin signalling cascade were assessed. There were no differences between phenotypes in the phosphorylation of IRS1 on tyrosine (Y)632 in the RG. However, there was an increase in IRS1 phosphorylation in the WG of HCR (P=0.002 vs. LCR; Figure 3.3A). The phosphorylation of Akt on threonine (T)308 and serine (S)473 was also greater in the white muscle from HCR compared to LCR with a 24% and 26% increase in the WG (P=0.016, P=0.03 vs. LCR; Figure 3.3B, 3.3C), but not RG. The phosphorylation of AS160,
a downstream substrate of Akt, was also greater in WG (21% increase, P=0.03 vs. LCR; Figure 3.3D) and tended to be higher in the RG. (P=0.12 vs. LCR; Figure 3.3D). There was a 46% increase in the expression of the Akt1 isoform in the RG of HCR (P=0.02 vs. LCR; Figure 3.4A) and a 95% increase in the WG (P=0.03 vs. LCR; Figure 3.4A). In accordance with these observations, the expression of Akt2 was also increased in the RG (35%, P=0.026 vs. LCR; Figure 3.4B) and in the WG (47%, P=0.008 vs. LCR; Figure 3.4B) of HCR. There was a decrease in the expression of AS160 in the RG (P=0.04 vs. LCR; Figure 3.4B), but no change in the WG of the HCR.

Figure 3.3 Insulin-stimulated cell signalling in skeletal muscle of LCR/HCR. Phosphorylation of IRS1 on Tyr632 (A), Akt on Thr308 (B) and Ser473 (C), and PAS160 were determined by western blotting on insulin-stimulated perfused red and white gastrocnemius muscle. To confirm equal loading α-tubulin was run as a loading control. Significant differences between groups (P<0.05) are indicated by the P value listed on the figure (n=7-8/group).
Figure 3.4 AS160 and isoform-specific Akt total protein content in skeletal muscle of LCR/HCR. Relative protein levels of Akt1 (A), Akt2 (B) and AS160 (C) were quantified using western blot analysis and densitometry in red and white gastrocnemius muscle. To confirm equal loading α-tubulin was run as a loading control. Significant differences between groups (P<0.05) are indicated by the P value listed on the figure (n=7-8/group).

**Mitochondrial oxidative capacity is enhanced in the WG, but not the RG of HCR.**

In order to determine whether there were differences in markers of mitochondrial capacity and density between phenotypes, citrate synthase activity, the expression of mitochondrial proteins and mitochondrial volume density were determined. There were no differences in the maximal activity of citrate synthase in RG of HCR and LCR, but citrate synthase activity was increased in WG of HCR (51%, P=0.0004 vs. LCR; Figure 3.5A). There were no differences in the expression of either PGC1 or CPTI in the RG for either phenotype. In contrast, the expression PGC1 was 55% higher in the WG of HCR (P=0.004 vs. LCR; Figure 3.5C) whereas CPTI was similar (P=0.098 vs. LCR; Figure 3.5D). There was no significant difference in mitochondrial volume density (MVD) in the SOL (a red, oxidative muscle) between groups (Figure
However, there was a ~70% higher mitochondrial volume density in the EDL (a white, glycolytic muscle) of the HCR (P=0.0002 vs. LCR; Figure 3.6B).

Figure 3.5 Mitochondrial enzyme activity and mitochondrial protein content in skeletal muscle of LCR/HCR. Citrate synthase activity (A) as determined by enzymatic assay and the relative protein levels of CPTI (B) and PGC1 (C) as quantified using western blot analysis and densitometry were analysed in red and white gastrocnemius muscle. To confirm equal loading, in western blots, α-tubulin was run as a loading control. Significant differences between groups (P<0.05) are indicated by the P value listed on the figure (n=7-8/group).
Figure 3.6 Mitochondrial volume density as determined by electron microscopy in soleus (n=4/group) and extensor digitorum longus (n=4/group). Representative micrographs (A) of soleus and extensor digitorum longus skeletal muscle on transverse sections. Mitochondrial volume density (B) was determined by point/count method. Significant differences between groups (P<0.05) are indicated by the P value listed on the figure (n=48 micrographs/group).

3.4 Discussion and Conclusions

The capacity of skeletal muscle to balance fuel oxidation to fuel availability (i.e., “metabolic flexibility”) is fundamental to metabolic health (Galgani et al, 2008; Kelley, 2005). In contrast, metabolic inflexibility of skeletal muscle is strongly associated with the development and progression of several chronic disease states such as obesity, type 2 diabetes and cardiovascular disease. Utilising hind-limb perfusion techniques, here it is demonstrated that genetic selection for high intrinsic running capacity (HCR) was
associated with enhanced rates of skeletal muscle glucose uptake (~30%; P=0.04; Fig 3.1A), glucose oxidation (~50%; P=0.04; Fig 3.1B) and lipid oxidation (~40%; P=0.02; Fig 3.2C). This study also provides novel evidence to show that HCR confers metabolic flexibility in skeletal muscle and is associated with improvements in whole-body markers of metabolic health (Table 3.1). In contrast, low intrinsic aerobic capacity (LCR) imparts metabolic inflexibility that is linked with an increase in whole-body metabolic markers of disease risk (Table 1). Artificial selection for low running capacity was further linked with altered molecular signaling, substrate storage and mitochondrial volume density in skeletal muscle in white rather than red skeletal muscle.

It has previously been established that increased skeletal muscle lipid stores are associated with metabolic inflexibility and insulin resistance (McGarry, 2002). Studies using models of insulin resistance show there is a defect in the synthesis of glycogen that could be attributed to a build-up of lipids in skeletal muscle (Colberg et al, 1996; Groop & Orho-Melander, 2008; Petersen et al, 2007). Furthermore, increased storage of lipids in insulin sensitive tissue, such as skeletal muscle, has been shown to be a primary contributor to metabolic inflexibility and insulin resistance (Kelley et al, 2002a; Lessard et al, 2007; Petersen et al, 2007). These defects are believed to precede type 2 diabetes and are thought to have a primary contribution to this disease state. In the present investigation it is reported that metabolic inflexibility in LCR was associated with lower glycogen storage in muscle (Figure 3.2B) but not increased lipid storage (Fig 3.2D). An increase in TAG in muscle from HCR animals (Fig 3.2C) is also observed. While this observation appears paradoxical, TAG levels are increased in the muscle of endurance
trained athletes: these individuals also have high muscle oxidative capacity and are highly insulin sensitive, a finding that has been termed an “athlete’s paradox” (Goodpaster et al, 2001). Bruce at al. (2003) previously observed that oxidative capacity in skeletal muscle is a better predictor of insulin sensitivity than intramyocellular lipid content in insulin resistant individuals (Bruce et al, 2003).

Impairments to insulin signal transduction are strongly associated with impaired carbohydrate and lipid metabolism in skeletal muscle (Corpeleijn et al, 2009; Galgani et al, 2008; Hawley & Lessard, 2008). Insulin resistance in skeletal muscle is associated with impairments in the activation, phosphorylation, and expression of major components of the insulin signalling cascade (Hawley & Lessard, 2008; Karlsson & Zierath, 2007; Petersen & Shulman, 2006). However, the exact mechanisms associated with impaired insulin-stimulated carbohydrate metabolism and signal transduction in metabolic inflexibility has yet to be determined. Here it is shown for the first time that metabolic inflexibility is associated with the reduced phosphorylation to components of the insulin signal cascade. Specifically, a fibre-type impairment in the phosphorylation of IRS1 on Y632, being lower in the WG of the LCR compared to HCR (Fig 3.3A) is observed. This effect was also seen downstream of IRS1 with the reduced phosphorylation of Akt on its T308 (Fig 3.3B) and S473 (Fig 3.3C) sites and reduced phosphorylation of its substrate AS160 (Fig 3.3D) in WG.

Differences in the physiological properties and function of red and white skeletal muscle have been well characterised (Hickson et al, 1976; Maltin et al, 1989; Vandenberghe et al, 1999). Red muscle has a higher oxidative capacity, partly because
of higher concentrations of mitochondria, and is more insulin sensitive than white muscle (James et al, 1986; Pande, 1971). In response to endurance exercise training, several kinases have been found to be differentially activated in red and white skeletal muscle (Ljubicic & Hood, 2008; Nader & Esser, 2001). For example, Ljubicic & Hood (2008) demonstrated that in situ contraction-induced oxygen consumption stimulated Akt activation in white, but not red muscle (Ljubicic & Hood, 2008) and concluded that the oxidative capacity of the skeletal muscle determines the activation of intracellular signalling as muscle oxygen consumption increases (Ljubicic & Hood, 2008). In the present study both red muscle, with a high oxidative capacity (the soleus and red gastrocnemius) and white muscle, with a low oxidative capacity (the extensor digitorum longus and white gastrocnemius) have been selected to determine whether the phenotypical differences in aerobic capacity can be attributed to differences in white and red skeletal muscle.

Akt is a critical protein whose isoforms, Akt1 and Akt2, have divergent roles in glucose, lipid and protein metabolism in skeletal muscle (Bouzakri et al, 2006; Cleasby et al, 2007; Kim et al, 2000). Full activation of Akt is required for the phosphorylation and inhibition of AS160 allowing the translocation of the Glut4 vesicle from the cytosol to the myocellular membrane (Thong et al, 2005). Both Akt isoforms were examined and found that expression of Akt1 (Fig 3.4A) and Akt2 (Fig 3.4B) were increased in both the RG and WG of HCR. Furthermore, the expression of AS160 (Fig 3.4C) was reduced in RG of HCR but unchanged in the WG. The reduced phosphorylation and expression, in the white skeletal muscle, of these important proteins of the insulin signalling cascade
could, in part, explain the decreased glucose uptake and oxidation associated with metabolic inflexibility.

In obesity and insulin resistance there is impairment to mitochondrial oxidative capacity in skeletal muscle (Miles et al, 2009; Simoneau & Kelley, 1997; Toledo et al, 2008). However, there is some contention about the role of mitochondrial oxidative capacity in insulin resistance. Some believe that impaired oxidative capacity is a consequence of insulin resistance (Hawley & Holloszy, 2009; Holloszy, 2009; Toledo et al, 2008) while others have suggested that mitochondrial dysfunction is the major defect that leads to insulin resistance (Petersen et al, 2004). Insulin resistant subjects with a family history of diabetes have low mitochondrial oxidative capacity that is correlated with decreased mitochondrial density in skeletal muscle as measured by electron microscopy (Befroy et al, 2007; Morino et al, 2005). In addition, Ukropcova et al. (2007) showed in the skeletal muscle of healthy individuals with a family history of type 2 diabetes that increasing dietary fat caused impairment to substrate switching and was related to decreases in their mitochondrial content and capacity (Ukropcova et al, 2007). Noland et al. (2007) previously reported an enhancement in PGC1 protein abundance and in the concentration of the oxidative enzyme, cytochrome c oxidase subunit 1 (COX1), in skeletal muscle from HCR (Noland et al, 2007). Reductions in the expression or activation of mitochondrial enzymes or proteins, like PGC1, CPTI or CS, are related to a decline in the oxidative capacity of skeletal muscle (Bruce et al, 2007; Morino et al, 2006; Schrauwen & Hesselink, 2004). Hence, a novel finding from the present study was that the most significant changes in mitochondrial volume density
occurred in white muscle rather than in red muscle between the divergent phenotypes (Fig 3.6B, Fig 3.6C).

To the surprise of the researchers, it was found that a significantly higher mitochondrial volume density in the EDL muscle from HCR (P<0.001 vs. LCR; Fig 3.6B), but no significant difference in the soleus (Fig 3.6B). These findings paralleled the increase in CS activity (Fig 3.5A) observed in the WG of HCR. It is also shown that artificial selection for high intrinsic running capacity imparts an increase in the expression of PGC1 protein content in WG but not RG of HCR (Fig 3.5C). The transcriptional coactivator PGC1 has previously been revealed to have a robust role in mitochondrial biogenesis and drives muscle fibre type determination (Lin et al, 2002). Lin et al. (2002), using transgenic mice expressing PGC1 under an MCK promoter (a promoter that is preferentially activated in white muscle), observed that the white muscle of these mice had red fibre like qualities (eg. increased myoglobin, fatigue resistant, etc.). Additionally, the fact that the most evident change in the increase of post-exercise mitochondrial content is observed in white muscle, may further explain this study’s findings (Hood et al, 2006).

In conclusion, selection for high intrinsic aerobic running capacity was associated with metabolic flexibility, superior insulin signalling, increased skeletal muscle oxidative capacity and elevated mitochondrial density in white rather than red fibre. The superior fuel-handling capacity in animals bred (but not exercise-trained) for superior running capacity can, in part, be explained by a fibre-type specific increase in insulin-stimulated phosphorylation of the insulin receptor substrate 1 (IRS1), Akt and the Akt substrate of
160 kDa (AS160) along with a substantial increase in mitochondrial volume density. Our results provide the first evidence that intrinsic running capacity, in the absence of exercise training, confers metabolic flexibility to skeletal muscle and is associated with improved whole-body metabolic health. The LCR/HCR model of divergent intrinsic aerobic capacity was developed to specifically study some of the potential mechanisms associated with the metabolic syndrome and cardiovascular disease in a system in which there is a degree of control over both environmental and genetic variables. The current study provides insight into the fundamental molecular events underlying the differences in metabolic flexibility between the LCR and HCR and provides guidance and large motivation for resolving these differences at the genetic level.
CHAPTER FOUR: Intrinsic aerobic capacity determines exercise induced insulin sensitisation and Nur77 expression in skeletal muscle.

4.1 Introduction

Low aerobic exercise capacity is a predictor of all-cause mortality (Myers et al, 2002), a relationship that is particularly strong in individuals with type 2 diabetes (Kokkinos et al, 2009). The importance of this association is highlighted by the observation that individuals with type 2 diabetes and their first degree relatives have lower aerobic exercise capacity than age and weight matched controls (Nadeau et al, 2009). Recent evidence suggests that adolescents with type 2 diabetes also exhibit impaired exercise capacity compared to age-matched peers, indicating that this impairment is present early in the onset of the disease (Nadeau et al, 2009). However, the complex interplay between environmental and genetic factors that contribute to both a reduced exercise capacity (Bouchard & Rankinen, 2001; Bouchard et al, 2000) and an increased risk for developing type 2 diabetes (Mootha et al, 2003) make it difficult to demonstrate a cause and effect relationship.

In order to study the contribution of aerobic exercise capacity to the etiology of complex disease states such as type 2 diabetes, unique animal models have been generated by artificial selection for low and high aerobic exercise capacity (Koch & Britton, 2008). In these models, 11 generations of selection resulted in a 347% difference in running capacity between low (LCR) and high (HCR) capacity runners (Wisloff et al, 2005). Importantly, selection for low aerobic capacity simultaneously resulted in metabolic dysfunction, including impaired cardiovascular function, increased adiposity, dyslipidaemia, and whole-body insulin resistance (Wisloff et al, 2005).
The molecular defect(s) that result in aberrant fuel metabolism in LCR are unclear. However, it has been recently reported that LCR have impaired skeletal muscle glucose and lipid metabolism (Lessard et al, 2009; Noland et al, 2007). Furthermore, it has also been demonstrated that impaired skeletal muscle metabolism is associated with reduced β2-AR content, impaired adrenergic signal transduction and reduced expression Nur77 in the skeletal muscle of LCR (Lessard et al, 2009). Nur77 is a nuclear receptor that is down-regulated in several models of insulin resistance and type 2 diabetes (Fu et al, 2007) and induces the transcription of important metabolic genes (i.e. GLUT4, CD36, UCP3) in response to β-adrenergic stimulation (Chao et al, 2009; Maxwell et al, 2005). Indeed, altered β-adrenergic signal transduction has been proposed to contribute to metabolic disease (Blaak et al, 2004), and variant alleles of the β2-adrenergic receptor have been identified as risk factors for obesity, dyslipidaemia and type 2 diabetes in humans (Ishiyama-Shigemoto et al, 1999; Meirhaeghe et al, 1999; Pinelli et al, 2006; Yamada et al, 1999). Therefore, defects to whole-body and skeletal muscle metabolism that occur following artificial selection for low aerobic capacity are similar to the impairments observed in individuals at risk for developing type 2 diabetes (Mootha et al, 2003; Perseghin et al, 1997). Accordingly, this model of divergent aerobic capacity offers a unique opportunity to investigate some of the intrinsic metabolic traits that link reduced exercise capacity to increased risk for the development of type 2 diabetes.

Although a genetic predisposition to the onset of obesity and type 2 diabetes is evident, lifestyle interventions, such as physical exercise, may be used to overcome the
increased risk for metabolic disease imparted via inheritance (Blaak et al, 2004; Perseghin et al, 1997). For example, the risk of diabetes in offspring of patients with type 2 diabetes is greatly reduced in physically fit individuals compared to their sedentary counterparts (Ahn et al, 2004). Accordingly, the aim of the present investigation was to determine the genetic (inherent exercise capacity) and environmental (exercise training) contributions to skeletal muscle metabolism in animal models of low and high intrinsic aerobic capacity. It was hypothesised that impaired skeletal muscle carbohydrate and lipid metabolism observed after artificial selection for low aerobic capacity would be reversed by an exercise training-induced restoration of Nur77.

4.2 Material and Methods

Experimental Animals

Forty male LCR/HCR rats (~20 wk old) from generation 22 (G22) were used in the present study. Rats models for high and low aerobic capacity were derived from genetically heterogeneous N:NIH stock rats by artificial selection for low and high treadmill running capacity as previously described in detail (Koch & Britton, 2001). Animals were phenotyped for intrinsic running capacity at 11 wk of age using an incremental treadmill running test and their average running capacity in metres was recorded (Koch & Britton, 2001). Rats were housed two per cage in a temperature-controlled animal room (21°C) maintained on a 12-hour reverse light-dark cycle. Animals were provided with standard chow diets and water ad libitum. All animal
experimentation procedures were carried out with the approval of animal ethics committees from California State University, Northridge and the University of Michigan.

**Experimental Design**

Following a one week acclimatisation period to laboratory conditions, age matched pairs of LCR/HCR rats were randomly assigned to one of four groups (n=10/group): LCR Sedentary (LCR SED), LCR Exercise Trained (LCR EXT), HCR Sedentary (HCR SED) and HCR Exercise Trained (HCR EXT). All exercise-trained animals followed a six week treadmill training protocol as detailed previously (Koch et al, 2005). Briefly, exercise training consisted of an incremental protocol (4d/wk) with the same absolute cumulative training distance (~10 km) for all animals. On days 1-3 the animals were familiarised to the treadmill starting at a speed of 10 m/min for 5 min at a 15% grade. During the training period the animals commenced treadmill training at a speed of 10m/min for 20 min at a 15% grade (session 1) and the time (0.5 min/session) and speed (1 m/ 2nd session) were gradually increased resulting in a final training session of 20 m/min for 31.5 min at a 15% grade (session 24). Exercise trained animals undertook their last training bout 48 hr before all experimental procedures.

**Blood Measures and Tissue Collection**

Following the six week intervention the animals underwent a 5 h fast after which fasting blood values were taken and glucose concentrations were determined with an Accu-Chek Go blood glucose meter (Roche Diagnostics; Sydney, Australia). Fasting serum non-esterified fatty acid measures were obtained using an enzymatic colorimetric method (NEFA C; Wako Pure Chemicals, Osaka, Japan). Animals were anesthetised
(Nembutal [1 ml/kg], Ovation Pharmaceutical, NJ, USA) and then muscle was dissected and used in either the *in vitro* incubations experiments (soleus) or quickly frozen in liquid N₂ (red gastrocnemius) with pre-cooled freeze clamps and stored at -80°C for later analysis.

**In vitro muscle incubations**

The soleus (SOL) muscle was excised and under magnification was carefully dissected into longitudinal strips from tendon to tendon. 6-8 strips were made from each muscle (15-20 mg/strip), with the SOL of one leg used for the measure of basal and insulin-stimulated glucose transport and the contralateral muscle used for the measure of lipid oxidation. Muscle strips were allowed to recover at 30°C for 30 min in continuously gassed (95% O₂/5% CO₂) Krebs-Henseleit (KHB) solution containing either 0.1% fatty-acid free BSA (Equitech-Bio, TX, USA), 8 mM glucose and 32 mM mannitol (glucose transport experiments) or 4% fatty-acid free BSA, 0.5 mM palmitate and 5 mM glucose (lipid oxidation experiments). Throughout the experiments, vials were gently shaken in a water bath at 30°C.

**Glucose transport**

Glucose transport was assessed using modified methods for determining 3-O-methylglucose transport in isolated rat soleus muscle as previously described (Thong et al, 2007). At the end of the 30 min preincubation the muscle was subsequently incubated with (INS) or without (BAS) 60 nm of insulin (Humulin, Eli Lilly, USA) at 30°C for 30 min in continuously gassed KHB containing 0.1% BSA, 8 mM glucose, 32 mM mannitol. Following the incubation the muscle was transferred to new vials containing
pre-gassed KHB with 2 mM pyruvate and 38 mM mannitol while shaking at 30°C for 10 min. Subsequent to the 10 min wash, muscle strips were transferred to vials containing well oxygenated KHB with 8 mM 3-MG, 32 mM mannitol, 0.1% fatty-acid free BSA, 1.0 μCi/ml 3-methyl-D-[1-3H] glucose (Perkin Elmer) and 0.1 μCi/ml D-[1-14C] mannitol (Perkin Elmer) as a extracellular space marker with (INS) or without (BAS) 60 nm insulin for the measure of glucose transport over 15 min. The reaction was stopped by rapidly washing three times in oxygenated KHB, blotting the muscle on filter paper, and freeze clamping with tongs pre-cooled in liquid N2. Under these experimental conditions 3-O-methylglucose remained linear for 15 min. The muscle strips were then weighed and digested in 10% TCA transferred to glass vials containing 5 ml of scintillation fluid (Opti-fluor, Perkin Elmer) and counted in a scintillation counter (Beckman Instruments, CA) set for simultaneous 3H and 14C counting. Rates of basal and insulin-stimulated skeletal muscle 3-O-methylglucose transport were calculated as previously described (Lessard et al, 2007) and results presented as fold change.

**Lipid oxidation**

Lipid oxidation was measured as previously described by Bruce et al. (2005) with minor modifications (Bruce et al, 2005). Following the 30 min preincubation the muscle was transferred into new vials containing pre-gassed KHB with 4% fatty-acid free BSA, 0.5 mM palmitate, 5 mM glucose, and 0.2 μCi / ml [1-14C] - palmitate. The incubation medium was subsequently covered with a 1 ml layer of mineral oil, to trap 14CO2 released during incubation, and then capped and incubated at 30°C for 30 min. The production of 14CO2 from the exogenous oxidation of [1-14C] - palmitate was measured
by injecting duplicate 1.0 ml aliquots of anaerobically collected incubation medium into a sealed flask containing an equal volume of 1M acetic acid and a plastic insert containing filter paper saturated with 500 µl of benzothonium hydroxide and quantified using liquid scintillation counting. In addition to complete oxidative products (\(^{14}\)CO\(_2\)), incomplete oxidative products (acid-soluble metabolites, ASM) were also measured as previously (Steinberg et al, 2004). Briefly, muscles were placed in borosilicate glass tubes containing 2.0 ml of ice-cold 2:1 chloroform-methanol (vol/vol), and were homogenised using a polytron (Kinematica AG, Switzerland). After homogenisation, samples were centrifuged at 2,000 x g (4°C) for 10 min. The supernatant was removed and transferred to a clean centrifuge tube. Distilled water (2.0 ml) was added, and samples were shaken for 10 min and centrifuged as before to separate the aqueous and lipophilic phases. Then, 200 µl of the aqueous phase was quantified by liquid scintillation counting to determine the amount of \(^{14}\)C-labeled oxidative intermediates resulting from isotopic fixation. The ratio of incomplete (ASM) to complete (\(^{14}\)CO\(_2\)) radiolabelled products were determined to provide an index of incomplete to complete fatty acid oxidation.

**Analysis of intramuscular substrate storage**

Portions of RG were freeze dried, powdered and analysed for the content of glycogen and intramuscular triacylglycerol as previously described (Lessard et al, 2007). Briefly, freeze-dried muscle was powdered and cleaned of all visible connective tissue and blood under magnification. Portions (4-5 mg) of freeze dried, powdered RG (9-10/group) were used to fluorometrically determine skeletal muscle triacylglycerol (total
glycerol) content, following Folch lipid extraction and saponification. Portions (3-4 mg) of freeze dried, powdered RG (8-10/group) and WG (8-10/group) were used to enzymatically analyse glycogen content by fluorometric detection after extraction with 2M HCl and neutralisation with 0.67M NaOH.

**Western blotting analysis**

Portions of muscle were cut from the RG were weighed frozen and homogenised in an ice-cold homogenisation buffer (1:8 wt/vol) containing 50 mM Tris-HCl (pH 7.5), 5 mM Na-pyrophosphate, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 10% glycerol (v/v), 1% Triton-X, 1 mM DTT, 1 mM benzamidine, 1 mM PMSF, 10 µg/ml trypsin inhibitor, and 2 µg/mL aprotinin. Following centrifugation (21,000 x g, 4°C) for 15 min the supernatant was collected and assayed for protein content. RG (60 µg) were solubilised in Laemmli buffer, separated by SDS-PAGE, and transferred to PVDF membranes. The membranes were then blocked (5% NFDM), and incubated overnight at 4°C with primary antibodies specific for either PGC1 (1:1000; Millipore, MA, USA), β2 adrenergic receptor (Santa Cruz Biotechnology, CA, USA; H-73), Nur77 (Cell Signaling, MA, USA; 3562), UCP3 (ABR, CO, USA; PA1-055), FAT/CD36 (Abcam, UK; FA6-152), or GLUT4 (Abcam, UK; ab654). Membranes were probed with α-tubulin (Sigma, MO, USA; T6074) antibody to monitor protein loading. The immunoreactive proteins were detected with enhanced chemiluminescence (Amersham Biosciences) and quantified by densitometry.

**Insulin Signalling**

After the 30 min incubation, with (INS, 60 nM) or without (BAS) insulin portions of the SOL strips were quickly frozen by freeze clamping with tongs pre-cooled in liquid N₂ and
stored at -80°C until later analyses. The determination of phospho-Akt Ser473 and phospho-Akt substrate (1:1000; Cell Signaling, MA, USA) with western blotting was conducted as above.

### 4.2.1 Statistical Analyses

Differences between treatment groups were determined using a one-way ANOVA with a Student-Newman-Keuls post hoc test using GraphPad Prism version 4.04 for Windows (GraphPad Software, CA, USA, www.graphpad.com). Results are expressed as mean ± SEM and statistical significance was accepted at P<0.05.

### 4.3 Results

*Exercise training improves whole body metabolic measures in LCR.*

There was a ~535% higher initial intrinsic treadmill running capacity in G22 of HCR compared to LCR (P<0.05; Table 4.1). HCR SED had significantly lower body and epididymal fat pad weights and fasting serum glucose and NEFA levels than LCR SED (P<0.05; Table 1). Exercise training significantly lowered the body and epididymal fat pad weights and fasting serum NEFA levels in LCR (P<0.05, LCR SED vs. LCR EXT; Table 4.1). There were no changes in whole body metabolic measures with exercise training in HCR (HCR SED vs. HCR EXT; Table 4.1).
Table 4.1 Whole-body metabolic health measures in sedentary and exercise trained LCR/HCR and initial running capacity. Significant differences between groups (* = P<0.05 vs. LCR SED; # = P<0.05 vs. LCR EXT) are indicated by the symbol listed on the table (n=10/group).

<table>
<thead>
<tr>
<th>Measure</th>
<th>LCR SED</th>
<th>LCR EXT</th>
<th>HCR SED</th>
<th>HCR EXT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Running Capacity (m)</td>
<td>282 ± 12</td>
<td>276 ± 17</td>
<td>1927 ± 57*#</td>
<td>1968 ± 64</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>497 ± 19</td>
<td>432 ± 10*</td>
<td>331 ± 8*#</td>
<td>317 ± 14</td>
</tr>
<tr>
<td>Fat Pad Weight (g)</td>
<td>6.4 ± 0.3</td>
<td>4.2 ± 0.5*</td>
<td>2.4 ± 0.1*#</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>Fasting Blood Glucose</td>
<td>6.2 ± 0.1</td>
<td>5.7 ± 0.2</td>
<td>5.3 ± 0.1*</td>
<td>5.3 ± 0.2</td>
</tr>
<tr>
<td>Non-Esterified Fatty Acids (mM)</td>
<td>0.35 ± 0.04</td>
<td>0.18 ± 0.03*</td>
<td>0.26 ± 0.03*</td>
<td>0.20 ± 0.02</td>
</tr>
</tbody>
</table>

**Exercise training enhances glucose metabolism in LCR but not HCR.**

In order to determine the effects of exercise training on skeletal muscle glucose metabolism, both basal and/or insulin stimulated glucose transport, glycogen content and insulin signalling were assessed. HCR SED had significantly higher rates of insulin-stimulated glucose transport (P<0.001; Fig 4.1A) in soleus than LCR SED. Exercise training increased the rates of insulin-stimulated glucose transport in LCR (P=0.001; LCR SED vs. LCR EXT; Fig 4.1A). In contrast, exercise training did not alter insulin-stimulated glucose transport in HCR (Fig 4.1A). Basal muscle glycogen content was higher in HCR SED than LCR SED (P=0.02; Fig 4.1B). Exercise training increased the muscle glycogen content in LCR (P=0.04; LCR SED vs. LCR EXT; Fig 4.1B) and there was tendency for increased glycogen storage in HCR EXT (P=0.06 vs. HCR SED; Fig 4.1B). The phosphorylation of Akt on its Ser473 site mirrored the results of the insulin-stimulated glucose transport measures. HCR SED had a ~60% increase in Ser473 Akt phosphorylation compared to LCR SED (P<0.01; Fig 4.1C). Exercise training significantly increased the Ser473 phosphorylation of Akt by ~45% in LCR (P<0.01, LCR SED vs. LCR EXT; Fig 4.1C). There were no changes in Ser473 phosphorylation of Akt in HCR EXT (Fig.
4.1C). There were no changes in the phosphorylation of AS160 in any of the groups (Fig. 4.1D).

![Figure 4.1](image)

**Figure 4.1** Skeletal muscle glucose metabolism in sedentary (SED) and exercise trained (EXT) LCR and HCR. $^3$H-3-O-methylglucose transport (A) was measured in the soleus muscle during *in vitro* incubations data is expressed as fold-change rates of µmol/g/h (mean ± SE, n=8-10), muscle glycogen (B) content were determined in red gastrocnemius on aliquots of freeze dried/powdered muscle and expressed per g of dry weight [dw] (mean ± SE, n=9-10). Basal and insulin-stimulated Akt (C) and AS160 (D) were determined by western blotting and to confirm equal loading α-tubulin was run as a loading control (mean ± SE, n=8-10). Significant differences between groups (P<0.05) are indicated by the P value listed on the figure (mean ± SE, n=8-10).

**Exercise training improves lipid metabolism in LCR but not HCR.**

Exercise training improves lipid metabolism in LCR but not HCR.

Total and partial lipid oxidation and intramuscular triacylglycerol were measured to determine the effect on exercise training in both phenotypes. HCR SED had significantly higher rates of total and partial palmitate oxidation in soleus muscle compared to LCR SED (P=0.001 and P<0.05, respectively; Fig 4.2A, 4.2B). Exercise
training increased the rates of total palmitate oxidation by 40%, but not partial oxidation in LCR (P=0.02, LCR SED vs. LCR EXT; Fig 4.2A, 4.2B). There were no changes in the rates of post-exercise training lipid oxidation in HCR (HCR SED vs. HCR EXT; Fig 4.2A, 4.2B). Intramuscular triacylglycerol content was unchanged in all groups (Fig 4.2C).

Figure 4.2  Skeletal muscle lipid metabolism in sedentary (SED) and exercise trained (EXT) LCR and HCR. (A) Complete fatty acid oxidation ($^{13}$CO$_2$ production from labeled palmitate) as measured in soleus during in vitro incubations data expressed as rates of μmol/g/h (mean ± SE, n=8-10). (B) Partial oxidation as determined by the amount of label in the acid-soluble metabolite (ASM) during the in vitro incubation data expressed as rates of μmol/g/h (mean ± SE, n=8-10). Intramuscular triacylglycerol (C) content were determined in red gastrocnemius on aliquots of freeze dried/powdered muscle and expressed per g of dry weight [dw] (mean ± SE, n=9-10). Significant differences between groups (P<0.05) are indicated by the P value listed on the figure (mean ± SE, n=8-10).
Exercise training increased the expression of β-adrenergic pathway components in LCR.

HCR SED had a ~45% higher expression of the β2-AR (P<0.01 vs. LCR SED; Fig 4.3A) and a ~90% higher expression of the transcription factor Nur77 (P<0.05 vs. LCR SED; Fig 4.3B). Exercise training increased the expression of β2-AR by 26% (P<0.05, LCR SED vs. LCR EXT; Fig 4.3A) and Nur77 by 85% (P<0.01, LCR SED vs. LCR EXT; Fig 4.3B) in LCR. Exercise training had no effect in the expression of β2-AR and Nur77 in HCR (HCR SED vs. HCR EXT; Fig 4.3A, 4.3B).

![Figure 4.3](image)

Figure 4.3 Total protein content of β-adrenergic pathway components in the skeletal muscle of sedentary (SED) and exercise trained (EXT) LCR/HCR. Relative protein levels of β2-Adrenergic receptor (β2-AR) (A) and the nuclear orphan receptor Nur77 (C) were quantified using western blot analysis and densitometry in red gastrocnemius muscle. To confirm equal loading α-tubulin was run as a loading control. Significant differences between groups (P<0.05) are indicated by the P value listed on the figure (n=8-10/group).
Exercise training enhanced the expression of Nur77 target proteins in LCR.

The nuclear orphan receptor Nur77 is a transcriptional regulator of target genes such as Glut4, UCP3 and FAT/CD36. There were no differences in the expression of Glut4 between HCR SED and LCR SED (Fig 4.4A), but there was a ~70% difference in the expression of UCP3 (P<0.01 vs. LCR SED; Fig 4.4B) and a 52% higher expression of FAT/CD36 (P<0.05 vs. LCR SED; Fig 4.4C) in HCR SED. Exercise training increased the expression of Glut4 by ~50% (P<0.01, vs. LCR SED; Fig 4.4A), UCP3 by ~45% (P<0.01, vs. LCR SED; Fig 4.4B) and FAT/CD36 by ~65% (P<0.05, vs. LCR SED; Fig 4.4C) in LCR. There were no changes in the expression of Glut4, UCP3 or FAT/CD36 in response to exercise training in HCR (Fig 4.4A, 4.4B and 4.4C; respectively).

**Figure 4.4** Total protein content of Nur77 target genes in the skeletal muscle of sedentary (SED) and exercise trained (EXT) LCR/HCR. Relative protein levels of the glucose transporter, Glut4 (A), the uncoupling protein, UCP3 (B) and the fatty acid transporter, FAT/CD36 (C) were quantified using western blot analysis and densitometry in red gastrocnemius muscle. To confirm equal loading α-tubulin was run as a loading control. Significant differences between groups (P<0.05) are indicated by the P value listed on the figure (n=8-10/group).
4.4 Discussion and conclusion

Exercise training is a potent environmental stimulus for the improvement of insulin sensitivity in both humans (Bruce et al, 2003; Kirwan et al, 2009) and animals (Lessard et al, 2007) with insulin resistance and type 2 diabetes. However, there is also a strong genetic contribution to the onset of these disease states (Bouchard & Rankinen, 2001). In the present investigation we hypothesised that exercise training would overcome the genetic predisposition to impaired metabolic function imparted by artificial selection for low aerobic capacity. We present novel data clearly demonstrating that exercise training improved both carbohydrate (Fig 4.1) and lipid metabolism (Fig 4.3) in the skeletal muscle of LCR, but not HCR. These changes were reflected by increases in the insulin-stimulated phosphorylation of Akt on Ser 473 (Fig 4.1C) and improvements in whole body measures such as circulating fatty acids levels and adiposity (Table 4.1). Exercise-induced improvements in skeletal muscle substrate metabolism coincided with enhancement to components of the β-adrenergic pathway (Fig 4.3), and known target proteins (Fig 4.4). Our results provide strong evidence to demonstrate that genetically-induced impairments to muscle glucose and lipid metabolism can be ameliorated by exercise training.

Exercise training is a highly effective treatment for the reversal of skeletal muscle insulin resistance (Hawley, 2004; Hawley & Lessard, 2008). This occurs, in part, through an increase in oxidative enzymes that facilitate the turnover and oxidation of fatty acid both at rest and during exercise (Goodpaster et al, 2003). Multiple research groups have
reported that low-intensity exercise increases rates of whole-body fat oxidation, decreases circulating lipids and enhances insulin sensitivity in previously untrained, obese and insulin resistant individuals (Hansen et al, 2009; Schrauwen et al, 2006; Venables & Jeukendrup, 2008). Venables & Jeukendrup (2008) recently reported that a six week low-intensity exercise training regimen enhanced whole-body fat oxidation and insulin sensitivity in obese individuals (Venables & Jeukendrup, 2008). In addition, it has been shown that low- to moderate-intensity exercise is as effective as moderate- to high-intensity exercise at improving whole-body metabolic risk factors (ie. HbA1c, BMI, LDL etc.) (Hansen et al, 2009). Furthermore, exercise training in LCR reduces several cardiovascular risk factors associated with the metabolic syndrome (Haram et al, 2009; Wisloff et al, 2005). In agreement with the results of these studies we now show that six weeks of endurance exercise training reversed impaired skeletal muscle lipid oxidation in LCR (Fig 4.2A). Exercise-induced enhancements to lipid oxidation in LCR were associated with increased insulin-stimulated glucose uptake and Akt Ser473 phosphorylation in skeletal muscle. Yet, despite increased lipid oxidation (Fig 4.2A) and improved insulin sensitivity (Fig 4.1A) in the skeletal muscle of LCR EXT, there was no change in intramuscular triglyceride content compared to LCR SED (Fig. 4.2C). This observation is in agreement with Perdomo et al (2004) who observed enhanced insulin sensitivity in skeletal muscle cells with increased lipid oxidation despite increased intramyocellular lipid accumulation in culture (Perdomo et al, 2004).

In the present investigation, both LCR and HCR underwent the same absolute exercise training protocol (i.e. the same frequency, intensity and volume). However,
there were only exercise training-induced metabolic improvements in animals with intrinsic impairments to skeletal muscle glucose and lipid metabolism (i.e. LCR; Fig 4.1 and Fig 4.2). While it is possible that a higher absolute (i.e. similar relative) training stimulus may have induced metabolic changes in HCR, our results are consistent with those of Barwell et al (2008) who observed exercise training-induced improvements in the insulin sensitivity index (ISI) in daughters of patients with type 2 diabetes, but not in women with no family history of diabetes (Barwell et al, 2008). Participants in that study (Barwell et al, 2008) were matched for aerobic capacity (VO2max) and adiposity and underwent exercise sessions at the same relative intensity (65-80% maximum heart rate). Taken collectively, the results from the present investigation along with those of Barwell et al. (2008) raise the intriguing possibility that individuals with a genetic predisposition to metabolic abnormalities may have enhanced responsiveness to the metabolic improvements induced by exercise training at a relative intensity (Barwell et al, 2008).

In the absence of exercise training, artificial selection for low intrinsic running capacity results in impaired skeletal muscle glucose and lipid metabolism (Fig 4.1 and Fig 4.2). However, it is unclear what specific molecular defect(s) are responsible for both reduced running capacity and impaired metabolism in LCR. We have previously conducted a comprehensive investigation of factors that may contribute to both exercise capacity and skeletal muscle insulin sensitivity in LCR/HCR and observed no differences in AMPK activation, or lipid (ceramide and diacylglycerol) storage in the muscle of these animals (Lessard et al, 2009). We did, however, observe a reduction in
β2-AR content and a concomitant impairment in β-adrenergic signal transduction and lipolysis in the skeletal muscle of LCR (Lessard et al, 2009). Given that β-adrenergic pathways are critical for the regulation of glucose and lipid metabolism in skeletal muscle and other tissues (Kraus et al, 1989; Martin et al, 1991; Wallberg-Henriksson, 1987), it follows that reduced activation of these pathways could contribute to metabolic disease. In support of this contention, an impaired response of β2-adrenoreceptors has been identified as the source of reduced adrenergic stimulated lipolysis in obese individuals (Blaak et al, 2004). Furthermore, variant alleles of the β2-AR have been associated with obesity, dyslipidaemia and type 2 diabetes in humans (Ishiyama-Shigemoto et al, 1999; Meirhaeghe et al, 1999; Pinelli et al, 2006; Yamada et al, 1999). However, the risk of obesity imparted by β2-AR alleles is reversed in physically active individuals (Meirhaeghe et al, 1999). Continuous contractile activity of skeletal muscle results in elevated levels of β-AR in skeletal muscle, which coincides with increased expression of genes that encode proteins important for oxidative metabolism (Kraus et al, 1989). In line with these observations, we now report that lower β2-AR content in LCR was normalized to the level of HCR by exercise training and this was associated with increased lipid oxidation in skeletal muscle (Fig 4.3A; Fig 4.2A).

One mechanism by which improved β-adrenergic signal transduction may improve skeletal muscle metabolism is via activation of the nuclear receptor, Nur77. Stimulation of the β-adrenergic pathway in skeletal muscle stimulates the expression of Nur77 and several of its target genes (i.e. GLUT4, CD36, UCP3) that are critical regulators of glucose and lipid metabolism in muscle (Kanzleiter et al, 2009; Lessard et al, 2009;
Maxwell et al, 2005). Skeletal muscle Nur77 expression is down-regulated in several animal models of obesity and type 2 diabetes (i.e. ob/ob, db/db, ZDF) (Fu et al, 2007) and is increased in response to insulin-sensitizing treatments (i.e. thiazolidinediones) (Fu et al, 2007) or a single exercise bout (Kawasaki et al, 2009a; Mahoney et al, 2005), indicating a possible role in the regulation of insulin sensitivity. Recent evidence suggests that Nur77 is also down-regulated in response to high-fat diet-induced obesity (Kanzleiter et al, 2009) and that genetic deletion of Nur77 results in insulin resistance and altered systemic glucose metabolism in mice (Chao et al, 2009). We have previously proposed that reduced mRNA and protein expression of Nur77 and its target genes in the skeletal muscle of LCR provides a potential mechanism for impaired metabolism in this model (Lessard et al, 2009). We now provide novel evidence to show that reduced Nur77, CD36 and UCP3 expression in LCR are normalised by exercise training and coincide with improved skeletal muscle insulin sensitivity and palmitate oxidation in this model. Exercise-induced expression of Nur77 and its target genes (GLUT4, FAT/CD36 and UCP3; Fig 4.4) provide a potential mechanism for improved skeletal muscle insulin sensitivity in LCR. In support of this contention, muscle-specific over-expression of FAT/CD36, UCP3, or GLUT4 improves insulin sensitivity in transgenic mice (Brozinick et al, 1996; Choi et al, 2007; Heron-Milhavet et al, 2004). Furthermore, independent investigations have observed an increase in the expression of FAT/CD36, UCP3, and GLUT4 following exercise training (Holloway et al, 2006; Lessard et al, 2007; Mensink et al, 2007). Thus, when taken collectively, the results of the present and previous investigations identify Nur77-mediated transcription as a potential mechanism for the
improvement of glucose and lipid metabolism in skeletal muscle following exercise training.

In summary, we have used a novel animal model of genetically-imparted endurance exercise capacity and metabolic health to study the genetic and environmental contributions to skeletal muscle glucose and lipid metabolism. Our results demonstrate that rats bred for low intrinsic aerobic capacity display skeletal muscle insulin resistance and reduced lipid oxidation. Decreases in glucose and lipid metabolism were associated with decreased β2-AR, and reduced expression of Nur77 target proteins that are critical regulators of muscle glucose and lipid metabolism (UCP3, FAT/CD36). Exercise training reversed the impaired glucose and lipid metabolism in the skeletal muscle of LCR, but not HCR. Exercise-induced improvements in skeletal muscle insulin sensitivity were associated with increased expression of β2-AR, Nur77, Glut4, UCP3 and FAT/CD36. Our investigation demonstrates that metabolic impairments resulting from genetic factors (low intrinsic aerobic capacity) may be overcome by environmental factors (exercise training) and identifies Nur77 as a potential mechanism for improved skeletal muscle metabolism in response to exercise training.
CHAPTER FIVE: Lipid-induced mammalian target of rapamycin activation in rat skeletal muscle reversed by exercise and 5′-aminoimidazole-4-carboxamide ribonucleoside.

5.1 Introduction

The insulin signal transduction cascade regulates many processes in skeletal muscle including glucose and lipid metabolism, cellular growth and differentiation and protein synthesis (Avruch, 1998; Kimball et al, 2002; Taniguchi et al, 2006). Accordingly, impaired insulin signalling has been implicated in several disease states including Type 2 diabetes (Taniguchi et al, 2006; Zamboni et al, 2005). Insulin action in muscle is a tightly regulated process with inputs from several diverse stimuli such as nutrient availability, hormonal milieu and exercise/muscle contraction (Hawley et al, 2006; Taniguchi et al, 2006). Specifically, chronic consumption of a high-fat diet increases the circulation of non-esterified fatty acids (NEFA) resulting in marked skeletal muscle insulin resistance in rodents and humans (Barnard & Youngren, 1992; Boden, 2002; Lessard et al, 2007). In contrast, one of the most potent means to improve insulin action in muscle is exercise training (Barnard & Youngren, 1992; Hawley, 2004; Hawley & Lessard, 2008). It has previously been demonstrated that exercise reverses the effects of a high-fat diet on skeletal muscle insulin resistance (Barnard & Youngren, 1992; Lessard et al, 2007). However, the precise mechanism(s) by which lipids induce insulin resistance and exercise training reverses these defects in skeletal muscle is currently unknown.

The serine/threonine protein kinase, mammalian target of rapamycin (mTOR) is a member of the phosphatidyl-inositol 3 kinase (PI3K) family of enzymes. This kinase is regulated by insulin and nutrient availability and plays a central role as a nutrient sensor in skeletal muscle (Marshall, 2006; Patti & Kahn, 2004). mTOR associates with its binding partners, raptor and rictor, to form two structurally and functionally distinct
complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), respectively (Bhaskar & Hay, 2007; Kim et al, 2002; Sarbassov et al, 2004). Activation of mTORC1 through a negative feedback loop inhibits insulin signalling via the activation of its downstream substrate ribosomal protein S6 kinase 1 (S6K1) and subsequent inhibitory serine phosphorylation of insulin receptor substrate 1 (IRS1) (Tremblay & Marette, 2001). In contrast, mTORC2 is a positive regulator of insulin signal transduction through its phosphorylation of protein kinase B/Akt (Akt) on its serine 473 activation site (Hresko & Mueckler, 2005; Sarbassov et al, 2005). Its ability to differentially modulate both proximal and distal components of the insulin signalling cascade raises the intriguing possibility that mTOR may be a critical regulator of insulin action in skeletal muscle.

Recent evidence demonstrates that in obese rodents or in rodents consuming a high-fat diet, over-activation of mTOR may be associated with impairments to skeletal muscle insulin action (Khamzina et al, 2005; Tremblay et al, 2007). In contrast, S6K1 null mice are protected from high-fat diet induced insulin resistance (Um et al, 2004). Taken collectively, these results (Khamzina et al, 2005; Tremblay et al, 2007; Um et al, 2004) implicate mTORC1 activation as a possible mechanism for impaired insulin action in response to elevated lipid availability. However, it is unclear what role, if any, mTORC2 activation plays in lipid-induced insulin resistance. Activation of Akt by mTORC2 has several potential consequences for myocellular metabolism, as the Akt pathway is responsible for mediating most of the metabolic actions of insulin (Taniguchi et al, 2006). At present, it is unknown whether in skeletal muscle mTORC1 & 2 are differentially regulated in response to increased lipid availability.
It was previously demonstrated that endurance training increases the activation of 5‘AMP-activated protein kinase (AMPK) in skeletal muscle of high-fat fed rodents (Lessard et al, 2007). Furthermore, exercise training has the ability to chronically elevate AMPK activation in skeletal muscle (Frosig et al, 2004; Sriwijitkamol et al, 2006). AMPK, because of its role in suppressing energy consuming processes, is a known physiological inhibitor of the energy consuming mTOR signalling pathway (Deshmukh et al, 2008; Kimball, 2006). At present, the effect of endurance training on mTOR activation in insulin resistant muscle has not been investigated.

mTOR complexes can positively or negatively affect insulin action and are regulated by nutrients and AMPK. Therefore, this study hypothesised that improvements in muscle insulin action following exercise training in high-fat fed animals would be associated with altered activation and formation of mTOR complexes 1/2. Accordingly, the effects of high-fat feeding and exercise training on the regulation of mTORC 1 & 2 and the activation of their downstream substrates (i.e. Akt, S6K1/IRS1) in was determined in skeletal muscle. In addition, it was determined whether acute palmitate exposure and the activation of AMPK by 5’-aminoimidazole-4-carboxamide-1- β-D-ribofuranoside (AICAR) regulate the formation and activation of mTOR complexes 1/2.
5.2 Material and Methods

Animals

Sprague Dawley rats (~4 wk old) were given *ad libitum* access to either control chow diet (n=8) (D12328, Research Diets Inc., NJ, USA, 73.1% carbohydrates, 10.5% fat, and 16.4% protein) or a high fat diet (n=16) (D12330, Research Diets Inc., 25.5% carbohydrates, 58% fat, and 16.4% protein). After a 4 wk dietary period to induce insulin resistance, high fat-fed animals were randomly assigned to either exercise training (HF EXT, n=8) or sedentary control (HF CON, n=8) groups. Exercise training consisted of treadmill running for 1 hr/day, 5 days/wk on a 15% incline at a speed that was progressively increased to 32 m/min during the first 5 days of training and maintained for a subsequent 4 wk. The third group of animals (CF CON) remained on the control chow diet for the duration of the study (8 wk). Following the experimental period animals were fasted for 8-12 hr before undergoing hind-limb perfusion (described subsequently). Exercise trained animals undertook their last exercise bout 36-48 hr prior to hind-limb perfusion. All experimental procedures were approved by the Institutional Animal Care and Use Committee at California State University, Northridge.

Hind-limb perfusion and 3-O-Methylglucose Transport

Rats were anaesthetised with an intraperitoneal injection of sodium pentobarbital (6.5 mg/100 g body mass) and surgically prepared for hind limb perfusion as previously described (Lessard et al, 2007). Briefly, cannulae were inserted into the abdominal aorta and vena cava, and the animals were sacrificed via an intracardiac injection of pentobarbital as the hind limbs were washed out with 30 ml of Krebs-
Henseleit buffer (KHB, pH 7.55). Immediately, the cannulae were placed in line with a non-recirculating perfusion system, and the hind limbs were allowed to stabilise during a 5 min washout period which consisted of a basic perfusate medium that contained 30% washed time-expired human erythrocytes (Ogden Medical Center, UT, USA) KHB, 4% dialysed bovine serum albumin (Fisher Scientific, Fair Lawn, NJ), 0.2 mM pyruvate and was continuously gassed with a mixture of 95% O₂- 5% CO₂ and warmed to 37°C. Perfusions were performed at a flow rate of 7.5 ml/min and glucose transport was measured over an 8 min period using 8 mM of the non-metabolised glucose analogue 3-

O-methylglucose (3-MG) (32 µCi 3-[³H] MG mM⁻¹, PerkinElmer Life Sciences, USA) in the presence (INSULIN) or absence (BASAL) of 500 µU/ml insulin. 2 mM mannitol (60 µCi-[1-

¹⁴C] mannitol mM⁻¹, PerkinElmer Life Sciences) was used as an extracellular space marker. Immediately following the perfusion, portions of the red gastrocnemius (RG), a muscle comprising 90-95% oxidative fibres (Wilson et al, 1998), were excised from both hind limbs, blotted on gauze dampened with cold KHB, freeze clamped in liquid N₂ and stored at -80°C for later analysis. Rates of basal and insulin-stimulated skeletal muscle 3-O-methylglucose transport were calculated as previously described (Lessard et al, 2007).

**Muscle Homogenisation**

Portions of muscle were cut from basal and insulin-stimulated RG, weighed frozen and homogenised in an ice-cold homogenisation buffer (1:8 wt/vol) containing 50 mM Tris-HCl (pH 7.5), 5 mM Na-pyrophosphate, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 10% glycerol (v/v), 1% Triton-X, 1 mM DTT, 1 mM benzamidine, 1 mM PMSF, 10 µg/mL
trypsin inhibitor, and 2 µg/ml aprotinin. Following centrifugation (21,000 x g, 4°C) for 25 min the supernatant was collected and assayed for protein content.

**Cell Culture**

Stock L6 myoblasts (American Type Culture Collection, VA, USA) were maintained at 37°C (95% O2-5% CO2) on 75 cm² collagen-coated flasks in α-modified Eagle's medium (α-MEM, Invitrogen, VIC, AUS) containing 10% foetal bovine serum (FBS, Sigma-Aldrich, NSW, AUS) culture medium, 1% penicillin-streptomycin (Sigma-Aldrich) and 5.5 mM glucose as previously described (Lessard et al, 2006). Differentiation was induced by switching to medium containing 2% horse serum (Sigma-Aldrich) when the myoblasts were ~90% confluent. Experimental treatments were started after 5 d, by which time nearly all of the myoblasts had fused to form myotubes. For experimental procedures the cells were maintained in 75 cm² flasks (co-immunoprecipitation experiments) or trypsinised and seeded in 6 well plates. All subsequent experiments were done after 4 hr serum starvation. To determine the effect of acute palmitate treatment, cells were incubated with 0.4 mM palmitate in ethanol vehicle for 0, 2 or 4 hr in 2% fatty-acid free BSA. The effect of AMPK activation was subsequently determined by incubating cells for 1 or 4 hr with or without 1 mM AICAR (Sigma-Aldrich). A control group was maintained for each experiment by incubating in the presence of 2% fatty-acid free BSA (Sigma-Aldrich, MO, USA) and the appropriate vehicle (ethanol and/or phosphate buffered saline). For the co-immunoprecipitation experiments cells were incubated with 0.4 mM palmitate for 4 hr with or without 1 mM AICAR. For insulin
stimulated conditions 100 nM insulin was added to appropriate wells during the last 30 min of incubations. All experiments were run in triplicate.

**Western Blotting**

Insulin-stimulated RG muscle lysates (60 µg) were solubilised in Laemmli buffer, separated by SDS-PAGE, and transferred to PVDF membranes. The membranes were then blocked (5% NFDM), and incubated overnight at 4°C with primary antibodies specific for either mTOR (mTab1), phospho-Akt1 Ser473, phospho-Akt1 Thr308, (1:1000; Upstate Biotechnology, NY, USA), AMPKα, Akt1, Akt2, S6K1, Raptor, Rictor (mAb), TSC2, phospho-mTOR Ser2448, phospho-S6K1 Thr389, phospho-TSC2 Thr1462, phospho-IRS1 Ser636/639, phospho-Akt substrate (1:1000; Cell Signaling, MA, USA) or phospho-AMPKα Thr172 which was raised against AMPKα peptide (KDGEFLRpTSCGAPNY) as described previously (Lessard et al, 2006). The immunoreactive proteins were detected with enhanced chemiluminescence (Amersham Biosciences) and quantified by densitometry.

**Akt2 Immunoprecipitation**

The Catch and Release v2.0 Reversible Immunoprecipitation System (Upstate Biotechnology, USA) was used for detection of phospho-specific Akt2 sites as per manufacturer’s instructions. Briefly, insulin-stimulated RG muscle lysates (600 µg) and Akt2 (rabbit) antibody (6 µg, Upstate Biotechnology) were rotated overnight at 4°C, immunocomplexes were eluted and subjected to SDS-PAGE electrophoresis. Antibodies specific for phospho-Akt Ser 473 (mouse) or phospho-Akt Thr308 (mouse) (Cell
Signaling, MA, USA) were used to detect the immunoreactive proteins as described above.

**Akt1 Kinase Activity Assay**

Akt1 Immunoprecipitation Kinase Assay Kit (Upstate Biotechnology) was used to determine Akt1 activation. Briefly, Akt1 antibody (4 µg/sample, Upstate Biotechnology) and insulin-stimulated RG muscle lysates (400 µg) were rotated overnight at 4°C. Protein G (50 µL, Amersham Bioscience) slurry was then added to the antibody-protein complex and rotated 120 min at 4°C. The kinase activity assay was then performed as per manufacturer’s instructions.

**mTOR Complex Co-immunoprecipitation**

The mTOR complexes have been previously been shown to be detergent sensitive (Sarbassov et al, 2004). Therefore, 0.3% CHAPS was substituted for 1% Triton-X in the homogenisation buffer for the co-immunoprecipitation of the mTOR complexes. Basal and insulin-stimulated RG homogenates (5 mg) or L6 cell lysates (500 µg) and mTOR (mTab1) antibody (8 µl/sample, Upstate Biotechnology) were rotated overnight at 4°C. Protein G (100 µl, Amersham Biotechnology) slurry was then added to the antibody-protein complex and rotated 120 min at 4°C. The beads were then washed five times with homogenisation buffer and aliquoted for western blotting. Aliquots of the bead-antibody-protein complex were resuspended in Laemmli Buffer, subjected to SDS-PAGE and transferred to PVDF membranes. The membranes were then blocked (5% NFDM) and incubated overnight at 4°C with primary antibodies specific for Raptor, Rictor (1:1000; Cell Signaling), and mTOR. Results are normalised to total mTOR.
5.2.1 Statistical Analyses

All results are presented as mean ± standard error (SE). Differences between treatment groups were determined using a one-way ANOVA with a Student-Newman-Keuls post hoc test.

5.3 Results

*Insulin-stimulated 3-O-methylglucose transport in red gastrocnemius skeletal muscle.*

Basal rates of 3-MG transport were similar among groups (Fig 5.1). The rates of insulin-stimulated glucose transport were decreased by the HF-diet (p<0.05, vs. CF CON; Fig 5.1) but were normalised to control levels by exercise training (p<0.05, vs. HF CON; Fig 5.1).

![Insulin-Stimulated Glucose Transport](image)

**Figure 5.1** Muscle glucose transport was measured in the red gastrocnemius muscle after an 8 min hindlimb perfusion with $^3$H-3-O-methylglucose in the absence (BASAL) or presence (INSULIN) of insulin (500 µU/ml) data expressed as rates µmol/g/h (mean ± SE, n=6-8). Significant differences between groups * = p<0.05 vs CF CON, # = p<0.05 vs. HF CON.
**Chronically increased lipid availability enhances mTOR complex formation.**

The basal and insulin-stimulated formation of the mTORC 1 & 2 complexes in response to increased chronic lipid availability and exercise were determined by immunoprecipitation of mTOR then probing for its binding partners (raptor and rictor, Fig 5.2A). In the insulin-stimulated muscle, there was a 70% increase in co-immunoprecipitation of raptor and mTOR in HF CON (p<0.01, vs. CF CON; Fig 5.2C), indicating higher mTORC1 complex formation in response to high-fat feeding. Exercise training completely abolished the high-fat diet-induced increase in mTORC1 formation such that values were restored to those observed in CF CON (p<0.01, HF EXT vs. HF CON; Fig 5.2C). The co-immunoprecipitation of rictor and mTOR (i.e. mTORC2 complex formation), in the insulin-stimulated muscle, mirrored mTORC1 complex formation. High-fat feeding increased mTORC2 formation by 64% (p<0.05, vs. CF CON; Fig 5.2B), while exercise training decreased mTORC2 formation by 79% (p<0.01, HF CON vs. HF EXT; Fig 5.2B). These results demonstrate that in response to insulin both mTORC1 and mTORC2 formation are stimulated by chronically increased lipid availability, but these effects are reversed by exercise training. There were no significant differences in the formation of mTORC1 (Fig 5.2C) and mTORC2 (Fig 5.2B) in the basal muscle. Changes of mTOR complex formation occurred in the absence of changes in the total protein content of mTOR, raptor and rictor (Fig 5.2D).
Figure 5.2 The basal or insulin-stimulated formation of the mTOR complexes in response to high-fat feeding or after exercise training was determined by immunoprecipitation of mTOR followed by Western blotting to detect its binding partners, rictor and raptor (A) rictor bound to mTOR (B) raptor bound to mTOR (C) are expressed as arbitrary units (mean ± SE, n=6-8). Relative protein levels of total mTOR, total rictor and total raptor (D) were quantified by densitometry. Representative blots are shown. Significant differences between groups * = p<0.05 vs CF CON, # = p<0.05 vs. HF CON.

**HF diet and exercise training differentially affects mTORC1 activity.**

In order to determine how mTORC1 complex formation translates to mTOR activation, the phosphorylation of its downstream substrates were measured under insulin-stimulated conditions. Increases in mTORC1 formation following a high-fat diet resulted in concomitant increases in mTOR activity, as demonstrated by phosphorylation of its downstream substrate S6K1 (Fig. 5.3A). In response to high-fat feeding phosphorylation of S6K1 was increased by 20% at Thr389 (p<0.001, CF CON vs. HF CON; Fig. 5.3A). Exercise training normalised S6K1 phosphorylation on Thr389 (p<0.001, HF CON vs. HF EXT; Fig 5.3A). Increased S6K1 activation by high-fat feeding resulted in an 18% increase in inhibitory phosphorylation of IRS1 at Ser636/639 (p<0.05,
CF CON vs. HF CON; Fig 5.3B), which was reversed in HF EXT (p<0.05, HF CON vs. HF EXT; Fig. 5.3B). These results indicate that a high-fat diet increased mTORC1 activity, which induced a negative feedback loop on insulin signal transduction at the level of IRS1. Importantly, these diet-induced effects were reversed by exercise training. There were no changes in the protein concentration in total S6K1 or total IRS1 (Fig. 5.3A, 5.3B, respectively).

![Diagram](image)

**Figure 5.3** The insulin-stimulated phosphorylation of S6K1 on Thr389 (A), IRS1 on Ser636/639 (B) were quantified using Western blot analysis and densitometry. Representative immunoblots are shown on each figure. Values are expressed as arbitrary units (mean ± SE, n=8). Significant differences between groups * = p<0.05 vs CF CON, # = p<0.05 vs. HF CON.

**Akt1 phosphorylation and activity mirrors the increase in mTORC2 complex formation.**

To assess mTORC2 activation in response to insulin stimulation, the isoform-specific phosphorylation of Akt1 and Akt2 at Ser473 were measured. In agreement with
changes in mTORC2 formation, the phosphorylation of Akt1 was increased by 26% on the Ser473 site in HF CON (p<0.05 vs. CF CON) but was decreased by 30% in HF EXT (p<0.05, vs. HF CON; Fig 5.4B). Changes in Akt1 Ser473 phosphorylation were accompanied by similar alterations in Akt1 kinase activity, which tended to increase in HF CON (12%, p<0.10 vs. CF CON, Fig 5.4C) and was decreased in HF EXT (p<0.05, HF EXT vs. HF CON; Fig 5.4C). Decreases in Akt1 Ser473 phosphorylation and activity following exercise training occurred despite an increase in total Akt1 protein (p<0.001 CF CON vs. HF EXT and p<0.001 HF CON vs. HF EXT; Fig 5.4A). In contrast, following high-fat feeding or exercise training there were no changes in Ser473 phosphorylation or total protein content of the Akt2 isoform, or the phosphorylation of its downstream target the Akt substrate of 160 kDa (AS160) (Fig 5.4D). These results indicate that mTORC2 may preferentially phosphorylate and activate the Akt1 isoform. In addition, the phosphorylation of Akt1 and Akt2 on Thr308 were similar in all groups (Fig 5.4B and 5.4D, respectively), demonstrating that the phosphorylation of Thr308 is independent of mTORC2 formation.
Figure 5.4 Total Akt1 (A) and its insulin-stimulated phosphorylation on Ser473 and Thr308 (B), were quantified using Western blot analysis and densitometry. Insulin-stimulated Akt1 Kinase activity was measured using the immunoprecipitation of 400 µg of protein (C). Site specific Akt2 phosphorylation was measured by immunoprecipitation of 600 µg of protein and Western blot analysis using antibodies specific for Akt Ser473 and Akt Thr308 phosphorylation sites (D). Total Akt2 and the phosphorylation of AS160 (D) were quantified using Western blot analysis and densitometry. Representative immunoblots are shown. Values are expressed as arbitrary units (mean ± SE, n=7-8). Significant differences between groups *= p<0.05 vs CF CON, # = p<0.05 vs. HF CON.

Exercise training regulates the phosphorylation of mTOR and its physiological inhibitors AMPK and TSC2.

AMPK and tuberous sclerosis complex 2 (TSC2) are known inhibitors of mTOR activation. Thus, their phosphorylation in response to high-fat feeding and exercise training was determined in response to insulin. The phosphorylation of AMPK on its Thr172 activation site was increased by 19% in HF EXT (p<0.05, vs. HF CON; Fig 5.5B). No changes were noted in the total protein concentration of the AMPKα catalytic subunit (Fig 5.5B). In contrast, exercise training decreased the phosphorylation of TSC2 on Thr1462 by 30% (p<0.01, vs. HF CON; Fig 5.5C). As TSC2 is a downstream target of Akt, these observed decreases in TSC2 phosphorylation likely reflect reduced Akt1 Ser473 phosphorylation and activation following exercise training (Fig 5.4A). There was no
difference in the total protein concentration of TSC2 between the groups (Fig 5.5C). Despite the increases in mTORC1 activation by high-fat feeding, the phosphorylation of mTOR on its Ser2448 site was increased in HF EXT by 37% (p<0.05, vs. HF CON) and 47% (p<0.01, vs. CF CON; Fig 5.5A).

**Figure 5.5** Insulin-stimulated phosphorylation of mTOR and its inhibitors AMPK and TSC2. The phosphorylation of mTOR on Ser2448 (A) AMPKα on Thr172 (B) and TSC2 on Thr1462 (C) were quantified using Western blot analysis and densitometry. Representative immunoblots are shown on each figure. Values are expressed as arbitrary units (mean ± SE, n=7-8). Significant differences between groups *= p<0.05 vs CF CON, # = p<0.05 vs. HF CON.

**Acute palmitate and AICAR activation of mTOR complexes in L6 cell culture.**

It has been previously demonstrated that within 3-5 hr circulating non-esterified fatty acids (NEFA) induce insulin resistance in skeletal muscle (Boden, 2002). Treatment with the AMPK activator AICAR has been shown enhance glucose transport and inhibit mTOR activation (Bolster et al, 2002; Ju et al, 2007). Therefore, this study determined if 4 hr palmitate incubation could activate mTOR signalling *in vitro* and if the stimulation of
AMPK by AICAR could inhibit the formation of mTOR complexes 1/2 and the phosphorylation of their downstream substrates. An incremental increase in palmitate-induced S6K1 and Akt1 phosphorylation after 2 and 4 hr of palmitate exposure in L6 myotubes (Fig 5.6A) was observed. AICAR decreased the phosphorylation of S6K1 after 1 and 4 hr of treatment in both the absence (CON) and presence of palmitate (Fig 5.6B). Similar to the results of the chronic in vivo experiments, 4 hr palmitate treatment increased the phosphorylation of S6K1 Thr389 and Akt1 Ser473 by 60% and 30%, respectively (p<0.05 vs. CON; Fig 5.6C). AICAR significantly increased the phosphorylation of AMPK on Thr172 (p<0.05 vs. CON, Fig 5.6C). AICAR-induced AMPK activation decreased palmitate-induced S6K1 Thr389 phosphorylation by 63% (p<0.05 vs. Palm, Fig 5.6C). There were no significant changes in the acute complex formation of either mTORC1 or mTORC2 in response to palmitate or AICAR (Fig 5.6E, 5.6F).
Figure 5.6 The basal and insulin-stimulated activation of mTORC1/2 substrates in response to palmitate and/or AICAR treatment was determined in L6 myotubes. Phosphorylation of S6K1 on Thr389 and Akt1 on Ser 473 was measured in response to treatment with 0.4 mM palmitate for 0 (control), 2 (2h Palm) or 4 (4h Palm) hr (A). The effect of 1 mM AICAR on the phosphorylation of S6K1 (Thr389), Akt1 (Ser473) and AMPK (Thr172) was measured in cells treated with (Palm) or without (Con) 0.4 mM palmitate during the entire 4 hr incubation (4h AICAR) or during the last 1 hr (1h AICAR) (B). In order to associate changes in mTORC activation with complex formation, 75 mm² flasks of cells were incubated in 0.4 mM palmitate and 1 mM AICAR for 4 hr to determine the phosphorylation of S6K1 on Thr389, Akt1 on Ser473 and AMPK on Thr172 (C). The formation of mTORC1/2 (D) was measured by immunoprecipitation of mTOR followed by western blot of mTOR binding partners’ raptor (E) and rictor (F) which were quantified by densitometry. Insulin (100 nM) was added to the appropriate groups during the last 30 min of incubation. Representative blots are shown. Actin was run as a loading control for each sample. Significant differences between groups *= p<0.05 vs CF CON, # = p<0.05 vs. HF CON; n=3/treatment.

5.4 Discussion and Conclusion

The aim of the present study was to determine the effect of increased lipid oversupply on the regulation of mTOR complexes, mTORC1 and mTORC2. Furthermore, it was sought to establish whether mTOR complex formation is linked to the activation
of downstream substrates involved in insulin signal transduction in skeletal muscle. It has previously been demonstrated that exercise training can reverse impairments to skeletal muscle insulin action in response to chronic high-fat feeding (Lessard et al, 2007). Given the potential for mTOR complexes to both positively and negatively regulate insulin action in skeletal muscle, it is plausible that high-fat feeding and exercise training divergently regulate mTOR complex formation. Accordingly, this study hypothesised that a potential mechanism by which exercise and high-fat feeding exert divergent effects on insulin action may involve the differential regulation of mTOR complex formation. For the first time, it is demonstrated in vivo that both mTORC1 and mTORC2 formation are up-regulated by chronic lipid availability (high-fat diet) and that these changes are completely reversed by exercise training. Additionally, increased mTORC1 and mTORC2 formation in response to a high-fat diet was associated with activation of their respective downstream substrates and altered insulin signal transduction.

The hyperactivation of mTORC1 by nutrient excess (amino acids, lipids, obesity) has been implicated in decreased insulin signal transduction at the level of IRS1 (Khamzina et al, 2005; Krebs et al, 2007; Patti et al, 1998; Tremblay et al, 2007; Tremblay & Marette, 2001; Um et al, 2004). It has been proposed that an S6K1-associated negative feedback loop results in the inhibitory serine phosphorylation of IRS1 in response to mTOR activation (Patti & Kahn, 2004; Um et al, 2006). Decreases in IRS1 associated PI3K activity in skeletal muscle after HF feeding have previously been observed (Lessard et al, 2007). However, the regulation of mTORC1 activation by acute
and chronic lipid availability is not well described. Recent *in vitro* work in skeletal muscle cell culture has directly implicated palmitate with the inhibition of insulin stimulated glucose uptake (Pimenta et al, 2008) likely through the activation of mTORC1 and its inhibition of insulin signalling (Mordier & Iynedjian, 2007). In support of a role for mTOR activation in insulin resistance, Miller et al. (2008) observed increased mTOR activation in the skeletal muscle of ob/ob mice (Miller et al, 2008). However, the authors did not observe improvements in whole-body insulin or glucose tolerance after acute treatment with rapamycin, a potent inhibitor of mTOR activation (Miller et al, 2008). In contrast, the current study shows an exercise-induced inhibition of mTOR signalling that corresponded with an increase in skeletal muscle specific insulin sensitivity (Fig 5.1).

A novel finding of the present study was that a high-fat diet increased the activation of mTORC1 in skeletal muscle, as evidenced by an increase Thr389 S6K1 phosphorylation (Fig 5.3A, Fig 5.6C) which led to a concomitant increase in the serine phosphorylation of IRS1 (Fig 5.3B). These results support the contention that one potential mechanism for the inhibition of proximal insulin signalling following acute and chronic lipid oversupply may be increased mTORC1 activation leading to IRS1 serine phosphorylation. In contrast, it is shown that exercise training reverses the effects of chronic lipid oversupply on mTORC1 activation (Fig 5.3A), as evidenced by normalisation of S6K1 phosphorylation and decreased serine phosphorylation of IRS1 (Fig 5.3B). It was previously shown that exercise training increases AMPK activation (Atherton et al, 2005; Hawley & Lessard, 2008; Lessard et al, 2007), suggesting that exercise training may reverse the effect of mTOR activation through increased AMPK activity. In agreement
with this hypothesis, Ju et al. (2007) have shown in cell culture that AMPK regulates insulin action through the inhibition of IRS1 serine phosphorylation by mTOR/S6K1 (Ju et al, 2007). Here it is demonstrated that treating palmitate incubated cells with the AMPK activator AICAR inhibits phosphorylation of S6K1 at its Thr389 site (Fig 5.6C).

Analogous to the effects of increased lipid availability on mTORC1, it is demonstrated that a HF-diet increases the formation of mTORC2, as indicated by increased mTOR/riktor association (Fig 5.1B). Again, exercise training reversed the effects of high-fat feeding on mTORC2 formation (Fig 5.1B). The simultaneous activation/deactivation of mTORC1 and mTORC2 in skeletal muscle seems paradoxical, as these two complexes have opposing effects on insulin signal transduction (Bhaskar & Hay, 2007). While mTORC1 activation inhibits IRS1 activation, it has recently been reported that mTORC2 is an upstream kinase of Akt at Ser473, which is required for its full activation in response to insulin (Hresko & Mueckler, 2005; Kumar et al, 2008; Sarbassov et al, 2005). The results of the present study indicate that in skeletal muscle, mTORC2 formation was coupled with Ser473 phosphorylation of the Akt1 isoform, which has primarily been studied with respect to its role in cell growth and differentiation (Cho et al, 2001; Wilson & Rotwein, 2007). Similar to the in vivo findings there is shown to be a significant increase in Ser473 Akt1 phosphorylation in L6 myotubes after 4 hr palmitate incubation (Fig 5.6C). In contrast, it was observed that there were no changes in Ser473 phosphorylation of the Akt2 isoform, which is an important regulator of glucose uptake in muscle (Fig 5.3D). In addition, no mTORC2-associated change in the phosphorylation of AS160, a downstream substrate of Akt,
with a putative role in insulin-stimulated glucose uptake, was detected (Fig 5.3D). The selective activation of Akt1 and the absence of Akt2 regulation by mTORC2 with high-fat feeding, may explain how a high fat diet impairs insulin stimulated glucose transport despite activation of mTORC2 in muscle. The converse argument would apply with respect to the ability of exercise training to enhance insulin-stimulated glucose uptake despite inhibiting mTORC2 formation.

The results of previous investigations that have examined the effects of endurance exercise on skeletal muscle mTOR activation are equivocal, with some studies demonstrating decreased (Glynn et al, 2008; Miranda et al, 2008; Williamson et al, 2006), no change (Atherton et al, 2005; Coffey et al, 2006; Mascher et al, 2007) or increased (Fujita et al, 2007) activation of mTOR following endurance exercise. In the present study an unexpected increase, was observed, in the exercise-induced phosphorylation of Ser2448 on mTOR despite decreased Akt activation (Fig 5.4B, 5.4C). These results are in agreement with recent studies that have demonstrated that the phosphorylation of mTOR on Ser2448 is independent of Akt activation (Allemand et al, 2009; Chiang & Abraham, 2005; Mothe-Satney et al, 2004; Parkington et al, 2003). The results of the present investigation are also consistent with those of Miranda et al. (2008) who demonstrated that insulin-stimulated S6K1 activation was decreased following endurance training (Miranda et al, 2008). Thus, it appears that unlike resistance training; which is a potent activator of mTOR (Atherton et al, 2005; Coffey & Hawley, 2007); endurance training may inhibit mTOR activation in skeletal muscle. Opposing roles for these two training modes on mTOR activation are not surprising
given the highly contrasting nature of muscle adaptations resulting from endurance and resistance exercise (Atherton et al, 2005; Coffey & Hawley, 2007).

One mechanism by which exercise training enhances insulin action in skeletal muscle is via the chronic activation of AMPK (Hawley & Lessard, 2008). AMPK is a known physiological inhibitor of the energy consuming mTOR signalling pathway (Kimball, 2006). Indeed, interventions that reduce intracellular ATP levels, or administration of the AMPK activator, AICAR, decrease mTOR activation as demonstrated by decreased phosphorylation of S6K1 (Deshmukh et al, 2008; Kimball, 2006). In the present study an exercise-induced increase in AMPK Thr172 phosphorylation was observed (Fig 5.5B), suggesting that AMPK activation may be one possible mechanism to explain the inhibition of mTOR activation in response to exercise training (Fig 5.5B). Indeed, when treating palmitate incubated cells with the AMPK activator AICAR it was observed to significantly decrease the phosphorylation of the mTORC1 substrate Thr389 S6K1 (Fig 5.6C). Given that such opposing stimuli and cellular responses are associated with the AMPK and mTOR pathways it seems logical that AMPK activation results in the inhibition of mTOR.

It is known that the activation of mTORC1 and mTORC2 are sensitive to increased nutrient availability (Jacinto et al, 2004; Kim et al, 2002; Marshall, 2006). Investigations using rapamycin, an mTOR inhibitor, have previously demonstrated that the formations of mTOR complexes are necessary for nutrient-induced activation of S6K1/IRS1 and Akt (Krebs et al, 2007; Sarbassov et al, 2006; Sipula et al, 2006; Tremblay & Marette, 2001). The results of this study provide novel evidence that the in vivo
formation of both mTOR complexes (mTORC1 and mTORC2) are responsive to both the chronic interventions of exercise training and a high-fat diet. Furthermore, it is demonstrated that changes in mTOR complex formation are associated with the activation of their respective downstream substrates. In agreement with the results from the current study, Schieke et al. (2006) have shown that increases in mTOR-raptor association after immunoprecipitation correlated with the phosphorylation and activation of S6K1 (Schieke et al, 2006). In contrast, acute in vitro palmitate application in cell culture had no effect on the formation of the mTOR complexes (Fig 5.6E, 5.6F) despite increased mTORC1/2 activation (Fig 5.6C).

In conclusion, this study demonstrates for the first time that a high-fat diet and exercise training have divergent in vivo effects on the formation and activation of mTORC1 and mTORC2. The data also suggests that changes in the activation of the mTOR complexes may be one mechanism to explain the altered insulin signal transduction in response to lipid availability. The reversal of mTORC 1/2 activation by exercise training was associated with the activation of AMPK in skeletal muscle. This observation is supported by this in vitro work demonstrating that AICAR abolishes the lipid-induced activation of S6K1. As impaired insulin action in skeletal muscle is pivotal at the onset of Type 2 diabetes these findings have implications for both the understanding of insulin signal transduction in skeletal muscle and the discovery of therapeutic targets for the treatment of its dysregulation.
CHAPTER SIX: General Summary and Discussion
The primary aim of the studies undertaken for this thesis was to enhance the understanding of some of the mechanisms by which lifestyle and genetic factors contribute to skeletal muscle metabolic flexibility/inflexibility. Several aspects of skeletal muscle metabolic flexibility were examined with respect to their potential to improve glucose homeostasis in two distinct animal models of metabolic flexibility.

The first investigation (described in Chapter three) was designed to determine the contribution of intrinsic oxidative capacity of skeletal muscle to metabolic flexibility in a rodent model in which aerobic running capacity was artificially manipulated to generate divergent phenotypes for this trait. The LCR/HCR model of divergent intrinsic aerobic capacity was developed to specifically study some of the potential mechanisms associated with the metabolic syndrome and cardiovascular disease in a system in which there is a degree of control over both environmental and genetic variables. The selection for high intrinsic aerobic running capacity was associated with metabolic flexibility, superior insulin signalling, increased skeletal muscle oxidative capacity and elevated mitochondrial density. The superior fuel-handling capacity in animals bred (but not exercise-trained) for superior running capacity can, in part, be explained by a fibre-type specific increase in insulin-stimulated phosphorylation of the insulin receptor substrate 1 (IRS1), Akt and the Akt substrate of 160 kDa (AS160) along with a substantial increase in mitochondrial volume density. These results provide the first evidence that intrinsically high running capacity, in the absence of exercise training, confers metabolic flexibility to skeletal muscle and is associated with improved whole-body metabolic health.
The observation that intrinsically low aerobic capacity can confer metabolic inflexibility to skeletal muscle, thus contributing to an increased risk for diseases associated with the metabolic syndrome, led to the next question of whether improving low skeletal muscle oxidative capacity could reverse these deleterious effects. Therefore, the second experimental chapter (described in Chapter four) was designed to determined if a six week programme of endurance exercise training could reverse the skeletal muscle metabolic ‘inflexibility’ observed in LCR (Chapter three). An incremental training protocol with the same absolute cumulative training distance (~10 km) was implemented, for both phenotypes, in order to maintain the same training environment (i.e. the same frequency, intensity and volume) for all animals. It was found that rats bred for low aerobic capacity had skeletal muscle insulin resistance and reduced lipid oxidation. Decreases in glucose and lipid metabolism were associated with decreased β2-AR and Nur77 protein content. These decreases were associated with the reduced expression of Nur77 target proteins that are critical regulators of muscle glucose and lipid metabolism (UCP3, FAT/CD36). Exercise training reversed the impaired glucose and lipid metabolism in the skeletal muscle of LCR. Exercise training-induced improvements in insulin sensitivity were associated with increased rates of fatty acid oxidation, increased phosphorylation of selected components of the insulin signalling pathway (Akt) and the increased expression of β-adrenergic components (β2-AR, Nur77) and target proteins (Glut4, UCP3, and FAT/CD36). This investigation identifies Nur77 as a potential target for improved metabolic health following exercise training in a model of low intrinsic exercise capacity.
The final experiment (described in Chapter five) was developed to ascertain a mechanism for skeletal muscle metabolic inflexibility by determining the independent and interactive effects of increased lipid availability and exercise training on the mTOR signalling cascade in a model of diet-induced metabolic inflexibility, the high-fat fed rat. A high-fat diet decreased insulin-stimulated glucose transport and was linked with an increase in the association of mTOR with its binding partners, rictor and raptor. These increases were coupled with the increased activation of its downstream substrates Akt1, S6K1 and the inhibitory serine phosphorylation of IRS1. Exercise training, restored skeletal muscle insulin sensitivity possibly through the activation of the AMP-activated protein kinase and thus the inhibition of the mTOR signalling pathway. In light of this data, it was determined whether acute palmitate exposure and the activation of AMPK by 5’-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) regulate the formation and activation of mTOR complexes 1/2 in cell culture. In this study it was demonstrated for the first time that a high-fat diet and exercise training have divergent in vivo effects on the formation and activation of mTORC1 and mTORC2. This data also suggests that changes in the activation of the mTOR complexes may be one mechanism to explain the altered insulin signal transduction in response to lipid availability. The reversal of mTORC 1/2 activation by exercise training was associated with the activation of AMPK in skeletal muscle. This observation is supported by the in vitro work demonstrating that AICAR abolishes the lipid-induced activation of S6K1. As impaired insulin action in skeletal muscle is pivotal at the onset of Type 2 diabetes these findings
have implications for both the understanding of insulin signal transduction in skeletal muscle and the discovery of therapeutic targets for the treatment of its dysregulation.

It is hoped the information resulting from the studies undertaken for this thesis will enhance our understanding of the mechanisms by which genetics, nutrition, and exercise interact and impact on skeletal muscle metabolic flexibility and whole-body metabolic health. However, several important questions remain that should be addressed in future work. For example, further translational investigation is needed to clarify the role of genetics and lifestyle on skeletal muscle metabolic flexibility in humans. Also, ongoing research is necessary to gain further insight into the molecular mechanisms by which nutrient sensors (ie. mTOR and AMPK) are affected by genetics and lifestyle. The continued discovery of the factors that lead to skeletal muscle metabolic inflexibility/flexibility and the mechanisms by which exercise acts to overcome these impairments may lead to the development of more effective treatments for diseases associated with the metabolic syndrome.
Chapter Seven: References


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