THERAPEUTIC INTERVENTIONS FOR LIPID-INDUCED INSULIN RESISTANCE IN SKELETAL MUSCLE: MECHANISMS OF ACTION

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

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Research Outcomes

The following publications have resulted from the work undertaken in this thesis:

1. **Peer-reviewed articles**


2. **Book Chapters**

3. **Abstracts**


Declaration

I, the candidate, Sarah Jane Lessard, certify that:

a) except where due acknowledgement has been made, the work is that of the author alone;

b) the work has not been submitted previously, in whole or in part, to qualify for any other academic award;

c) 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.

Sarah J. Lessard

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**Abbreviations**

**ACC:** acetyl CoA carboxylase  
**AICAR:** 5-aminoimidazole-4-carboxamide ribonucleoside  
**AMP:** adenosine monophosphate  
**AMPK:** AMP-activated protein kinase  
**ANOVA:** analysis of variance  
**AS160:** Akt substrate of 160 kilodaltons  
**ATP:** adenosine triphosphate  
**BMI:** body mass index  
**CF:** chow-fed  
**CoA:** coenzyme A  
**CPT:** carnitine palmitoyl transferase  
**DAG:** diacylglycerol  
**d.m.:** dry mass  
**EX:** exercise training  
**FA:** fatty acid  
**FABPpm:** plasma membrane-bound fatty acid binding protein  
**FAT/CD36:** fatty acid translocase  
**GLUT:** glucose transport protein  
**G-6-P:** glucose-6-phosphate  
**HF:** high-fat diet  
**HK:** hexokinase  
**HSL:** hormone-sensitive lipase  
**IMTG:** intramuscular triacylglycerol  
**IR:** insulin receptor
IRS: insulin receptor substrate
LN: lean Zucker rat
OB: obese Zucker rat
PGC: peroxisome proliferator-activated receptor gamma coactivator
PI3K: phosphatidylinositol 3-kinase
PIP2: phosphatidylinositol-3,4-bisphosphate
PIP3: phosphatidylinositol-3,4,5-triphosphate
PKC: protein kinase C
PPAR: peroxisome proliferator-activated receptor
PUFA: polyunsaturated fatty acid
R_d: rate of insulin-stimulated glucose disposal
RSG: Rosiglitazone
SE: standard error
TAG: triacylglycerol
TZD: thiazolidinedione
UCP3: uncoupling protein 3
w.w.: wet weight
Abstract

It has long been known that in addition to disruptions in glucose homeostasis, individuals with insulin resistance have a breakdown in lipid dynamics, often manifested by elevated levels of circulating fatty acids (FA) together with accumulation of lipids in insulin-sensitive tissues, including skeletal muscle. However, little is known about how common therapies used to treat insulin resistant individuals (such as Rosiglitazone and exercise training) improve skeletal muscle lipid and glucose metabolism. Thus, the primary aim of the studies undertaken for this thesis was to enhance our understanding of the mechanisms by which Rosiglitazone and exercise training improve skeletal muscle lipid metabolism and insulin sensitivity in two distinct models of insulin resistance. The first investigation determined the effect of chronic Rosiglitazone treatment on the accumulation of lipid metabolites and enzymatic regulators of lipid metabolism in the skeletal muscle of obese Zucker rats. The observation that Rosiglitazone treatment exacerbated the accumulation of muscle ceramide and diacylglycerol in skeletal muscle, while improving glucose tolerance led to the conclusion that this insulin sensitising drug improves insulin sensitivity by mechanisms other than reduction of fatty acid metabolites in this tissue. Accordingly, the second investigation sought to identify an alternative mechanism by which Rosiglitazone treatment may improve skeletal muscle insulin sensitivity. It was found that Rosiglitazone restored AMP-activated protein kinase (AMPK) α2 activity in the skeletal muscle of obese Zucker rats, providing a potential peroxisome proliferator activated receptor (PPAR) γ-independent mechanism by which this drug may mediate its insulin-sensitising actions. The final experiment undertaken for this thesis determined the independent and interactive effects on Rosiglitazone and exercise training on
various aspects of skeletal muscle glucose and lipid metabolism in a model of diet-induced insulin resistance, the high-fat fed rat. Exercise training, but not Rosiglitazone treatment restored skeletal muscle insulin sensitivity in high-fat fed rats. Improvements in insulin sensitivity with exercise training were associated with increased FA oxidation, increased AMPK activity and a normalisation of the expression of the Akt substrate, AS160. In contrast, Rosiglitazone treatment was associated with increased FA uptake and decreased insulin-stimulated glucose uptake in skeletal muscle. Exercise prevented the accumulation of skeletal muscle lipids in Rosiglitazone-treated animals when the two treatments were combined. In summary, the results from the studies undertaken for this thesis provide novel information regarding the mechanisms by which two insulin-sensitising therapies, exercise training and Rosiglitazone treatment, act to improve glucose and lipid metabolism in skeletal muscle.
CHAPTER ONE: Literature Review
1.1 The Role of Skeletal Muscle in Type 2 Diabetes

The prevalence of type 2 diabetes mellitus is increasing at an alarming rate with the total number of cases expected to reach 300 million world-wide by 2025 (Zimmet et al. 2001). Consequently, intense research efforts have focused on the development of improved treatments for this disease. Type 2 diabetes is a progressive metabolic disorder that results from the interplay of many environmental and genetic factors (Malecki and Klupa 2005). Its progression involves several organs that are responsible for whole-body metabolic control, from central regulators (i.e. the hypothalamus) to peripheral organs (including adipose tissue, the liver, pancreas and skeletal muscle). Metabolic defects in any one or a combination of these organs can lead to impaired delivery of glucose from the circulation to insulin-sensitive tissues, which eventually results in the accumulation of glucose in the blood. Chronic hyperglycemia leads to the secondary complications of diabetes, which include cardiovascular disease, kidney failure, blindness and neuropathy. Accordingly, current interventions for the treatment of patients with diabetes are targeted to restore euglycemia, either by improving glucose uptake by tissues in response to insulin, or by stimulating glucose uptake by insulin-independent mechanisms (Clement 2004).

Several organs act to dispose of blood glucose in response to insulin. However, skeletal muscle is the principal site of glucose uptake, accounting for up to 80% of glucose disposal under insulin-stimulated conditions (DeFronzo et al. 1985). Although the primary defect(s) leading to the development of type 2 diabetes is unknown, impairment of muscle glucose uptake in response to insulin is thought to be a major contributor to this disease. Skeletal muscle also has the capacity to take up and store significant amounts of glucose via insulin-
independent mechanisms (Richter et al. 2001). Therefore, the potential for this
tissue to dispose of, and store large amounts of glucose by multiple pathways
makes it an excellent target for the treatment of type 2 diabetes.

1.2 Skeletal Muscle Glucose Uptake: Molecular Pathways

Glucose uptake into skeletal muscle is mediated by two distinct molecular signalling pathways. One pathway requires insulin for its initiation and is termed “insulin-dependent”. The other pathway is “insulin-independent”, and can activate glucose transport in response to other stimuli such as exercise and hypoxia. Both pathways are important targets for improved glucose homeostasis.

1.2.1 Insulin-dependent glucose uptake

The initial steps in the molecular pathway that lead to increased skeletal muscle glucose uptake in response to insulin have been well-characterised (Thong et al. 2005), and are shown in Figure 1.1. When insulin is secreted by the pancreas in response to an elevated blood glucose concentration, it signals the need for glucose uptake in skeletal muscle by binding to the extramyocellular α-subunit of the insulin receptor (IR). The signal is then transmitted intracellularly to the β-subunit by a conformational change, which enables autophosphorylation of the IR, followed by tyrosine phosphorylation of intracellular proteins known as insulin-receptor substrates (IRS). Tyrosine-phosphorylated IRS binds to a specific group of proteins containing src-homology-2 domains (SH2), the most important of which for insulin’s metabolic actions is phosphatidylinositol 3-kinase (PI3K).
Figure 1.1 A schematic representation of the molecular pathway leading to insulin-stimulated glucose uptake in skeletal muscle.

The binding of insulin to the insulin receptor initiates a series of conformational changes and phosphorylation events involving several intracellular proteins. IRS, Insulin receptor substrates; PI3-K, phosphatidylinositol-3-kinase; PIP2, phosphatidylinositol-3,4-bisphosphate; PIP3, phosphatidylinositol-3,4,5-triphosphate; PDK, phosphatidylinositol-dependent protein kinase; PKC, protein kinase C; Akt, protein kinase B (PKB); AS160, Akt substrate of 160 kDa; GLUT4, Glucose Transporter 4; HK, hexokinase.

PI3K promotes glucose uptake and several other processes involved in carbohydrate and lipid metabolism, via its lipid product 3, 4, 5-phosphatidylinositol (PIP3). In turn, PIP3 activates a number of serine and threonine kinases, including PI-dependent protein kinases (PDK), Akt/protein kinase B, and protein kinase C (PKC). Activation of Akt and/or PKC leads to
the translocation of glucose transporters (GLUT4) from intracellular stores to the plasma membrane, allowing for the facilitated diffusion of glucose into the cell from the intercellular compartment.

Although the signalling mechanisms proximal to the insulin receptor have been well-established, the signalling pathways downstream of Akt/PKC that lead to GLUT4 translocation to the plasma membrane are still under debate (Dugani and Klip 2005). The precise mechanisms that lead to the cycling of GLUT4 vesicles between the intracellular compartment and the plasma membrane are complex and outside the scope of this review [for review see (Dugani and Klip 2005)]. Briefly, it is likely that Akt [predominantly the Akt2 isoform; (Bae et al. 2003)] is recruited to GLUT4-containing vesicles, where it phosphorylates several vesicle component proteins, including GLUT4 itself (Kupriyanova and Kandror 1999). In particular, the Akt substrate of 160-kDa (AS160) appears to be an important regulator of vesicle translocation (Miinea et al. 2005) by retaining GLUT4 in the basal state until phosphorylation by Akt signals the need for vesicle mobilisation to the plasma membrane (Eguez et al. 2005).

There is also evidence that the atypical PKC (aPKC) isoforms, PKC ζ and λ, play an important role in the translocation of GLUT4. It has been demonstrated in muscle cells that increasing the expression of PKCζ stimulates glucose transport, whereas decreasing the expression of this isoform has an inhibitory effect on this process (Bandyopadhyay et al. 1997). More recently, aPKC has been implicated in both the mobilisation of GLUT4 (Imamura et al. 2003) and its fusion to the plasma membrane (Hodgkinson et al. 2005; Lizunov et al. 2005).
1.2.2 Insulin-independent glucose uptake: AMP-activated protein kinase

In the absence of insulin, skeletal muscle glucose transport can be initiated in response to increased demand and/or depletion of energy stores. A drop in myocellular energy status through events such as muscle contraction or hypoxia can activate pathways that reduce cellular energy expenditure and increase its production in order to restore energy balance. The key enzyme responsible for these metabolic changes is the AMP-activated protein kinase (AMPK).

AMPK is a heterotrimeric protein that is comprised of a catalytic α subunit and regulatory β and γ subunits (Kahn et al. 2005). Two isoforms of the catalytic subunit, α1 and α2, are expressed in skeletal muscle and encoded by different genes. Studies using rodent knockout models indicate that the α1 and α2 isoforms play distinct roles in the regulation of whole-body glucose homeostasis (Viollet et al. 2003), though their precise roles in skeletal muscle glucose metabolism remain unclear.

As depicted in Figure 1.2, 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 (Winder and Hardie 1999; Fujii et al. 2006). 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.
Figure 1.2 A schematic representation of the insulin-independent regulation of AMP-activated protein kinase (AMPK) pathway in skeletal muscle.

Energy-depleting events such as muscle contraction or pharmacological treatment with AICAR activate a number of signal transduction pathways that lead to AMPK activation and insulin-independent GLUT4 translocation. AICAR, 5-aminoimidazole-4-carboxamide; ZMP, AICAR monophosphate; CaMKK, calmodulin-dependent protein kinase kinase; LKB1, AMP kinase kinase

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 AMPK signalling cascade is further amplified during muscle contraction by the Ca2+/calmodulin system (Hawley et al. 2005). The rise in intracellular [Ca2+] that occurs during contraction activates calmodulin-dependent protein kinase kinase (CaMKK), which then phosphorylates AMPK at its Thr172 residue (Hawley et al. 1995). Ca2+/calmodulin-dependent AMPK activation is additive to the effects of AMP (Salt et al. 1998; Hawley et al. 2005) and is fully functional in cells that are deficient in LKB1 (Hawley et al. 2005).

Although the precise mechanism by which AMPK stimulates glucose transport is currently unknown, it is believed that activation of this kinase results in the phosphorylation of unknown target proteins, which leads to the translocation of GLUT4 protein to the plasma membrane (Winder and Hardie 1999; Fujii et al. 2006). Evidence to support this theory has come from studies using the pharmacological activator of AMPK, 5-aminoimidazole-4 carboxamide (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). More mechanistic 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). Evidence that PKC may be involved in AICAR-induced glucose transport comes from work demonstrating that PKCζ activity is increased in myotubes treated with AICAR, and AICAR-induced glucose transport is inhibited by a PKCζ inhibitor (Chen et al. 2002). Interestingly, the activation of PKCζ by AICAR in that study (Chen et al. 2002) was similar in magnitude to the activation induced by insulin, but was not sensitive to wortmannin, indicating a point of convergence in the insulin-dependent and -independent pathways.

Although this review will focus on the role of AMPK in facilitating glucose transport into skeletal muscle, it should be noted that the AMPK signalling pathway is not the only contributor to insulin-independent glucose uptake in this tissue. Muscle contraction induces the activation of several signalling mechanisms that have been associated with increased GLUT4 translocation including Ca2+/calmodulin, nitric oxide and PKC (Jessen and Goodyear 2005). Jorgensen et al. (2004) recently reported no effect on contraction-induced glucose transport in transgenic mice with a gene knockout of either the AMPK α1 or α2 catalytic subunits. These workers concluded that other contraction-induced mechanisms may compensate for the loss of a single AMPK isoform. It is also likely that the remaining AMPK isoform compensates for the loss of its counterpart, as evidenced by the finding that the expression of the AMPKα1 isoform is doubled in AMPK α2 knockout mice (Jorgensen et al. 2004). The simultaneous knockout of both isoforms has not yet been studied.

Although its importance in contraction-stimulated glucose uptake is debatable, the ability of AMPK to stimulate skeletal muscle glucose uptake makes it an attractive target for the treatment of type 2 diabetes (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). Furthermore, 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 together, 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 Insulin Resistance in Skeletal Muscle

1.3.1 General definition/ characteristics

Insulin mediates a number of cellular processes, making insulin resistance a multifaceted syndrome (Wilcox 2005). Insulin resistance is generally defined as an attenuated physiological response to a standard or maximal dose of insulin. However, for the purposes of this review, the definition of insulin resistance will be narrowed to the inability to maintain glucose homeostasis in response to normal circulating insulin levels.

In individuals with insulin resistance, the failure of insulin to induce adequate glucose uptake and storage in peripheral tissues such as skeletal muscle and adipose, and to inhibit hepatic glucose output, leads to a rise in blood glucose concentration. Initially, the body may compensate for impaired insulin sensitivity by increasing pancreatic insulin secretion, thus allowing blood glucose concentration to be maintained. If this state of insulin resistance progresses, hyperinsulinemia may no longer compensate for impaired insulin
action, resulting in hyperglycemia and eventually the development of type 2 diabetes and its secondary complications (Saltiel 2000; Unger and Orci 2001).

The initial defect(s) that result in impaired glucose homeostasis secondary to insulin resistance are not clear. However, since skeletal muscle is responsible for the majority of insulin-stimulated glucose disposal (DeFronzo et al. 1985), impaired insulin action in this tissue likely plays a major role in the impaired glucose homeostasis associated with insulin resistance. Indeed, muscle from insulin resistant individuals with type 2 diabetes (Andreasson et al. 1991) and rodent models of insulin resistance (Zierath et al. 1997) demonstrates marked reductions in insulin-stimulated transport of the non-metabolised glucose analogue 3-O-methylglucose.

1.3.2 Molecular mechanisms associated with skeletal muscle insulin resistance

In individuals with type 2 diabetes, impaired insulin-stimulated glucose uptake in skeletal muscle is associated with inadequate recruitment of GLUT4 to the plasma membrane (Bjornholm and 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 downregulation of one or more components of the insulin signal transduction pathway, or from disruption of mechanical events that allow for the translocation/insertion of GLUT4 in the plasma membrane. It is possible that 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 equivocal, 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 insulin resistant skeletal muscle. 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 individuals with type 2 diabetes (Bjornholm et al. 1997; Krook et al. 2000; Bjornholm and Zierath 2005). 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 and Zierath 2005). Further downstream, Akt activation in response to physiological insulin concentrations is normal in individuals with type 2 diabetes (Krook et al. 1998; Kim et al. 1999), but appears to be blunted at higher (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. 2005b). In addition to impairments in the classic insulin signalling cascade, there is also evidence that downregulation of aPKC isoforms may be involved in the aetiology of skeletal muscle insulin resistance (Farese 2002). Defective insulin-stimulated PKCλ/ζ activity has been observed in insulin resistant and diabetic
individuals (Beeson et al. 2003; Kim et al. 2003b), as well as rodent models of insulin resistance (Kanoh et al. 2001).

It is apparent that defects in insulin resistant muscle have been found at virtually every known step of the signalling pathway that ultimately leads to GLUT4 translocation. However, the extent to which these alterations in signalling are causative in the overall development of insulin resistance is 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 (So et al. 2000; Beck-Nielsen et al. 2003). In fact, environmental factors are believed to outweigh those from genetic contributions in causing 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 and Ybarra 2005), has prompted some to refer to this co-existence of conditions as “diabesity” (Shafrir 1996).

### 1.4 Intramyocellular Lipids and Insulin Resistance

#### 1.4.1 Ectopic lipid deposition and lipotoxicity

Given the strong epidemiological association between obesity and diabetes (Beck-Nielsen et al. 2003; Golay and Ybarra 2005), it is plausible that one or more of the physiological abnormalities associated with obesity is causally related to the onset of type 2 diabetes. Obesity is associated with
alterations in lipid metabolism such as hyperlipidemia and the increased storage of lipids in insulin-sensitive tissues such as liver and skeletal muscle (Sharma 2006). The observation that increased lipid availability may be causally related to impaired glucose metabolism and insulin resistance in skeletal muscle was made some time ago (Randle et al. 1963). Since then, evidence has accumulated to suggest that defects in skeletal muscle insulin signalling may be secondary to altered lipid metabolism (Schmitz-Peiffer 2000).

Lipid uptake and storage by skeletal muscle is essential as a source of energy and for normal muscle function (Kiens 2006). However, conditions which promote excessive intracellular lipid accumulation due to lipid oversupply (such as obesity, high fat diet or lipid infusion) can induce insulin resistance in this tissue [(McGarry 2002) see figure 1.3]. Impaired cellular function as a result of excessive lipid accumulation is referred to as “lipotoxicity” (Russell 2004). Bachmann et al. (2001) demonstrated that increased lipid availability due to either acute lipid infusion or a chronic high-fat diet increased intramuscular lipid content and impaired insulin sensitivity. More recently, impaired insulin signalling in skeletal muscle following lipid infusion in healthy individuals was observed to be dose-dependent (Belfort et al. 2005). Similar observations have been made in rodent models of insulin resistance, where both high-fat feeding or pre-incubation of muscle strips in 1 mM palmitate were associated with accumulation of lipids and impaired insulin action (Thompson et al. 2000).
Figure 1. 3  Inverse correlation between intramyocellular lipid (IMCL) and the rate of insulin-stimulated glucose disposal ($R_d$).

Soleus muscle IMCL was measured using $^1$H-magnetic resonance spectroscopy (MRS) in healthy individuals. $R_d$ was assessed during a hyperinsulinemic-euglycemic clamp. BMI, body mass index. Reproduced from McGarry (2002).

There is compelling evidence linking impaired lipid metabolism such as elevated plasma and tissue lipid levels, and impaired glucose homeostasis in individuals with obesity and type 2 diabetes (McGarry 2002). However, it is unclear whether altered lipid metabolism is coincidental with the insulin resistant state, or causative in its development. It has been hypothesised that the accumulation of lipid metabolites may directly or indirectly interfere with insulin signal transduction, thereby reducing skeletal muscle GLUT4 translocation and glucose uptake (Schmitz-Peiffer 2000). The accumulation of lipid species within skeletal muscle is a result of the balance between FA uptake and utilisation. Therefore, a better understanding of the regulation of lipid transport and
metabolism may provide insight into the defects that lead to lipid-induced insulin resistance in skeletal muscle.

1.4.2 Regulation of lipid transport and storage in skeletal muscle

Transport of FA from the circulation to the cytosol of skeletal muscle cells is likely the combined result of passive diffusion across the sarcolemma and protein-mediated membrane transport (Kiens 2006). To date, three putative FA transport proteins have been identified in skeletal muscle: 1) fatty acid translocase (FAT/CD36), 2) plasma membrane fatty acid binding protein (FABPpm), and 3) fatty acid transport protein (FATP). Of these transporters, FAT/CD36 has the most well-defined role in skeletal muscle lipid metabolism. Muscle-specific overexpression of FAT/CD36 in mice results in reduced plasma lipids as well as increased palmitate oxidation (Ibrahimi et al. 1999). Furthermore, skeletal muscle FAT/CD36 and triacylglycerol storage are increased in healthy, endurance trained athletes following consumption of a high fat diet (Cameron-Smith et al. 2003), in obese individuals (Bonen et al. 2004), as well as in animal models of type 2 diabetes (Luiken et al. 2001). In addition to chronic changes in the expression of this protein, it is likely that plasma membrane content of FAT/CD36 is regulated acutely via its translocation from intracellular compartments (Koonen et al. 2005). Taken together, these observations indicate that FA transport into skeletal muscle is a highly regulated process that may influence whole-body lipid metabolism.

Once inside the cytosol, FA 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) [see figure 1.4]. 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 oxidation must first cross the mitochondrial membrane in a process regulated by carnitine palmitoyl transferase (CPT1). Increased FA uptake, without a similar increase in oxidation would result in the accumulation of FA within the myocyte. The capacity of skeletal muscle to oxidise FA is reduced in obesity and type 2 diabetes (Blaak 2004). Decreased muscle FA oxidation combined with increased FA transport (described above) in obesity and diabetes provides a potential mechanism for the accumulation of lipid species in these conditions.

Figure 1.4 A schematic representation of the regulation of lipid uptake and storage in skeletal muscle.
Fatty acids (FA) are transported across the sarcolemma and activated via formation of FA-CoA before entering the mitochondria for oxidation, or being transformed by the sphingolipid or glycerolipid synthesis pathways. FAT/CD36, fatty acid translocase; ACS, acyl coenzyme A synthetase; GPAT, glycerol-3-phosphate acyltransferase; DGAT, diacylglycerol acyltransferase; HSL, hormone-sensitive lipase; CPT1, carnitine palmitoyl transferase; PLC, phospholipase C; DAG, diacylglycerol; TAG, triacylglycerol
Excess FA-CoA that is not required to meet immediate energy demands through oxidation may be channelled through the glycerolipid or sphingolipid synthesis pathways. An important intermediate product in the sphingolipid pathway is the signalling molecule ceramide, which is involved in the regulation of various cell functions (Summers 2006). FA-CoA that is targeted for storage within the myocyte enters the glycerolipid synthesis pathway. Glycerol-3-phosphate acyltransferase (GPAT) catalyses the first committed step in this pathway, which results in the formation of the metabolically active lipid intermediate DAG. DAG may also be produced from the hydrolysis of membrane phospholipids such as PI-4, 5-bisphosphate (PIP$_2$) by the enzyme phospholipase C (PLC) (Nishizuka 1995). The final step in glycerolipid synthesis that allows for the synthesis of TAG from DAG is regulated by diacylglycerol acyltransferase (DGAT). TAG, which acts as the main storage form of lipids in muscle is deposited into lipid droplets where it may be hydrolysed back to DAG, and eventually to FA-CoA if required in other pathways. The rate-limiting enzyme for TAG hydrolysis is hormone-sensitive lipase (HSL), which is a highly regulated protein thought to play an important role in the control of whole-body lipid metabolism (Holm et al. 2000).

In summary, there is evidence that individuals with insulin resistance have increased uptake and possibly decreased FA oxidation which may result in the accumulation of lipid species in muscle. Furthermore, it has been well-established that excess lipid deposition in skeletal muscle is linked to insulin resistance. Lipid storage in skeletal muscle is a highly regulated process, and disregulation of any of the steps involved in FA transport and storage may lead to the accumulation of one or more intramuscular lipid species. Moreover, demonstrating the capacity of a specific lipid species to interfere with insulin
signal transduction would provide evidence for its accumulation to play a
causative role in insulin resistance.

1.4.3 Fatty acid-induced impairment of insulin signalling

Triacylglycerol

Skeletal muscle TAG content is increased in obesity (Szczepaniak et al.
1999) and type 2 diabetes (Goodpaster et al. 1997), and is inversely related to
whole-body insulin sensitivity (Phillips et al. 1996; Pan et al. 1997a; Kelley et al.
2002). However, the mechanism by which stored TAG may interfere with
insulin signal transduction remains elusive. Evidence demonstrating a
dissociation between muscle lipid content and insulin sensitivity suggests that
TAG accumulation \textit{per se} may not responsible for skeletal muscle insulin
resistance (Goodpaster and Kelley 2002; van Loon and Goodpaster 2006).
Endurance trained athletes are highly insulin sensitive (Staudacher et al. 2001)
despite exhibiting elevated intramuscular triacylglycerol levels (Goodpaster et
al. 2001). Furthermore, Perdomo et al., (2004) demonstrated that increased
palmitate oxidation due to CPT1 overexpression enhanced insulin sensitivity in
palmitate-treated L6 myotubes despite intramyocellular lipid accumulation
(Perdomo et al. 2004). The capacity to oxidise lipids has also been
demonstrated to be a better predictor of insulin sensitivity than muscle lipid
status in type 2 diabetic [(Bruce et al. 2003) see figure 1.5] and obese
individuals (Goodpaster et al. 2003). Such findings have lead to the proposal
that the correlation between TAG content and insulin resistance may not
represent a functional relationship (van Loon and Goodpaster 2006). Rather, it
has been suggested that intramuscular TAG acts as a marker for other, more
metabolically active lipid metabolites that may directly impair insulin action in
skeletal muscle (Goodpaster and Kelley 2002).
Figure 1.5 Relationship between glucose infusion rate (GIR) and markers of lipid status and skeletal muscle oxidative capacity. Measurements of A) % body fat; B) body mass index (BMI); C) plasma free fatty acid (FFA) concentration; D) maximal oxygen consumption (VO$_{2\text{max}}$); E) citrate synthase activity and F) β-hydroxyacyl CoA dehydrogenase (HAD) activity were correlated to GIR during a euglycemic-hyperinsulinemic clamp. Reproduced from Bruce et al. (2003).

Fatty acyl CoA

FA-CoA accumulates in skeletal muscle in response to lipid oversupply and has the potential to inhibit insulin signal transduction. Unlike muscle TAG,
which is considered to be relatively inert, FA-CoA esters play an important role in the regulation of glucose and lipid metabolism as well as gene transcription (Faergeman and Knudsen 1997). Studies in humans (Ellis et al. 2000; Houmard et al. 2002) and animals (Oakes et al. 1997a) have demonstrated a strong inverse correlation between FA-CoA levels and whole body insulin action. There is evidence that FA-CoA may impair glucose metabolism through the inhibition of hexokinase (Thompson and Cooney 2000), which catalyses the first step in glucose storage and oxidation (see figure 1.6). It has also been suggested that FA-CoA may inhibit insulin signal transduction through the activation of PKC isoforms either directly, or via the production of DAG (Hegarty et al. 2003). Although the activation of atypical PKC (ζ, λ) isoforms are known to facilitate insulin-stimulated glucose uptake (see Figure 1.1), the conventional (α, β, γ) and novel (δ, ε, η, θ) isoforms contain lipid regulation domains (Newton 2003), and their activation is associated with lipid-induced insulin resistance (Schmitz-Peiffer 2000).

Diacylglycerol

As with FA-CoA, the production of DAG is associated with the activation of PKC isoforms that may inhibit insulin action. The activation of conventional (cPKC) and novel (nPKC) PKC isoforms involves translocation and binding of the enzyme to the plasma membrane where it may interact with membrane-embedded DAG and initiate downstream signalling events (Newton 2003). Thus, the measurement of membrane-associated PKC in muscle acts as a surrogate marker for PKC activation. In healthy volunteers undergoing lipid infusion for 6 h, reduced rates of insulin-stimulated glucose disappearance were associated with increased muscle DAG content and membrane-associated PKCβII content (Itani et al. 2002). Similarly, lipid infusion in rats resulted in
elevated muscle FA-CoA and DAG content concomitant with increased membrane-associated PKCθ (Yu et al. 2002). Elevated sarcolemmal PKCθ content observed by Yu et al. (2002) was associated with increased phosphorylation of IRS-1 at ser307 which resulted in decreased IRS-1 tyrosine phosphorylation and IRS-1-associated PI3-kinase activity, providing a potential mechanism by which FA acutely impair insulin signal transduction.

Overexpression of the cPKC isoforms α, βI, and γ provides evidence for PKC-induced impairments upstream of IRS-1 as demonstrated by an increase in inhibitory serine and threonine phosphorylation of the IR in response to the DAG analogue, phorbol ester, under these conditions (Chin et al. 1993).

Ceramide

In addition to DAG-induced inhibition of IR and IRS-1 activation, defects in insulin signal transduction at the level of Akt have been attributed to elevated muscle ceramide content (Summers 2006). Skeletal muscle ceramide content is negatively correlated with insulin sensitivity (Straczkowski et al. 2004) and is increased in the skeletal muscle of obese insulin-resistant humans (Adams et al. 2004) and diabetic rodents (Gorska et al. 2004). Evidence from investigations using cultured cells has demonstrated that ceramide's inhibitory effect on insulin signalling is attributable to downregulation of Akt via mechanisms that are independent of IRS-1 or PI3-kinase (Zhou et al. 1998; Schubert et al. 2000). This may occur as a result of dephosphorylation of Akt at ser473 due to ceramide-induced activation of protein phosphatase 2A (PP2A) (Chavez et al. 2003), or via PKC-induced inhibitory phosphorylation of Akt (Powell et al. 2004). Further evidence for the involvement of ceramide in lipid-induced insulin resistance has come from experiments using C2C12 myotubes that over-express the enzyme acid ceramidase, which catalyses the conversion
of ceramide to sphingosine. Prevention of ceramide accumulation in this model abolished FA-induced impairment of insulin sensitivity in C2C12 myotubes, indicating that impairments to insulin action were ceramide-specific (Chavez et al. 2005).

Figure 1.6 Potential mechanisms by which lipid species inhibit insulin signal transduction in skeletal muscle. The accumulation of FA-CoA, DAG or ceramide may promote inhibitory phosphorylation events, or prevent stimulatory phosphorylation of insulin signalling proteins. G-6-P, glucose-6-phosphate; P_i, inhibitory phosphorylation; PP2A, protein phosphatase 2A.

1.4.4 Models of lipid-induced insulin resistance

Lipid oversupply leads to impairments in skeletal muscle insulin-signalling and consequently, insulin resistance. However, due to the
progressive nature of insulin resistance, it is likely that its clinical onset involves chronic metabolic adaptations involving gene transcription and protein expression, in addition to acute signalling events. More detailed study of the impairments to lipid and glucose metabolism that result from chronically elevated lipid availability is made possible through the use of appropriate animal models of insulin-resistance. The metabolic syndrome associated with the development of type 2 diabetes in humans is characterised by a number of chronic physiological conditions including obesity, dislipidemia, elevated skeletal muscle lipid storage, fasting hyperinsulinemia and hyperglycemia (Stumvoll et al. 2005). Thus the results from investigations that employ animal models that exhibit some or all of these characteristics can be used to extrapolate to the human condition. Such models may be obtained through the modification of genetic and/or lifestyle conditions, both of which are thought to contribute to the human disease state (Beck-Nielsen et al. 2003). Two animal models that are commonly used in the study of lipid-induced insulin resistance are the obese (fa/fa) Zucker rat and high-fat fed rat.

**Obese (fa/fa) Zucker rat**

The adipokine leptin is involved in the regulation of a number of physiological processes including the control of appetite, body mass and the storage and utilisation of lipids (Fruhbeck 2006). The actions of leptin are mediated by its receptor (OB-R) which is present in nearly all tissues (Fruhbeck 2006). The leptin-unresponsive obese Zucker rat is homozygous for the *fa* mutation in the leptin receptor, and consequently exhibits a number of metabolic abnormalities. Among these are obesity, dislipidemia, hyperinsulinemia, hyperglycemia and skeletal muscle insulin resistance (Unger and Orci 2001).
Compared with lean littermates, the obese Zucker rat also exhibits increased lipid deposition in non-adipose tissues such as skeletal muscle, making it one model for the study of lipid-induced insulin resistance in this tissue (Unger and Orci 2001).

*High-fat fed rodent*

The consumption of a diet where fat comprises >30% of total energy intake 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 and Chicco 2006). Similarly, mice and rats fed diets high in saturated, monounsaturated or polyunsaturated FA develop glucose intolerance, hyperinsulinemia and obesity (Lombardo and Chicco 2006). High-fat fed animals also exhibit increased skeletal muscle lipid storage and muscle-specific insulin resistance (Oakes et al. 1997c). Although genetic components may play a role in the onset 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.

### 1.5 Treatments for Lipid-Induced Insulin Resistance

In humans, insulin resistance precedes type 2 diabetes by several decades, and the complications due to diabetes are often irreversible [(Saltiel 2000) see figure 1.7]. Accordingly, it would seem prudent to attempt to improve
insulin resistance before the onset of diabetes and its secondary complications. Since skeletal muscle is the major source for insulin-stimulated glucose uptake, then any treatment targeted to improve glucose uptake in this tissue will improve whole-body glucose homeostasis. Furthermore, given the causative link between abnormal lipid metabolism and impairments to insulin-stimulated glucose uptake, effective treatments to improve insulin resistance should have the ability to ameliorate aberrant lipid and glucose metabolism.

Currently, there are several treatment options for patients with type 2 diabetes, depending on the stage and severity/progression of their symptoms (Inzucchi 2002; Wysowski et al. 2003). Sulfonylurea drugs (glyburide) and other secretagogues (repaglinide) act to improve glucose homeostasis by stimulating insulin secretion by the pancreatic β-cell. Other treatment modalities include biguanides (metformin) and α-glucosidase inhibitors (acarbose), which decrease hepatic glucose production and decrease gut carbohydrate absorption, respectively. A recent therapeutic option for individuals with insulin resistance or type 2 diabetes is the use of thiazolidinediones (TZDs), which act by increasing peripheral insulin sensitivity.

**Figure 1.7** Timeline for the progression of metabolic impairments that lead to type 2 diabetes.
Adapted from Saltiel, 2000.
and glucose disposal (Malinowski and Bolesta 2000). All of these therapies are effective at lowering blood glucose concentrations, although they have distinct mechanisms of actions and molecular targets (Inzucchi 2002; Wysowski et al. 2003). However, none are without adverse side-effects, and the consequences of their long-term use, especially in the case of the TZDs, are largely unknown. Thus, significant effort is being dedicated to the identification of new metabolic targets, as well as uncovering the precise mechanisms of action of existing molecular targets for the treatment of type 2 diabetes.

1.5.1 Molecular targets for the treatment of insulin resistance

AMP-activated protein kinase (AMPK)

AMPK has the ability to stimulate skeletal muscle glucose uptake in an insulin-independent manner, making this enzyme a potential therapeutic target for improving glucose uptake in the face of impaired insulin signal transduction (see section 1.2.2). In addition to its contribution to insulin-independent glucose uptake, AMPK activation has been linked to increased insulin sensitivity in skeletal muscle. Fisher et al. (2002) demonstrated that activation of AMPK by prior treatment with AICAR resulted in increased insulin-stimulated glucose transport in isolated rat skeletal muscle, while Smith et al. (2005) observed similar effects in C2C12 myotubes (Fisher et al. 2002; Smith et al. 2005). However, others have observed no enhancement of insulin-stimulated glucose uptake in human primary myocytes that were pre-incubated with AICAR (Al-Khalili et al. 2004). In insulin-resistant, high-fat fed rats a single dose of AICAR (250 mg/kg) enhanced muscle and liver insulin sensitivity during a euglycemic-hyperinsulinemic clamp (Iglesias et al. 2002). Activation of AMPK via overexpression of LKB1 was found to inhibit serine phosphorylation of IRS-1
thereby enhancing insulin-stimulated Akt phosphorylation, providing a potential mechanism for AMPK-induced amelioration of insulin signalling (Tzatsos and Kandror 2006).

Recent work has identified the Akt substrate AS160 as a target of AMPK in skeletal muscle (Kramer et al. 2006; Treebak et al. 2006). It has also been observed that muscle contraction can stimulate AS160 phosphorylation by an unknown mechanism that does not involve AMPK or Akt activation (Bruss et al. 2005; Kramer et al. 2006). Thus, AS160 may be a possible point of convergence for the insulin-dependent and –independent glucose transport pathways. In addition to its acute effects on insulin signalling, chronic AMPK activation increases GLUT4 protein expression which may also contribute to amplified insulin-stimulated GLUT4 translocation (Buhl et al. 2001).

Aside from its role in regulating both insulin-dependent and –independent glucose uptake in skeletal muscle, AMPK is also a regulator of lipid metabolism. As depicted in figure 1.8, AMPK activation results in the upregulation of FA oxidation in skeletal muscle via phosphorylation of its target protein, acetyl CoA carboxylase (ACC), the enzyme that catalyses the rate-limiting step in the conversion of acetyl CoA to malonyl CoA. AMPK-induced phosphorylation at ser-218 inhibits the action of ACC and results in decreased cellular malonyl CoA levels. Since malonyl CoA is a potent inhibitor of CPT1, a reduction in malonyl CoA alleviates the inhibition of CPT1 and consequently increases the transfer of FA-CoA into the mitochondria for oxidation. Fatty acid uptake and oxidation are thought to be mismatched in type 2 diabetes and obesity, and increased capacity to oxidise lipids is associated with improved insulin sensitivity (Bruce et al. 2003; Goodpaster et al. 2003; Perdomo et al. 2004; Bruce et al. 2006). Therefore, it seems plausible that AMPK-induced increases
in FA oxidation may be an additional mechanism by which AMPK activation improves skeletal muscle insulin sensitivity.

**Figure 1.8** Mechanism by which AMP-activated protein kinase (AMPK) stimulates fatty acid (FA) oxidation in skeletal muscle.

Inhibitory phosphorylation of acetyl CoA carboxylase (ACC) by AMPK results in decreased malonyl CoA production. This, in turn results in decreased malonyl CoA-mediated inhibition of carnitine palmitoyl transferase (CPT1), allowing for increased mitochondrial oxidation of FA-CoA.

Although chronic activation of AMPK in muscle would likely have beneficial effects to glucose and lipid metabolism, the consequences of long-term, pharmacological systemic AMPK activation are unknown. Chronic (7 weeks) administration of AICAR (0.5 mg/g) reduced plasma triacylglycerol and free fatty acids, and increased glucose tolerance in obese Zucker rats (Buhl et al. 2002). Similarly, chronic (15 wk, 3 days/week) AICAR administration in leptin-receptor deficient (obese Zucker rats, diabetic fatty rats) and leptin-deficient (ob/ob mice) rodent models of insulin resistance attenuated the progression of hyperglycemia and diminished lipid accumulation in the liver,
skeletal muscle and pancreatic islets (Yu et al. 2004). More recently, 8 weeks of chronic AICAR treatment improved insulin sensitivity and prevented the onset of type 2 diabetes in Zucker diabetic fatty (ZDF) rats (Pold et al. 2005). Interestingly, Pold et al. (2005) observed that exercise training (treadmill running) for 8 weeks produced similar improvements in the same model. Although the data regarding chronic AICAR treatment in animal models appears promising, the doses required to produce a beneficial effect (up to 1000 mg/kg) are believed to be too high for use in humans and would likely result in adverse side-effects (Winder 2000; Musi and Goodyear 2002). Thus, the development of more potent pharmacological AMPK activators may be needed.

In summary, activation of AMPK results in 1) increased insulin-independent glucose transport, 2) improved insulin-dependent glucose transport, and 3) increased FA oxidation. All of these actions may contribute to improved glucose and lipid metabolism in skeletal muscle, making AMPK a good target for the treatment of insulin resistance.

Peroxisome proliferator-activated receptor γ (PPARγ)

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the family of nuclear hormone receptors (Issemann and Green 1990; Rosen and Spiegelman 2001). The name PPAR was derived from the initial isoform, PPARα, which was observed to induce proliferation of peroxisomes in the liver. Since then, two other members of this transcription factor family, PPARδ and γ, have been described (Kliewer et al. 1994). Because of its involvement in many critical physiological functions, and its potential for pharmacological regulation, PPARγ has been the most extensively studied isoform. As displayed in figure 1.9, in order to initiate
transcription of target genes PPARs form a heterodimer with a retinoid X receptor (RXR) and bind to DNA at specific sequences known as PPAR response elements. Further regulation of this process is mediated by the binding of endogenous ligands such as fatty acids, and protein-protein interactions with coactivators and corepressors (Forman et al. 1995; Kliewer et al. 1997; Spiegelman 1998; Willson et al. 2000; Rosen and Spiegelman 2001; Yu et al. 2005). The presence of multiple PPARγ isoforms (PPARγ1 and PPARγ2) resulting from alternative promoter use and splicing may also increase the diversity of ligand and tissue-specific PPARγ-induced transcription (Zhu et al. 1995; Fajas et al. 1997).

**Figure 1.9 Regulation of gene transcription by peroxisome proliferator-activated receptor (PPAR)-γ.**

The transcription of target genes related to adipogenesis and lipid metabolism by PPARγ is regulated by several ligands, coactivators and corepressors. The binding of ligands such as thiazolidinediones (TZDs) to PPARγ facilitates the binding of protein coactivators and impedes the binding of corepressors, which upregulates transcriptional activity. RXR, retinoid X receptor; PPAR, peroxisome proliferator-activated receptor; PGC, PPAR gamma coactivator; SRC, steroid receptor coactivator; NCoR, nuclear receptor corepressor; SMRT, silencing mediator of retinoid and thyroid hormone receptors.
Although it has many physiological functions, the role that PPARγ plays in the regulation of lipid metabolism is of specific interest because of its potential involvement in the treatment of insulin resistance. PPARγ was initially identified as a transcription factor important in adipose tissue differentiation and gene transcription (Argmann et al. 2005). In adipocytes, PPARγ upregulates the transcription of genes that promote FA storage, while repressing those that facilitate lipolysis (Auwerx 1999). As mentioned above, the ability of PPARγ to induce transcription of its target genes is partially regulated by the binding of endogenous ligands. However, several high-affinity synthetic ligands have been generated that can pharmacologically regulate PPARγ activity. Most notably the TZDs, which were developed for use as insulin-sensitisers without knowledge of their molecular target (Spiegelman 1998; Willson et al. 2000; Rosen and Spiegelman 2001), but were later discovered to be potent PPARγ agonists (Lehmann et al. 1995).

The whole-body insulin sensitising effect of TZDs and their ability to activate PPARγ raises the possibility that their insulin-sensitising actions may be attributable to altered lipid metabolism. The potential role of PPARγ in insulin resistance is underscored by the fact that several epidemiological studies have linked allelic variations of PPARγ to obesity and type 2 diabetes (Argmann et al. 2005). The link between the insulin-sensitising effects of TZDs and their potential to alter lipid metabolism was made inadvertently in experiments that involved treating 3T3-L1 adipocytes or ob/ob mice with the TZD, pioglitazone (Kletzien et al. 1992a; Kletzien et al. 1992b). These researchers noted that improved insulin sensitivity as a result of pioglitazone treatment occurred in concert with increased expression of adipocyte fatty acid...
binding protein (Kletzien et al. 1992b) and enhanced adipocyte differentiation (Kletzien et al. 1992a). It was concluded that the insulin-sensitising effects of TZDs may be attributable to their regulation of gene transcription in adipose tissue (Kletzien et al. 1992b).

Although the initial observations regarding the insulin-sensitising effects of PPAR agonists were observed in adipose tissue where PPARγ-1 and -2 isoforms are expressed at high levels, these isoforms are also expressed at lower levels in other tissues, including skeletal muscle (Braissant et al. 1996; Vidal-Puig et al. 1997). The function of PPARγ in skeletal muscle is not well-understood. However, its expression in this tissue correlates with that of genes involved in the regulation of lipid metabolism such as lipoprotein lipase (LPL), carnitine palmitoyl transferase (CPT1) and fatty acid binding protein (FABP) suggesting at least permissive function as a regulator of lipid metabolism (Lapsys et al. 2000). It is also believed to play a role in skeletal muscle insulin resistance, as gene expression of PPARγ is elevated in muscle biopsy samples and primary muscle cell cultures taken from obese individuals and type 2 diabetics (Park et al. 1997). Furthermore, PPARγ mRNA expression is acutely regulated by insulin and is positively correlated to body mass index (BMI) and fasting insulin concentration (Park et al. 1997). In contrast, downregulation of PPARγ in C2C12 myocytes using antisense RNA reduced insulin sensitivity, whereas overexpression of PPARγ resulted in enhanced insulin-stimulated glucose uptake (Verma et al. 2004). Thus, it appears that PPARγ expression in skeletal muscle is elevated in insulin resistant states such as obesity and type 2 diabetes, whereas overexpression of this transcription factor results in increased insulin-sensitivity in cultured cells.
More insight into the tissue-specific effects of PPARγ activation has been gained from animal models with gene deletions specific to adipose and muscle (Kintscher and Law 2005). Whole-body deletion of PPARγ cannot be easily studied, since homozygous mutation of PPARγ is lethal at an early embryonic stage, highlighting its importance in development (Kubota et al. 1999). Mice developed with a muscle-specific deletion of PPARγ exhibit increased adiposity and whole-body insulin resistance (Hevener et al. 2003; Norris et al. 2003). Impaired glucose tolerance observed in the muscle-specific PPARγ knockout was at least partly attributable to the secondary development of insulin resistance in adipose tissue and liver (Hevener et al. 2003; Norris et al. 2003). However, the effect of muscle-specific PPARγ deletion on insulin sensitivity in this tissue is equivocal, as one study has reported a reduction (Hevener et al. 2003), while another observed no change in skeletal muscle insulin-stimulated glucose uptake (Norris et al. 2003). With respect to muscle PPARγ knockouts, Norris et al. (2003) noted that reduced insulin sensitivity in adipose and liver was secondary to altered muscle lipid metabolism, and noted that the insulin-sensitising effects of TZDs were normal in these mice, suggesting that muscle PPARγ is not required for the insulin-sensitising effects of TZDs. In contrast, Hevener et al. (2003) attributed a large portion of decreased glucose tolerance in this same model to reduced skeletal muscle glucose uptake which was not ameliorated by TZD therapy, leading these workers to conclude that muscle PPARγ may act as a direct target for TZDs.

Contrary to muscle-specific knockouts, mice with an adipose-specific deletion of PPARγ exhibit decreased adiposity due to the role of PPARγ in adipogenesis, and do not display whole-body insulin-resistance (He et al. 2003; Koutnikova et al. 2003; Jones et al. 2005). The relatively mild metabolic
consequences observed in adipose-specific PPARγ knockout mice were attributed to a compensatory upregulation of genes involved in the catabolism of FA in skeletal muscle (i.e. PPARδ, PPARγ coactivator 1 [PGC1], uncoupling protein 3 [UCP3]) providing an increased capacity to oxidise excess lipids (Koutnikova et al. 2003). Despite normal whole-body glucose and insulin tolerance compared to controls, adipose tissue PPARγ knockout animals display hyperlipidemia and liver insulin resistance that was attributed to the accumulation of lipids in this tissue (He et al. 2003; Jones et al. 2005). Treatment with the TZD Rosiglitazone restored liver insulin sensitivity in these mice, suggesting that adipose PPARγ is not essential for the liver insulin-sensitising effects of this drug (He et al. 2003). Taken together, the results of these studies suggest that aside from the essential role that PPARγ plays in adipose tissue, the expression of this transcription factor in muscle may be important in the maintenance of whole-body insulin sensitivity.

In summary, it is likely that the interplay between aberrant glucose and lipid metabolism provides a functional link in the development of skeletal muscle insulin resistance. The potential of AMPK and PPAR-γ to alter both of these metabolic pathways makes them good targets for the treatment of insulin resistance in skeletal muscle. However, further study is required to elucidate their precise mechanisms of action.

1.5.2 Rosiglitazone

In response to the rising prevalence of type 2 diabetes, the prescription and use of oral antidiabetic drugs is rapidly increasing (Wysowski et al. 2003). Since it was originally marketed for clinical use in the United States in 1999, the TZD Rosiglitazone has been widely prescribed for the treatment of type 2
diabetes because of its insulin-sensitising ability (Inzucchi 2002; Wysowski et al. 2003). In individuals with type 2 diabetes, 26 weeks of Rosiglitazone therapy (4-8 mg/day) improved glycemic control (as indicated by decreased haemoglobin A1c), and insulin sensitivity (from fasting glucose and insulin concentrations using the homeostasis model assessment [HOMA]) (Lebovitz et al. 2001). Rosiglitazone treatment was also effective in delaying the onset of type 2 diabetes in individuals with insulin resistance and impaired glucose tolerance (Durbin 2004).

![Chemical structure of Rosiglitazone](image)

**Figure 1. Chemical structure of Rosiglitazone.**
The peroxisome proliferator-activated receptor agonist Rosiglitazone is a widely used insulin-sensitising agent with the ability to alter lipid and glucose metabolism.

As a TZD, Rosiglitazone is also a PPARγ agonist and displays a higher affinity for PPARγ than other members of this family (i.e. troglitazone and pioglitazone), allowing it to be effective at lower doses (Young et al. 1998). Consequently, Rosiglitazone has the ability to alter lipid metabolism in addition to its insulin-sensitising effects. In this regard, improved insulin sensitivity following 12 weeks of Rosiglitazone treatment (8 mg/day) has been associated with increased body weight and fat mass but decreased plasma non-esterified fatty acid (NEFA) concentration and turnover in individuals with type 2 diabetes (Miyazaki et al. 2001). The improved plasma lipid status attributed to TZD therapy is likely due to decreased hepatic TAG production and increased TAG clearance combined with enhanced insulin-induced suppression of lipolysis.
Increased adiposity associated with Rosiglitazone treatment is well-documented, with the increased fat deposition appearing in subcutaneous, rather than visceral compartments (Carey et al. 2002).

Improved insulin sensitivity in the face of increased adiposity seems paradoxical given the strong relationship between obesity and insulin resistance. This apparent contradiction has generated great interest in the mechanism by which Rosiglitazone mediates its insulin sensitising actions. However, despite extensive study, the precise mode of action of this agent remains unknown. The “lipid steal hypothesis” of TZD action suggests that PPARγ agonists may prevent lipid-induced insulin resistance by upregulating FA uptake and storage in adipose tissue, thereby reducing plasma lipids and preventing excessive ectopic lipid deposition in other insulin sensitive tissues such as liver and skeletal muscle (Hockings et al. 2003; Jucker et al. 2003; Ye et al. 2004). The sequestration of FA into adipose tissue would have the effect of sparing other tissues from lipotoxicity, thereby allowing them to maintain their normal roles in maintaining glucose homeostasis. Early support for this hypothesis came from work demonstrating that in addition to improving plasma lipid availability, Rosiglitazone treatment (10 µmol/kg/day for 4 days) significantly reduced liver triglyceride content in high-fat-fed rats (Oakes et al. 1994). More direct evidence came from the observation that treating healthy rats with Rosiglitazone (4 mg/kg) for 7 days resulted in increased adipose tissue lipid uptake and, consequently reduced liver and muscle FA uptake during acute lipid infusion (Ye et al. 2004). Thus, it is apparent that Rosiglitazone treatment has the potential to improve whole-body glucose and lipid metabolism. Since skeletal muscle lipid accumulation is thought to be a major contributing factor to whole-body insulin-resistance, it is possible that the
insulin-sensitising effects of Rosiglitazone are, at least in part, attributable to the prevention of lipid accumulation in this tissue. However, it is currently unclear whether the insulin-sensitising effects of long-term Rosiglitazone treatment are directly attributable to insulin-sensitisation in skeletal muscle secondary to reduced lipid accumulation.

The effect of Rosiglitazone treatment on skeletal muscle lipid storage is equivocal, as previous investigations have reported a decrease (Hockings et al. 2003; Jucker et al. 2003; Ye et al. 2003), an increase (Muurling et al. 2003; Todd et al. 2006), or no effect (Oakes et al. 1994; Oakes et al. 1997b; Mayerson et al. 2002; Coort et al. 2005) on muscle TAG content. Regardless of its effect on skeletal muscle lipid storage, all of these studies demonstrated whole-body insulin-sensitisation in response to Rosiglitazone treatment, indicating that reduction of muscle lipid storage may not be its primary mode of action. In support of this contention, Muurling et al. (2003) reported that treatment of ob/ob mice with Rosiglitazone (3 mg/kg/day) for 10 weeks resulted in improved skeletal muscle 2-deoxyglucose uptake during a hyperinsulinemic-euglycemic clamp, despite an increase in skeletal muscle TAG content. It is important to note that the use of a chemical extraction method to measure muscle TAG content in Muurling et al. (2003), can not distinguish between intramyocellular (IMCL) and extramyocellular (EMCL) lipid content. However, when determined by $^1$H-nuclear magnetic resonance spectroscopy (NMR), a technique that can distinguish between these two lipid pools, EMCL was shown to increase following 3 months of Rosiglitazone treatment (8 mg/kg/day), while IMCL did not change in the muscle of individuals with type 2 diabetes (Mayerson et al. 2002). Furthermore, in the study of Mayerson et al. (2002) insulin sensitivity was improved by Rosiglitazone treatment and was associated
with a decrease in liver TAG content, suggesting that “lipid steal” from liver may be more important than reduced muscle lipid accumulation in Rosiglitazone-induced insulin sensitisation. It is also possible that in the face of increased or unchanged total muscle TAG content, there is a decrease in those lipid species that have been linked to impairments in insulin sensitivity such as FA-CoA, ceramide and DAG. Further work is needed to determine whether Rosiglitazone treatment results in changes in lipid species other than TAG.

Although it seems likely that Rosiglitazone’s insulin-sensitising effects are at least partly attributable to PPARγ activation, it is also possible that it acts via PPAR-independent mechanisms (Feinstein et al. 2005). Treatment of H-2Kb skeletal muscle cells with Rosiglitazone resulted in stimulation of both the AMPK-α1 and –α2 isoforms via the AMP/ATP-dependent pathway (Fryer et al. 2002). The timeframe of this response (<30 min) was believed to be too short to elicit PPARγ-dependent changes in gene transcription, leading the authors to conclude that activation of AMPK may play a role in Rosiglitazone’s insulin-sensitising action. It remains unclear whether Rosiglitazone’s insulin-sensitising effects in vivo are attributable to skeletal muscle AMPK activation.

Due to its relatively recent release as a therapy for insulin resistance, the long-term consequences of Rosiglitazone treatment are unknown. However, several adverse side-effects have been associated with the use of this drug including increased adiposity, oedema and increased low-density lipoprotein cholesterol (Malinowski and Bolesta 2000). The first TZD approved for the treatment of insulin-resistance, Troglitazone, was removed from the market due to concerns regarding its potential to induce hepatotoxicity (Malinowski and Bolesta 2000). Clarification of the mechanisms by which chronic Rosiglitazone treatment exerts its insulin-sensitising effects may provide insight for the
development of new, more specific treatments for insulin resistance that produce fewer adverse side-effects.

1.5.3 Exercise Training

The limited effectiveness and adverse side-effects associated with current oral antihyperglycemic agents has prompted a search for new drug targets for the treatment of type 2 diabetes (Moller 2001). However, since lifestyle factors leading to obesity appear to be major contributors to the increased world-wide prevalence of type 2 diabetes, it follows that lifestyle modification may be prescribed as an effective therapy for this disease (Moller 2001). Indeed, lifestyle modification through increased physical activity and dietary control has been demonstrated as a successful means for the prevention of type 2 diabetes in individuals with impaired glucose tolerance (Pan et al. 1997b; Eriksson et al. 1999; Tuomilehto et al. 2001; Molitch et al. 2003). In a randomised, placebo controlled trial of >3000 individuals with impaired glucose tolerance, lifestyle intervention (reduced caloric intake combined with 150 min of moderate physical exercise per week) was ~two-fold more effective in reducing the incidence of type 2 diabetes than metformin treatment (850 mg twice daily) over a period of ~2.8 years (Knowler et al. 2002). Although these investigations (Pan et al. 1997b; Eriksson et al. 1999; Tuomilehto et al. 2001; Knowler et al. 2002; Molitch et al. 2003) studied a combination of lifestyle adjustments that may have contributed reduced incidence of type 2 diabetes, there is convincing evidence to support the prescription of exercise as a therapy for insulin resistance and type 2 diabetes (Booth et al. 2000; Booth et al. 2002; Hawley 2004; Pedersen and Saltin 2006). In this regard, Houmard et al. (2004) demonstrated a “dose-response” relationship between volume of exercise training (min/week) and insulin
sensitivity in sedentary, obese and overweight individuals that were randomly assigned to exercise programs of varying volumes and intensities for 6 months (Houmard et al. 2004). These workers concluded that an 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).

The observation that exercise training increases both insulin-dependent and -independent glucose transport in skeletal muscle is well-established (Holloszy and Narahara 1965; Richter et al. 1982; Ivy et al. 1983; Garetto et al. 1984; Richter et al. 1984; Wallberg-Henriksson et al. 1988; Holloszy 2005). Similar observations regarding exercise-induced improvements in skeletal muscle glucose transport have been made in insulin-resistant rats (Cortez et al. 1991), diabetic rats (Wallberg-Henriksson and Holloszy 1984) and humans with insulin resistance and type 2 diabetes (Christ-Roberts et al. 2003). Acutely, increased glucose transport in response to a bout of exercise or muscle contraction may be mediated by a variety of intramyocellular signalling events including AMPK activation, Akt phosphorylation, nitric oxide production, and calcium-mediated mechanisms involving Ca2+/calmodulin-dependent protein kinase (CaMK) and PKC (Sakamoto and Goodyear 2002; Hawley 2004; Richter et al. 2004; Jessen and Goodyear 2005). However, the insulin-sensitising effects of an acute exercise bout are short-lived, and may only persist for up to 48 hours if another bout of exercise is not undertaken (Ivy et al. 1983; Etgen et al. 1993). In contrast, chronic exercise training may produce metabolic adaptations that result in sustained improvements in whole-body and muscle insulin sensitivity (Booth et al. 2000; Booth et al. 2002; Hawley 2004; Pedersen
and Saltin 2006). The remainder of this review will focus on the chronic adaptations in skeletal muscle that may result in improved insulin sensitivity following chronic exercise training. A summary of these improvements is shown in Figure 1.11.

**Figure 1.11 Chronic adaptations that may contribute to exercise training-induced improvements in skeletal muscle insulin sensitivity.**

AMPK, AMP-activated protein kinase; HK, hexokinase; CS, citrate synthase; GLUT, glucose transporter; PI3K, phosphatidylinositol-3-kinase; AS160, Akt substrate of 160 kDa; NRF, nuclear respiratory factor; PPAR, peroxisome proliferator-activated receptor; PGC, PPARγ Coactivator; DAG, diacylglycerol

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 (Zierath 2002). Perhaps the most consistently observed effect of exercise training in healthy and insulin
resistant skeletal muscle is increased expression of GLUT4 protein (Henriksen 2002; Zierath 2002; Holloszy 2005). In addition, insulin sensitivity following 7 days of exercise training in previously sedentary men was associated with increased insulin-stimulated PI-3 kinase activity (Houmard et al. 1999). However, the mechanism for this increase is unknown, as it was not accompanied by increased protein levels of PI-3 kinase or upstream components of the insulin-signalling cascade.

The upregulation of AMPK is another potential mechanism by which exercise training improves insulin sensitivity. In addition to acute activation of AMPK due to muscle contraction (see section 1.2.2), chronic exercise training may result in an upregulation of AMPK protein. In healthy individuals, 3 weeks of endurance training increased the skeletal muscle protein content of the AMPK α1, β2 and γ1 subunits (Frosig et al. 2004). Similarly, 7 weeks of 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 8 weeks of treadmill running in ZDF rats produced similar improvements in insulin sensitivity as daily AICAR administration. 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 exercise training induces a chronic upregulation of AMPK (Frosig et al. 2004; Wojtaszewski et al. 2005; Sriwijitkamol et al. 2006). Furthermore, it has been previously described that chronic activation of AMPK through AICAR results in improved skeletal muscle insulin sensitivity (see section 1.5.1). Thus, it is plausible that the insulin-sensitising effects of exercise training are at least partially attributable to increased skeletal muscle AMPK 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 et al. 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. 2006; Treebak et al. 2006). 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. 2006). The effects of chronic exercise training on AS160 expression and phosphorylation are currently unknown.

The regulation of lipid turnover and utilisation is another potential mechanism by which exercise training may improve insulin sensitivity (Bruce and Hawley 2004). Exercise training results in an increase in the oxidative
capacity of skeletal muscle by upregulating the expression of proteins involved in mitochondrial biogenesis such as PGC1, PPARα and nuclear respiratory factor 1 (NRF1) (Gollnick and Saltin 1982; Irrcher et al. 2003). Oxidative enzyme capacity is low in individuals with insulin resistance, which is thought to contribute to a “metabolic inflexibility” that does not allow for easy transition between fasting and postprandial states (Kelley 2002). 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). Furthermore, increased oxidative capacity following exercise training was recently associated with increased CPT1 activity and decreased ceramide and DAG content in the muscle of obese individuals (Bruce et al. 2006). The findings by Bruce et al. (2006) suggest that 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.

Although there appears to be a strong association between improved muscle oxidative capacity and whole-body insulin sensitivity, this relationship may be dependent on age. 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 Pruchnic et al. (2004) who observed an increase in oxidative capacity following 12 weeks of 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.

In summary, there are several ways in which exercise training may improve skeletal muscle glucose uptake. These include upregulation of GLUT4 expression, chronic activation of AMPK, facilitation of insulin signal transduction at the level of PI3-kinase and AS160, 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 (Roberts and Barnard 2005; Pedersen and Saltin 2006). Therefore, exercise training is an effective therapy for the treatment of insulin-resistance in skeletal muscle.

Given the potential for Rosiglitazone and exercise training to improve insulin sensitivity by different mechanisms, it is plausible that their combined treatment would produce additive beneficial effects. In support of this contention, Hevener et al., (2000) observed that treating obese Zucker (fa/fa) rats with the TZD Troglitazone combined with exercise training (treadmill running) for 3 weeks was more effective in improving insulin sensitivity than either treatment alone (Hevener et al. 2000). Troglitazone and exercise training
produced an additive increase in skeletal muscle GLUT4 content, suggesting that the whole-body insulin sensitisation was at least partially attributable to increased muscle glucose uptake (Hevener et al. 2000). More investigation into the individual and combined effects of exercise and TZD therapy may provide insight into the respective mechanisms of action of these therapies.
CHAPTER TWO: Aims of thesis
The review of literature (chapter one) highlighted the strong association between aberrant skeletal muscle lipid metabolism and whole-body insulin-sensitivity. However, it was also noted that some important research questions remained unanswered. Specifically, little is known about the efficacy by which current treatments for insulin resistance improve glucose and lipid metabolism in skeletal muscle. Accordingly, the primary aim of the investigations undertaken for this thesis was to enhance our understanding of the mechanisms by which two common therapies for the treatment of insulin resistance, Rosiglitazone and exercise training, reverse aberrant lipid handling and improve insulin sensitivity in this tissue.

The objective of the initial investigation which is described in chapter three, was to determine the changes to skeletal muscle lipid metabolism that occur as a result of chronic Rosiglitazone treatment in a genetic model of insulin resistance, the obese Zucker rat. It was hypothesised that increased intramuscular lipid storage would be associated with impaired glucose tolerance in the obese Zucker rat relative to lean control animals, and that Rosiglitazone treatment would improve glucose tolerance and reduce muscle lipid content.

The subsequent investigation (chapter four) examined an alternative and PPARγ-independent mechanism by which Rosiglitazone may improve insulin sensitivity in the same animal model. It was hypothesised that AMPK activity would be increased by chronic Rosiglitazone treatment, providing an alternative mechanism for Rosiglitazone’s insulin-sensitising actions in muscle.

The final study described in chapter five compared the independent and interactive effects of Rosiglitazone treatment and exercise training on muscle glucose and lipid metabolism in a less severe model of insulin resistance, the fat-fed rat. Given that both treatments have the potential to improve skeletal
muscle insulin sensitivity; it was hypothesised that the combination of exercise and Rosiglitazone treatment would result in additive improvements in insulin-stimulated muscle glucose transport. Furthermore, it was hypothesised that changes in insulin-stimulated glucose transport following exercise training or Rosiglitazone treatment would be associated with changes in fatty acid transport and storage.

It is hoped that information gathered from experiments undertaken for this thesis will enhance our understanding of the mechanisms by which these common therapies for insulin resistance improve skeletal muscle insulin sensitivity. Continued discovery of the factors that lead to skeletal muscle insulin resistance, and the mechanisms by which available insulin-sensitising agents act to overcome these impairments may lead to the development of more effective treatments for this disease in the future.
CHAPTER THREE: Rosiglitazone Enhances Glucose Tolerance by Mechanisms Other Than Reduction of Fatty Acid Accumulation within Skeletal Muscle

3.1 Introduction

Insulin resistance in skeletal muscle is a characteristic feature of obesity, and precedes the development of type 2 diabetes and its secondary complications (DeFronzo 1992). The direct cause of skeletal muscle insulin resistance in obese individuals remains unclear; however, accumulation of intramuscular triacylglycerol (IMTG) is strongly associated with both whole-body and skeletal muscle insulin resistance (Pan et al. 1997a; Kelley et al. 2002; Krssak and Roden 2004). The molecular mechanisms linking IMTG accumulation and impaired insulin sensitivity have not been fully elucidated, but it has been proposed that IMTG acts as a marker for the presence of other, more metabolically active lipid intermediates, which are directly linked to defects in insulin signalling, and may play a causative role in obesity-induced insulin resistance (Shulman 2000).

One such lipid intermediate is diacylglycerol, which is elevated in both genetic and diet-induced insulin resistance (Kim et al. 2001; Itani et al. 2002; Yu et al. 2002). Diacylglycerol is proposed to induce insulin resistance by activating diacylglycerol-sensitive protein kinase C (PKC) isoforms (Schmitz-Peiffer et al. 1997; Qu et al. 1999; Montell et al. 2001), which results in serine phosphorylation of insulin receptor substrate (IRS)-1 (Cortright et al. 2000; Lin et al. 2001). Ceramide, which is a second messenger in the sphingomyelin signalling pathway, is also elevated in the muscle of obese, insulin resistant humans (Adams et al. 2004; Straczkowski et al. 2004), and rodents (Turinsky et al. 1990), and its accumulation affects downstream insulin signalling by preventing insulin-induced Akt phosphorylation and activation (Schmitz-Peiffer et al. 1999; Chavez et al. 2003).
Rosiglitazone belongs to a class of insulin sensitizing drugs that are ligands for peroxisome proliferator activated receptor gamma (PPAR-γ). PPAR-γ is a critical transcription factor that influences numerous genes related to lipid homeostasis, suggesting that changes in lipid metabolism may mediate the therapeutic effects of Rosiglitazone. In addition to improving insulin sensitivity, chronic Rosiglitazone treatment results in decreased blood triglyceride (Jucker et al. 2003; Muurling et al. 2003), and free fatty acid (FA) levels (Miyazaki et al. 2003; Buse et al. 2004), and increased FA uptake and oxidation in cultured skeletal muscle (Wilmsen et al. 2003). Since there is strong evidence for the role of several lipid species in the development of skeletal muscle insulin resistance, and PPAR-γ agonism results in altered lipid homeostasis, the primary aim of the current investigation was to determine the effects of Rosiglitazone on the skeletal muscle lipid profile.

In this regard, previous studies examining IMTG content following chronic Rosiglitazone treatment have reported increased (Muurling et al. 2003), decreased (Hockings et al. 2003; Kim et al. 2003a; Kuhlmann et al. 2003), and unchanged (Mayerson et al. 2002; Kuhlmann et al. 2003) levels. The changes observed in IMTG content with Rosiglitazone treatment may result from alterations in regulatory enzymes of lipid turnover such as hormone sensitive lipase (HSL) and glycerol 3-phosphate acyltransferase (GPAT). However, the effect of Rosiglitazone treatment on the protein content and activities of these regulatory enzymes are unresolved.

The present investigation examined the effect of chronic Rosiglitazone treatment on both glucose tolerance, the skeletal muscle content of lipid metabolites and the activities of lipid regulatory enzymes in a rodent model of insulin resistance. It was hypothesized that muscle lipids would be elevated in
obesity, and that improved glucose tolerance following Rosiglitazone treatment would coincide with decreased levels of IMTG, diacylglycerol and ceramide.

3.2 Materials and Methods

Animals

Female obese Zucker (fa/fa) and age-matched lean (fa/?i) rats were obtained from Monash Animal Services (Victoria, Australia) at 19 wk of age. Rats were housed under controlled light (12:12 light:dark) at an ambient temperature of 21°C. Animals had free access to standard rat chow and water except when overnight fasting was required for blood measurements. All procedures were approved by the RMIT University Animal Ethics Committee. The obese rats were randomly divided into two experimental groups: control (OB CON, n=9) and Rosiglitazone (OB RSG, n=9). The lean rats were treated with vehicle (LN CON, n=9) and acted as a control group for OB CON. Rats were dosed daily, for 6 wk, by oral gavage with either vehicle, which consisted of 0.5% carboxymethylcellulose (CMC; Sigma-Aldrich, Inc., MO, USA) (100 µL/100 g body mass), or 3 mg/kg Rosiglitazone (GlaxoSmithKline, UK) suspended in an equal volume of CMC.

Oral glucose tolerance test (OGTT) and Surgery

Following the 6 wk experimental period, rats were overnight fasted, weighed and underwent an OGTT. To assess oral glucose tolerance, glucose (70% w/v solution) was administered by oral gavage at a dose of 2.5 g/kg body weight, and tail blood was obtained at time -1, 10, 15, 30, 60 and 90 min after administration of the glucose load. Three days later, rats were euthanized by CO₂ asphyxia, followed by exsanguination. The soleus and red gastrocnemius
muscles were rapidly dissected from the rat hindlimbs, snap-frozen in liquid nitrogen and stored at –80°C for later analysis.

**Blood Metabolite Measurements.**

Blood was analysed for fasting concentrations of glucose, insulin, triglyceride and cholesterol. Plasma was obtained by centrifugation of whole blood collected at the time of euthanasia, and stored at –80°C until analysis. Blood glucose levels were monitored with the MediSense2™ Blood Glucose Testing System (MediSense Australia Pty. Ltd, Victoria, Australia). Plasma insulin levels were determined using an enzyme immunoassay kit (Ultrasensitive Rat Insulin ELISA, Mercodia AB, Uppsala, Sweden) and plasma triglycerides and total cholesterol were measured using the enzymatic assay kits GPO-PAP and CHOD-PAP (Roche, Basel, Switzerland), respectively.

**Analysis of intramuscular muscle lipids**

IMTG content was analysed as previously described (Frayn and Maycock 1980). Freeze-dried muscle was powdered, and cleaned of all visible connective tissue and blood under magnification. Lipid was extracted by a Folch extraction (Folch et al. 1957), the triacylglycerol was saponified in an ethanol / KOH solution (Sigma-Aldrich, Inc., MO, USA) at 60°C, and glycerol content was determined fluorometrically.

The fatty acid composition of muscle triacylglycerols was determined on a separate portion of powdered skeletal muscle. Skeletal muscle lipids were extracted (Folch et al. 1957) and triacylglycerols were separated from phospholipids by solid phase extraction on Sep-Pak® Silica cartridges (Waters™ Division, Millipore Corporation, MA, USA) as described previously (Pan and Storlien 1993). Triacylglycerol fractions were transmethylated with 14% (w/v) boron trifluoride in methanol and fatty acid methyl esters were
separated by gas-liquid chromatography on a Hewlett-Packard 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) with a fused silica capillary column. Individual fatty acids were identified by comparing each peak’s retention time to those of external standards, and are expressed as the mole percentage of total fatty acids. The five major fatty acids in the triacylglycerol pool (16:0, 16:1, 18:0, 18:1, 18:2), which represent ~95% of total muscle fatty acids are presented.

Diacylglycerol and ceramide were extracted and quantified according to the methods of Preiss et al. (Preiss et al. 1986). Briefly, lipids were extracted from freeze-dried, powdered soleus muscle using chloroform: methanol: PBS+0.2% SDS (1:2:0.8). Diacylglycerol kinase and [γ-32P] ATP (Amersham Biosciences, NSW, Australia; 15 µCi/µmol cold ATP) were added to extracts, and the reaction was stopped using chloroform:methanol (2:1; Sigma-Aldrich, Inc., MO, USA). Samples were spotted onto thin-layer chromatography plates and developed to two thirds of the total plate length. Bands corresponding to diacylglycerol and ceramide were identified against standards after phospho-imaging, dried, scraped from the TLC plate and counted in a liquid scintillation analyser (Tri-Carb 2500TR, Packard, Canberra, Australia).

Analysis of hormone-sensitive lipase (HSL) activity

An aliquot of freeze-dried muscle was used to determine HSL activity as described previously (Watt et al. 2003). Briefly, the powdered muscle was homogenized and after centrifugation the supernatant was removed and analysed for HSL activity against a triolein substrate. All measurements were made in triplicate and the mean of these values is reported.
Analysis of glycerol 3 phosphate acyltransferase (GPAT) activity

Skeletal muscle GPAT activity was determined as described by Muoio et al., (Muoio et al. 1999). After homogenisation, total GPAT activity was measured with 900 mM [3H] glycerol-3-phosphate (ARC, St. Louis, USA) and 90 mM palmitoyl CoA. The reaction was run for 20 min at 37°C and stopped with 1% HClO₄ (Sigma-Aldrich, Inc., MO, USA) and chloroform : methanol (2:1; Sigma-Aldrich, Inc., MO, USA). After a series of washes with HClO₄, 1 mL of the organic phase containing the labeled G-3-P incorporated into lysophosphatidic acid was dried and 4 mL scintillation fluid was added. Radioactivity was determined with a liquid scintillation analyser.

Subcellular fractionation and Western blotting protocol

Soleus muscle was homogenized in 8x wt/vol ice cold buffer containing 20 mM HEPES (pH 7.4), 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM DTT, 1 mM Na₃VO₄, 10% glycerol, 3 mM benzamidine, 10 µM leupeptin, 5 µM pepstatin-A and 1mM PMSF (all from Sigma-Aldrich Inc, MO, USA). The homogenate was centrifuged at 100,000 g for 30 min at 4°C, and the supernatant was collected as the cytosolic fraction. The pellet was resuspended by agitation in 4x wt/vol of ice-cold homogenisation buffer to which 1% Triton-X (Sigma-Aldrich Inc., MO, USA) was added. The resuspended pellet was then centrifuged at 15,000 g for 10 min at 4°C. The supernatant, representing the total membrane fraction, was collected. Both the membrane and cytosolic fractions were stored at –80°C. A separate portion of soleus muscle was homogenized, centrifuged at 16,000 g for 60 min and later analysed for total cellular HSL protein.

Protein concentration of the muscle lysates was determined (Pierce, Rockford, IL, USA). Muscle lysates (100 µg) from both fractions were solubilized.
in Laemelli buffer and separated by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (BioRad Laboratories, CA, USA) by electrophoresis, and the membranes were blocked, and incubated overnight at 4°C with antibodies specific for HSL (kindly provided by Fredric Kraemer, Stanford University, CA, USA) or for PKC-θ, -α/β, or -δ isoforms (1:1000; Cell Signaling, Beverley, MA, USA). The immunoreactive proteins were detected with enhanced chemiluminescence (Perkin Elmer, Rowville, Victoria, Australia) and quantified by densitometry.

3.2.1 Statistical Analysis

Results are presented as means ± standard error (SE). All statistical analyses were performed using the unpaired Student’s t-test. A P value of less than 0.05 was considered significant.

3.3 Results

Obese control rats at 25 wk of age had a greater body mass and elevated fasting plasma insulin, triglyceride, and cholesterol levels compared with lean control rats (Table 3.1, P<0.05). Rosiglitazone treatment in obese rats resulted in further increases in body mass and plasma cholesterol, but lower plasma insulin and triglyceride levels compared with OB CON (Table 3.1, P<0.05). Compared with lean rats, obese rats had elevated (P<0.05) plasma glucose levels throughout the oral glucose challenge (Figure 3.1). The area under the curve (AUC) during the oral glucose challenge averaged 123 ± 3 and 152 ± 8 mM for lean and obese control rats (P<0.05), respectively. Rosiglitazone treatment improved glucose tolerance in obese rats, as indicated by a reduced AUC (126 ± 4 mM, P<0.05; Figure 3.1).
Table 3.1  Body mass and plasma characteristics of lean (LN CON), obese (OB CON) and rats treated with Rosiglitazone (OB RSG).

<table>
<thead>
<tr>
<th></th>
<th>LN CON</th>
<th>OB CON</th>
<th>OB RSG</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mass (g)</strong></td>
<td>211 ± 7.6</td>
<td>388 ± 9.4*</td>
<td>444 ± 13.3#</td>
</tr>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td>6.3 ± 0.5</td>
<td>7.1 ± 0.5</td>
<td>6.4 ± 0.3</td>
</tr>
<tr>
<td>Insulin (pM)</td>
<td>62 ± 18</td>
<td>986 ± 291*</td>
<td>103 ± 49#</td>
</tr>
<tr>
<td>Triglyceride (mM)</td>
<td>0.42 ± 0.07</td>
<td>2.93 ± 0.64*</td>
<td>1.15 ± 0.19#</td>
</tr>
<tr>
<td>Cholesterol (mM)</td>
<td>1.13 ± 0.06</td>
<td>1.57 ± 0.05*</td>
<td>1.84 ± 0.11#</td>
</tr>
</tbody>
</table>

LN CON vs OB CON, P<0.05; # OB CON vs OB RSG, P<0.05; n=9/group.

Figure 3.1  Oral glucose tolerance test.

Glucose was administered by oral gavage at a dose of 2.5 g/kg body weight, and blood glucose was measured for 90 min. P < 0.01, OB CON vs. OB RSG (n=9/group).

IMTG content in soleus muscle was higher in obese compared with lean rats (70.5 ± 5.1 vs 27.5 ± 2.0 µmol/g dm, P<0.001), and increased a further 30% (P=0.04) following Rosiglitazone treatment (Figure 3.2). Soleus muscle diacylglycerol and ceramide levels were not elevated when comparing lean with obese control rats, but were increased by 65% (P<0.001) and 100% (P=0.02), respectively, following Rosiglitazone treatment (Figure 3.2). IMTG content in a glycolytic muscle, the extensor digitorum longus (EDL), was also increased with
obesity (LN CON: 9.3 ± 2.4 vs OB CON: 35.2 ± 8.4, P<0.05), but was not increased following Rosiglitazone treatment.

Figure 3. 2 Intramyocellular lipids.
Changes in skeletal muscle IMTG (A), diacylglycerol (B), and ceramide (C) with obesity (LN CON vs. OB CON), and RSG treatment (OB CON vs. OB RSG). Significant differences (P < 0.05; n=9/group) between groups are indicated by the P values listed on the figure.

In order to determine the potential mechanism(s) mediating the changes in intramuscular IMTG and diacylglycerol levels with Rosiglitazone treatment, the basal activities of HSL and GPAT were assessed. There was no difference
in HSL activity between lean and obese control rats; whereas HSL activity was decreased by ~30% following Rosiglitazone treatment in obese rats (Figure 3.3 A, P=0.02). The decreased HSL activity in OB RSG was not associated with changes in total HSL protein as determined by Western blot analysis (Figure 3.3 B and C). Skeletal muscle GPAT activity was not altered by obesity, or Rosiglitazone treatment (Figure 3.3 D).

Figure 3.3  Activity and protein levels of lipid regulatory enzymes.
A) HSL activity with obesity (LN CON vs. OB CON, not significant) and RSG treatment (OB CON vs. OB RSG, P = 0.02). B) A representative Western blot of total HSL protein. C) Mean values [arbitrary units (AU)] ± SEM of HSL protein with obesity (LN CON vs. OB CON, not significant) and RSG treatment (not significant [n.s.]). D) GPAT activity with obesity (LN CON vs. OB CON, n.s.) and RSG treatment (OB CON vs. OB RSG, n.s.; n=9/group).
Diacylglycerol is known to translocate PKC from the cytosolic to the membrane fraction resulting in its activation, and PKC has been implicated in the insulin resistance of skeletal muscle (10,11). Accordingly, the cytosolic and membrane fraction protein content of several diacylglycerol-sensitive PKC isoforms were measured. There was no change in either total PKC-θ, -α/β, or -δ protein, or in the membrane fractions of these PKC isoforms with obesity, or Rosiglitazone treatment (Figure 3.4, Table 3.2).

**Table 3.2 Membrane/total protein ratio of diacylglycerol-sensitive PKC isoforms from LN CON, OB CON, and OB RSG rats.**

<table>
<thead>
<tr>
<th></th>
<th>LN CON</th>
<th>OB CON</th>
<th>OB RSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKC α/β</td>
<td>0.31 ± 0.05</td>
<td>0.35 ± 0.04</td>
<td>0.38 ± 0.04</td>
</tr>
<tr>
<td>PKC θ</td>
<td>0.90 ± 0.01</td>
<td>0.95 ± 0.02</td>
<td>0.93 ± 0.02</td>
</tr>
<tr>
<td>PKC δ</td>
<td>0.76 ± 0.02</td>
<td>0.80 ± 0.01</td>
<td>0.82 ± 0.03</td>
</tr>
</tbody>
</table>

![Cytosol and Membrane Proteins](image)

**Figure 3.4 Protein kinase C (PKC) distribution.**

Representative immunoblots of PKC-θ, -δ, and -α/β. Soleus muscles were homogenized, and the cytosolic and membrane fractions were separated using subcellular fractionation (see section 3.2). Proteins were quantified by Western blot analysis and densitometry. There were no significant differences between groups (n=9/group).
Increased skeletal muscle lipid content with obesity and Rosiglitazone were associated with changes in FA composition (Figure 3.5). The proportion of monounsaturated fatty acids (MUFA) was elevated in obese (38.0 ± 0.8 %) compared with lean rats (29.1 ± 0.8 %, P<0.0001), and was further elevated by Rosiglitazone (43.7 ± 2.5 %, P=0.05). The elevation in % MUFA observed in obese rats was due to specific increases in palmitoleate [16:1(n-7), P<0.0001] and oleate [18:1(n-9)] (P<0.0001) fatty acids, in conjunction with decreases in the proportions of stearate (18:0, P<0.0001) and linoleate [18:2 (n-6), P<0.0001]. The additional increase in % MUFA observed following Rosiglitazone treatment was solely due to an increase in 16:1 (n-7) (Figure 3.5, P=0.02).

**Figure 3.5** Fatty acid (FA) composition of muscle triacylglycerol.
A) Molar percentage of total FA for specific fatty acyl species (palmitate 16:0, palmitoleate 16:1, stearate 18:0, oleate 18:1, linoleate 18:2). B) Proportion of polyunsaturated species (PUFA), monounsaturated species (MUFA), and saturates within the muscle lipid pool. *, P < 0.05, LN CON vs. OB CON; #, P < 0.05, OB CON vs. OB RSG (n=9/group).
3.4 Discussion and Conclusions

The novel finding of the current study was that despite a normalisation of glucose tolerance, six weeks of Rosiglitazone treatment increased IMTG (1.3-fold), diacylglycerol (1.7-fold) and ceramide (2-fold) in the skeletal muscle of obese, insulin resistant rats. These results suggest, therefore, that the improvements in glucose tolerance seen with Rosiglitazone treatment are not mediated by reductions in the total content of these muscle lipids. Furthermore, increased diacylglycerol was not associated with increased membrane-associated PKC-θ, -α/β, or -δ content (Fig. 3.4, Table 3.2). Since diacylglycerol-induced insulin resistance in skeletal muscle is thought to be mediated by PKC translocation to the sarcolemma (Schmitz-Peiffer et al. 1997; Qu et al. 1999; Montell et al. 2001), this provides further evidence that the insulin-sensitising effects of Rosiglitazone in skeletal muscle are not lipid-mediated. It is important to note that the improved glucose tolerance with Rosiglitazone observed in the current study was not necessarily due to increased skeletal muscle glucose uptake, and may involve increased hepatic or adipose glucose uptake, decreased gluconeogenesis and/or increased insulin secretion during the glucose challenge. However, direct measurement of insulin-stimulated glucose uptake following chronic Rosiglitazone treatment has previously shown an increase in skeletal muscle glucose transport despite increased IMTG content (Muurling et al. 2003).

The effect of Rosiglitazone treatment on skeletal muscle lipid storage is unclear, as previous investigations have reported a decrease (Hockings et al. 2003; Jucker et al. 2003; Kim et al. 2003a; Koh et al. 2003; Kuhlmann et al. 2003), an increase (Muurling et al. 2003), or no effect (Mayerson et al. 2002;
Kuhlmann et al. 2003) on IMTG content. Most studies demonstrating decreased IMTG content with Rosiglitazone have analysed muscle with a large proportion of type II fibres (Hockings et al. 2003; Jucker et al. 2003; Kim et al. 2003a). In contrast, in the present study muscle with predominantly (>90%) type I composition was analysed because type I muscle fibres contain more ectopic lipids (He et al. 2001), have a greater role in lipid utilisation (Dyck et al. 1997) and are more insulin sensitive than type II fibres (Henriksen et al. 1990). The conflicting reports among laboratories analysing different muscle groups present the possibility that Rosiglitazone action may be fibre-type dependent. In order to test this, the IMTG content of a more highly glycolytic muscle (EDL; >50% type IIB fibers) was also analysed, and no increase in IMTG with Rosiglitazone treatment was found. This suggests that oxidative fibres may be more responsive to Rosiglitazone treatment than glycolytic fibers. This also raises the possibility of contamination by extramyocellular adipocytes when using chemical extraction to measure IMTG, as a previous study using $^1$H-nuclear magnetic resonance spectroscopy demonstrated increased extramyocellular lipid content in human skeletal muscle following RSG treatment (Mayerson et al. 2002). However, contamination of a muscle sample by extramyocellular adipocytes can be avoided if a thorough removal of adipose tissue under magnification is performed (Guo 2001). It is believed that our samples were not contaminated with extramyocellular adipocytes since thorough microdissection was performed. Furthermore, HSL activity, which was measured using the same aliquot of freeze-dried tissue as IMTG, was decreased in obese Rosiglitazone treated rats. If there was significant adipocyte contamination, one would expect HSL activity to be elevated because of its high expression in adipocytes compared with myocytes. Similarly, GPAT is
also highly expressed in adipose tissue and was not different between treatment groups.

The decrease in HSL activity (Fig. 3.3) is a potential mechanism for increased IMTG and diacylglycerol following RSG treatment. HSL is a regulatory enzyme for skeletal muscle IMTG degradation (Holm et al. 2000) and Rosiglitazone treatment caused a significant decrease in HSL activity that was not associated with changes in total protein content. GPAT activity was also examined because it is the first committed step in glycerolipid synthesis and was found to be similar in all groups and was unaffected by Rosiglitazone. Taken together, these data are consistent with the increased IMTG observed after Rosiglitazone treatment. HSL is also an important diacylglycerol lipase as demonstrated by diacylglycerol accumulation in HSL -/- mice (Haemmerle et al. 2002). The downregulation of HSL in the present study is also likely to be responsible for diacylglycerol accretion following Rosiglitazone treatment.

The consistent observation of increased lipid levels with Rosiglitazone may also be due to altered fatty acid (FA) transport and oxidation. Rosiglitazone increases sarcolemmal FA transport in primary skeletal muscle cell culture, which is associated with increased fatty acid translocase expression (Wilmsen et al. 2003). Such increases in sarcolemmal FA transport, if present during chronic Rosiglitazone treatment, would likely result in skeletal muscle lipid deposition if they were not matched by similar increases in FA oxidation. Rosiglitazone does not appear to alter whole-body FA oxidation (Mayerson et al. 2002); however, other PPAR-γ agonists (eg: Troglitazone) have been reported to increase (Ide et al. 2000; Cha et al. 2001), or cause no change (Sreenan et al. 1999) in skeletal muscle FA oxidation in isolated preparations. The effect of Rosiglitazone on skeletal muscle FA oxidation has yet to be
examined, and may provide further insight into the observed changes in lipid levels following Rosiglitazone treatment.

Elevated intramyocellular lipid levels, in conjunction with increased insulin sensitivity appears counterintuitive given the abundance of evidence linking lipid accumulation and insulin resistance. However, the composition of intramuscular lipid stores may provide a protective effect against insulin resistance in the face of increased lipid levels. Since skeletal muscle insulin resistance has been directly correlated with the proportion of saturated fatty acids present in the triacylglycerol pool (Manco et al. 2000), the present study also investigated whether changes in specific fatty acid species occur in the muscle lipid pool with Rosiglitazone treatment. Although there was no decrease in total saturated species with RSG, a shift towards a higher proportion of MUFA was observed, which was specifically due to an increase in palmitoleate [16:1 (n-7)]. Incubation of C2C12 myocytes with 16:1(n-7) had no deleterious effect on insulin-stimulated Akt phosphorylation (Chavez et al. 2003), however, incubation with the saturated species palmitate (16:0) and oleate (18:0) resulted in the incorporation of these fatty acids into ceramide (Chavez et al. 2003) and diacylglycerol (Montell et al. 2001; Chavez et al. 2003), and a decrease in insulin sensitivity. Therefore, a shift towards the storage of monounsaturated species may provide protection from ceramide- and diacylglycerol- induced insulin resistance. Further investigation of the effect of Rosiglitazone treatment on the incorporation of fatty acids into specific diacylglycerol and ceramide molecular species are warranted.

In summary, improved glucose tolerance after six weeks of Rosiglitazone treatment was associated with increased skeletal muscle IMTG, diacylglycerol and ceramide content. Rosiglitazone-induced increases in lipid levels were
associated with decreased HSL activity and changes in the fatty acid composition of muscle lipids. These results suggest that reducing intramuscular lipid accumulation is not the mechanism by which Rosiglitazone improves insulin-sensitivity.
CHAPTER FOUR: Chronic Rosiglitazone Treatment Restores AMPKα2 Activity in Insulin Resistant Skeletal Muscle

4.1 Introduction

Skeletal muscle insulin resistance is a common state associated with inactivity, ageing, genetic predisposition and environmental factors and is a hallmark feature of a variety of disease states including obesity, hyperlipidemia, hypertension and type 2 diabetes (Saltiel 2000). Rosiglitazone is a member of the Thiazolidinedione (TZD) class of oral antidiabetic agents that improves insulin sensitivity in a range of insulin-resistant states (Inzucchi et al. 1998; Hallsten et al. 2002). TZDs are peroxisome proliferator-activated receptor-\(\gamma\) (PPAR\(\gamma\)) agonists that, upon activation by fatty acids (FA) or FA-derived compounds, bind to responsive elements located in the promoter regions of many genes and modulate their transcriptive activities (Saltiel and Olefsky 1996; Auwerx 1999). Although PPAR-\(\gamma\) plays a role in the TZD-induced insulin sensitization, metabolic responses to TZDs can be dissociated from PPAR\(\gamma\)-induced gene transcription (Fujiwara et al. 1988; Ranganathan and Kern 1998; Brunmair et al. 2001). Furthermore, the study described in chapter three reported that Rosiglitazone improves glucose tolerance by mechanisms other than a reduction of fatty acid accumulation within skeletal muscle. Hence, it is likely that there exist PPAR\(\gamma\) independent mechanisms by which TZDs improve insulin sensitivity.

The AMPK-activated protein kinase (AMPK) is a cellular energy sensor that regulates glucose and lipid metabolism by phosphorylating key regulatory enzymes (Winder and Hardie 1999; Kemp et al. 2003). AMPK activation causes many metabolic changes that would be beneficial to individuals with type 2 diabetes and the metabolic syndrome including increased glucose uptake and metabolism by muscle, decreased glucose production by the liver, and
decreased synthesis and increased oxidation of fatty acids (Winder and Hardie 1999; Hardie 2003). As TZDs increase insulin-stimulated glucose uptake and utilization, and decrease circulating levels of free fatty acids and triglycerides [for reviews see (Saltiel and Olefsky 1996; Olefsky 2000)], it is reasonable that a component of the insulin sensitizing effect of these drugs could be mediated through the action of the AMPK pathway. In this regard, Fryer et al. (2002) reported that acute incubation of H-2Kb muscle cells with Rosiglitazone increased the AMP/ATP ratio and activated both $\alpha_1$- and $\alpha_2$- containing AMPK isoforms, which lead to a marked increase in the phosphorylation of acetyl-CoA carboxylase (ACC). Furthermore, AMPK has been reported to phosphorylate IRS1 at Ser789 and enhance p85 docking, consistent with improving insulin signalling (Jakobsen et al. 2001).

In the present study, the effects of acute Rosiglitazone administration in L6 muscle cells was compared with chronic Rosiglitazone treatment in skeletal muscle from obese, glucose intolerant Zucker rats on AMPK regulation and its downstream phosphorylation target, ACC. It was found that Rosiglitazone treatment normalises AMPK$\alpha_2$ activity in the skeletal muscle of obese Zucker rats to a level comparable to their lean litter-mates.

### 4.2 Materials and Methods

#### Animals

Female obese Zucker ($fa/fa$) and age-matched lean ($fa/?$) rats were obtained from Monash Animal Services (Victoria, Australia) at 19 wk of age. Rats were housed under controlled light (12:12 light:dark) at an ambient temperature of 21°C. Animals had free access to standard rat chow and water except when overnight fasting was required for blood measurements. All
procedures were approved by the RMIT University Animal Ethics Committee. Obese rats were randomly divided into two experimental groups: control (OB CON, n=9) and Rosiglitazone (OB RSG, n=9). The lean rats were treated with vehicle (LN CON, n=9) and acted as a control group for OB CON. Rats were dosed daily, for 6 wk, by oral gavage with either vehicle, which consisted of 0.5% carboxymethylcellulose (CMC) (100 µL/100 g body mass; Sigma-Aldrich Inc, MO, USA), or 3 mg/kg Rosiglitazone (GlaxoSmithKline, Stevenage, UK) suspended in an equal volume of CMC. Following the 6 wk experimental period, rats were overnight fasted and euthanized by CO₂ asphyxia, followed by exsanguination. The time interval between CO₂ administration and muscle harvesting was ~1-2 min, and the interval between the last Rosiglitazone treatment and tissue collection was ~24 h. The red gastrocnemius muscles were rapidly dissected from the rat hindlimbs, snap-frozen in liquid nitrogen and stored at –80°C for later analysis.

**Muscle metabolite and adenine nucleotide contents**

Red gastrocnemius muscle was freeze-dried, powdered, and cleaned of all visible connective tissue and blood under magnification. A ~2 mg aliquot was then taken and extracted in 0.5 M HClO₄ (1 mM EDTA; Sigma-Aldrich Inc., MO, USA) and neutralised with 2.2 M KHCO₃ (Merck Pty Ltd., VIC, Australia). Muscle ATP, PCr, creatine and lactate were measured, and muscle ADP and AMP calculated as described for cells. Muscle for glycogen analysis was extracted in 2M HCl (Merck Pty Ltd., VIC, Australia) and neutralised with 0.67 M NaOH (Sigma-Aldrich Inc., MO, USA) and glycogen content was determined by fluorometric techniques (Passonneau and Lauderdale 1974).
**AMPK activity and AMPK subunit protein expression**

Approximately 80 mg of wet muscle was homogenized in buffer A (50 mM Tris HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 50 mM NaF, 5mM Na pyrophosphate, 10% glycerol, 1% Triton X-100, 10mg/mL trypsin inhibitor, 2 mg/mL aprotinin, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride) [all from Sigma-Aldrich Inc., MO, USA]. The muscle homogenate was then centrifuged at 20,000 x g for 25 min, and the supernatant was collected. The supernatant was aliquoted for determination of i) protein concentration (Pierce, Rockford, IL, USA), ii) immunoprecipitation (AMPK α1, α2), iii) ACC affinity purification, and iv) protein expression by Western blotting (UCP3). Aliquots were stored at −80°C until use, and immunoprecipitates were used to determine protein content and enzyme activity (AMPK -α1, -α2) [described below].

**Immunoprecipitation**

Approximately 5 mg of protein from the supernatants was incubated with AMPK-α1 or AMPK-α2 antibody-bound protein A sepharose beads (6MB, Amersham Biosciences, Uppsala, Sweden) for 2 h at 4°C. The polyclonal antipeptide antibodies to AMPK α1 and α2 were raised to non-conserved regions of the AMPK isoforms -α1 (rat 231-251) and -α2 (rat 351-366). Immunocomplexes were washed with PBS and suspended in 50 mM Tris (pH 7.5) buffer for the AMPK activity assay (Christopher et al. 2003). The AMPK activities in the immune complexes were measured in the in the presence of 200 mM AMP (Sigma-Aldrich Inc., MO, USA). Activities were calculated as picomoles of phosphate incorporated into the SAMS peptide per min, per milligram of protein subjected to immunoprecipitation (pmol/min/mg). Acetyl CoA carboxylase (ACC) was affinity purified by incubating the post-AMPK
immunoprecipitation supernatants in Streptavidin Sepharose High Performance beads (Amersham Biosciences, Uppsala, Sweden) for 1 h at 4°C.

**Western Blotting**

The affinity-purified ACC fraction was electrophoresed on 7.5% SDS-PAGE and detected by immunoblotting with anti-phospho-Ser218-ACC polyclonal antibody (Chen et al. 2000). The blots were then stripped (Restore Western Blot Stripping Buffer, Pierce Biotechnology, Inc.) at 37°C for 20 min, blocked, and incubated with horseradish peroxidase conjugated Streptavidin (Amersham Pharmacia Biotech UK, Little Chalfont, UK) to determine total ACC. Aliquots of immunoprecipitated AMPK -α1, and -α2 proteins were electrophoresed on 10% SDS-PAGE and detected by immunoblotting with anti-phospho-Thr172 antibody, raised against AMPK alpha peptide (KDGEFLRpTSCGSPNY) as described previously (Clark et al. 2004). The membranes were then stripped (see above), washed in PBS, blocked for 60 min in 5% skim milk powder/PBS, and reprobed with antibodies specific for the AMPK α-1, and α-2 isoforms. Aliquots containing 80 µg total protein were electrophoresed on 15 % SDS-PAGE and detected by immunoblotting with anti-UCP3 antibody (Alpha Diagnostic Intl. Inc., San Antonio, TX).

**Cell Culture**

L6 myoblasts were maintained at 37°C (95% O2 / 5% CO2) on 60 mm collagen-coated plastic dishes in α-modified Eagle’s medium (α-MEM; SAFC Biosciences, VIC, Australia) containing 10% fetal bovine serum (FBS; SAFC Biosciences, VIC, Australia) culture media and 1% penicillin/streptomycin (Invitrogen Australia Pty Ltd., VIC, Australia), and 5mM glucose (Sigma-Aldrich Inc., MO, USA). Differentiation was induced by switching to medium containing 2% horse serum (Invitrogen Australia Pty Ltd., VIC, Australia) when the
myoblasts were ~90% confluent. Experimental treatments were started after 4 days, by which time nearly all of the myoblasts had fused to form myotubes.

**Experimental Protocol**

To determine the effect of Rosiglitazone treatment on AMPK activity, AMPK Thr172 phosphorylation and ACC Ser218 phosphorylation, L6 myotubes were incubated with 0 (DMSO; Sigma-Aldrich Inc., MO, USA), 5, 50, or 200 µM Rosiglitazone for 30 min at 37°C. Following incubation, cells were rinsed 3× with PBS and lysed with 200 µL of buffer A. Lysates were immediately frozen in liquid N₂ and kept at –80°C until further analysis. AMPKα1 and α2 subunits, and ACCβ protein were isolated by immunoprecipitation and affinity chromatography, respectively, as described above. AMPK activity was determined using the SAMS peptide assay, and protein expression and phosphorylation were determined (described above).

**Palmitate oxidation**

To examine the effects of Rosiglitazone on fat oxidation, cells were grown in 60 mm plates and were incubated for 1 h in the absence (DMSO, n=6) or presence of 5 (n=6), or 200 (n=6) µM Rosiglitazone in 3 mL of media consisting of α-MEM, 2% BSA and 0.5 mM ¹⁴C-palmitate (Amersham Biosciences, Castle Hill, NSW, Australia). Following the incubation period the reaction was stopped, and 2 mL of media was added to an equal volume of 1 M acetic acid and the released ¹⁴CO₂ was collected in benzothonium hydroxide (Sigma-Aldrich Inc., MO, USA). The cells were rinsed twice with PBS, methanol was added to the plate, and cells were scraped for subsequent analysis of the acid soluble metabolite (ASM) fraction. The ¹⁴C content of both fractions were determined using scinitillation counting and the oxidation rates (CO₂ + ASM) were calculated.
Metabolite Analysis

L6 myotubes were incubated with DMSO (Vehicle), 200 µM Rosiglitazone, 500 µM DNP, or 200 µM AICAR for 30 min. The medium was removed and the cells were rinsed 3× with PBS, lysed with 100 µl 0.5 M HClO₄ (in 1 mM EDTA; Sigma-Aldrich, Inc., MO, USA), and neutralized with 2.2 M KHCO₃ (Sigma-Aldrich, Inc., MO, USA). This extract was used for the determination of adenosine triphosphate (ATP), phosphocreatine (PCr), creatine and lactate by enzymatic fluorometric assays (Bergmeyer 1974). Free ADP and AMP concentrations were calculated with the assumption of equilibrium of the adenylate kinase and creatine kinase reactions (Dudley et al. 1987). Free ADP was calculated using the measured ATP, creatine and PCr values, an estimated H⁺ concentration (Sahlin et al. 1976) and the creatine kinase equilibrium constant of 1.66 x 10⁹. Free AMP concentration was calculated from the estimated free ADP and measured ATP with the adenylate kinase constant of 1.05.

4.2.1 Statistical Analysis

Results are presented as means ± SE. Statistical analysis of skeletal muscle measures was performed using an unpaired Student’s t-test. Analysis of cell culture experiments was undertaken using one-way ANOVA. A P value of <0.05 was considered significant. Where ANOVA revealed significant differences, Neuman Keuls post-hoc test was used to locate such differences.

4.3 Results

Effects of acute incubation of muscle cells with Rosiglitazone

Table 4.1 displays the AMP:ATP ratio of L6 myotubes following incubation with DMSO (control), 200 µM Rosiglitazone, 500 µM DNP, or 200 µM
AICAR for 30 min. Incubation with DNP caused a 2.4-fold increase in AMP: ATP (P=0.02 vs. control). No change in AMP: ATP was observed following Rosiglitazone or AICAR treatment.

Table 4.1 AMP (free)/ATP ratios from L6 myotubes treated with Rosiglitazone, AICAR, or DNP

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AMP (free) : ATP ratio (× 1000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (DMSO, n=6)</td>
<td>0.010 ± 0.003</td>
</tr>
<tr>
<td>200 µM Rosiglitazone (n=6)</td>
<td>0.007 ± 0.001</td>
</tr>
<tr>
<td>500 µM DNP (n=4)</td>
<td>0.024 ± 0.006*</td>
</tr>
<tr>
<td>200 µM AICAR (n=4)</td>
<td>0.011 ± 0.001</td>
</tr>
</tbody>
</table>

Values are means ± SEM. *P<0.05 vs. Control.

Figure 4.1 shows the effect of varying concentrations of Rosiglitazone on the activity of AMPKα1 isoform (4.1A) and Thr172 phosphorylation of AMPK α1 (4.1B), while Figure 4.2 displays the ACC Ser218 phosphorylation [Figure 4.2A] and palmitate oxidation (Figure 4.2B) in L6 myotubes following acute incubation. The dose-response trend of increasing AMPKα1 activity was significant at a dose of 200 µM Rosiglitazone (P<0.05), and corresponded with similar increases in Thr172 phosphorylation of the AMPKα1 isoform (Figure 4.1B). Increased AMPKα1 activity in L6 myotubes at the highest dose of Rosiglitazone was also associated with a 3-fold increase in the ACC Ser218 phosphorylation compared to control (0 vs. 200 µM Rosiglitazone; P<0.05; Figure 4.2A) and a significant increase in palmitate oxidation (0 vs. 200 µM RSG; 391 ± 20 vs 455 ± 19 pmol/h, respectively; Figure 4.2B). AMPKα2 activity in L6 myotubes was undetectable.
Figure 4.1 Activation of AMP-activated protein kinase (AMPK) by phosphorylation in Rosiglitazone-treated L6 myotubes.

(A) AMPK activity measured using the SAMS peptide assay of AMPKα1 protein isolated by immunoprecipitation from L6 myotubes incubated with 0 (DMSO), 5, 50, or 200 µM RSG for 30 min. (B) Ratio of AMPKα1 protein phosphorylated at Thr172 (pT172) to total levels of AMPKα1 protein, as determined by Western blotting (representative blots shown) in L6 myotubes incubated with increasing concentrations of RSG. *P < 0.05 vs. 0 µM RSG (n=6/group).
Figure 4.2 Increased acetyl-CoA carboxylase (ACC) phosphorylation and palmitate oxidation in Rosiglitazone-treated L6 myotubes.

(A) ratio of ACC protein phosphorylated at Ser\textsuperscript{218} to total ACC protein (pACC/total ACC) in L6 myotubes treated with 0 (DMSO), 5, 50, or 200 µM RSG for 30 min. (B) rate of [\textsuperscript{14}C]-palmitate oxidation in L6 myotubes treated with 0, 5, or 200 µM RSG for 1 h. *P < 0.05 vs. 0 µM RSG (n=6/group).

Effects of chronic Rosiglitazone treatment in skeletal muscle

Table 4.2 displays the resting nucleotide and metabolite concentrations for the three groups under investigation. There were no differences in ATP,
AMP, or ADP concentrations between muscle from lean or obese control animals, or obese muscle treated with Rosiglitazone. Consequently, there were no changes in the AMP/ATP ratio for any group (Table 4.2). Resting concentrations of phosphocreatine, creatine and lactate were also similar between groups. However, glycogen content was elevated in muscle from obese compared to lean animals (P<0.05), and this remained high in Rosiglitazone treated rats.

Table 4.2 Resting skeletal muscle nucleotide and metabolite concentrations from LN CON, OB CON, and OB RSG rats.

<table>
<thead>
<tr>
<th></th>
<th>LN CON</th>
<th>OB CON</th>
<th>OB RSG</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nucleotides</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP (mmol/kg dm)</td>
<td>19.3 ± 0.7</td>
<td>21.5 ± 1.2</td>
<td>18.2 ± 2.1</td>
</tr>
<tr>
<td>Free AMP (µmol/kg dm)</td>
<td>0.42 ± 0.08</td>
<td>0.56 ± 0.16</td>
<td>0.49 ± 0.11</td>
</tr>
<tr>
<td>Free ADP (µmol/kg dm)</td>
<td>80.7 ± 7.1</td>
<td>95.4 ± 18.2</td>
<td>90.1 ± 15.2</td>
</tr>
<tr>
<td>AMP:ATP (× 1000)</td>
<td>0.022 ± 0.004</td>
<td>0.028 ± 0.008</td>
<td>0.027 ± 0.005</td>
</tr>
<tr>
<td><strong>Metabolites</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphocreatine</td>
<td>67.1 ± 4.5</td>
<td>58.3 ± 5.1</td>
<td>60.5 ± 8.9</td>
</tr>
<tr>
<td>Creatine</td>
<td>28.0 ± 2.0</td>
<td>23.2 ± 4.0</td>
<td>27.8 ± 2.0</td>
</tr>
<tr>
<td>Lactate</td>
<td>35.9 ± 2.0</td>
<td>33.0 ± 2.7</td>
<td>39.6 ± 1.5</td>
</tr>
<tr>
<td>Glycogen</td>
<td>43.2±3.6</td>
<td>87.9±7.8*</td>
<td>91.4±7.2</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n = 5-7. * P<0.05, vs. LN CON (n=9/group).

The activities of the AMPK-α1 and -α2 isoforms along with the ACC Ser218 phosphorylation from skeletal muscle of animals treated for 6 wk with Rosiglitazone are shown in Figure 4.3. The activity of AMPKα1 was similar in lean and obese animals and was unaffected by 6 wk Rosiglitazone treatment (Figure 4.3A). In contrast, the activity of the AMPKα2 isoform was two-fold lower in obese versus lean animals (7.45 ± 1.0 vs. 15.7 ±2.2 pmol/mg/min; P<0.05), but was restored to lean control values following Rosiglitazone treatment (OB RSG: 14.2 ± 1.5 pmol/mg/min ; Figure 4.3B). The observed changes in AMPKα2 activity were most likely due to corresponding changes in
protein expression, rather than changes in phosphorylation of the protein at the T172 residue, which was similar in all groups (data not shown). Altered AMPKα2 activity with obesity and after Rosiglitazone treatment paralleled changes in the phosphorylation state of its target protein, ACC. The ratio of phosphorylated ACC to total ACC protein (pACC/Total ACC) was significantly decreased in muscle from obese animals (P<0.05), but was restored to lean control levels following Rosiglitazone treatment (Figure 4.3C). AMPKα1 protein expression tended to be higher (p=0.06) in muscle from obese than lean control animals, but was significantly decreased after Rosiglitazone treatment (P<0.05; OB CON vs OB RSG; Figure 4.4A). There were reciprocal changes in the protein expression of the AMPKα2 isoform, which was lower in muscle from obese animals, but restored to lean control values after chronic Rosiglitazone treatment (Figure 4.4B).
Figure 4. 3 Increased skeletal muscle AMP-activated protein kinase (AMPK)-α2 activity in obese rats after chronic Rosiglitazone (RSG) treatment.

Activity of AMPKα1 (A) and AMPKα2 (B) isoforms in muscle taken from lean (LN CON), obese (OB CON), and RSG-treated (OB RSG) rats. Activity was measured using the SAMS peptide assay in protein isolated by immunoprecipitation of muscle lysates. C: pACC/total ACC ratio from the skeletal muscle of LN CON, OB CON, and OB RSG rats. *P < 0.05 vs. LN CON; #P < 0.05 vs. OB CON (n=9/group).
Figure 4.4 Isoform-specific changes in AMP-activated protein kinase (AMPK) protein with obesity and Rosiglitazone (RSG) treatment.

Mean levels [arbitrary units (AU), n = 8] of AMPKα1 (A) and AMPKα2 (B) protein from LN CON, OB CON, and OB RSG rats. Proteins were isolated from skeletal muscle lysates by immunoprecipitation and quantified using Western blot analysis and densitometry. *P < 0.05 vs. LN CON; #P < 0.05 vs. OB CON (n=9/group).

Figure 4.5 displays the protein levels of UCP3 in skeletal muscle of lean and obese animals after Rosiglitazone treatment. UCP3 protein abundance was not different between lean and obese animals. However, Rosiglitazone treatment resulted in a significant increase in UCP3 protein levels (P<0.05).
4.4 Discussion

TZD’S are known to improve insulin sensitivity by activating PPAR-γ and inducing adipogenesis in adipose tissue (Day 1999) but also via PPAR-γ independent mechanisms. In the present study, Rosiglitazone increased AMPK activity leading to phosphorylation of ACC in vitro and in vivo.

Maximal activation of AMPK was observed in L6 myotubes when treated with 200 µM Rosiglitazone, due to an increase in the activity of the AMPKα1 isoform. Moreover, the increased AMPKα1 activity at the highest dose of Rosiglitazone was associated with a marked increase in the phosphorylation of ACC and a concomitant increase in palmitate oxidation. Recently, Cha et al. (Cha et al. 2005) reported cultured muscle cells from type 2 diabetic patients had lower rates of basal β-oxidation, but that this defect was normalized after
chronic (4 d) TZD treatment. Accordingly, an AMPK-stimulated increase in lipid oxidation may be one mechanism by which Rosiglitazone exerts its insulin-sensitising effect.

Only one previous study has examined the effect of Rosiglitazone on AMPK activity in skeletal muscle myotubes. Fryer et al. (2002) observed that treating muscle cells derived from heterozygous H-2Kb tsA58 transgenic mice with Rosiglitazone lead to increases in the AMP/ATP ratio and a concomitant increase in AMPK activity. In the present study an increase in AMPK activity in Rosiglitazone treated cells was also observed. However, no difference in the energy charge (AMP/ATP) of the cell was detected, suggesting that Rosiglitazone’s action was exerting an energy charge independent effect on AMPK. The reasons for the differences in results between the present and previous study (Fryer et al. 2002) in terms of AMP: ATP ratio, are not readily apparent. The H-2Kb cell line contains both AMPK -α1 and -α2 isoforms (Fryer et al. 2002) and we cannot rule out the possibility that this is a more sensitive cell line to treatments that affect energy charge. However, it should be noted that the H-2Kb line are immortalized cells derived from the simian virus 40 (SV40) large tumor (T) antigen (TAg) transgenic mouse (Jat et al. 1991) and may have some limitations with respect to their physiological significance. In order to be consistent in the present investigation, L6 myotubes derived from rat skeletal muscle were used so that the acute effects of Rosiglitazone might be compared with the chronic effects of this treatment regimen in obese Zucker rats.

Previous studies have suggested that altered skeletal muscle AMPK activity may not play a role in obesity and diabetes (Musi et al. 2001; Hojlund et al. 2004; Steinberg et al. 2004; Wojtaszewski et al. 2005). However, a recent
study (Chen et al. 2005) found that although basal AMPK activity was not altered, AMPK activation was impaired in cultured human skeletal muscle from obese type 2 diabetics. There is also evidence that basal AMPK phosphorylation is reduced in the cardiac muscle of both Zucker diabetic fatty rats and \( ob/ob \) mice (Wang and Unger 2005), and that this defect is normalized by TZD treatment. Further study is clearly needed in order to draw general conclusions on the role of skeletal muscle AMPK defects in obesity and diabetes.

To date, no studies have determined the effects of chronic Rosiglitazone treatment on the responses of AMPK in skeletal muscle or, more importantly, the effect of Rosiglitazone on skeletal muscle from insulin resistant animals. Our finding that chronic Rosiglitazone treatment increased both AMPK\( \alpha 2 \) and ACC phosphorylation in obese Zucker rats is of major clinical significance given the widespread use of TZDs as an anti-diabetic drug. The altered AMPK\( \alpha 2 \) activity with obesity and Rosiglitazone treatment corresponded with changes in the phosphorylation state of its target protein, ACC, which was decreased with obesity, but was restored to the level of lean controls after Rosiglitazone administration. Of interest was the reciprocal response of the AMPK protein levels to the chronic Rosiglitazone administration: AMPK\( \alpha 2 \) protein was reduced in obesity but restored to lean control values after Rosiglitazone treatment, while AMPK\( \alpha 1 \) protein abundance was suppressed with treatment. Consistent with our in vitro cellular data, the muscle nucleotide concentrations were unaltered by chronic Rosiglitazone treatment in vivo (Table 4.2).

Since hypoxia stimulates AMPK activity and a catecholamine response (Borovsky et al. 1998; Hallsten et al. 2002), the combination of euthanasia by \( CO_2 \) asphyxiation and the elapsed time between tissue collection and freezing
may have resulted in an altered intracellular environment that could contribute to changes in AMPK activity. These factors are potential limitations of the current study. However, we are confident that the observed AMPK activation in the present study was not due to hypoxia for the following reasons. First, muscle metabolites were within the expected ranges for all treatment groups and were similar to those obtained by other groups studying Obese Zucker rats (Klein et al. 1994; Ranganathan and Kern 1998). Second, the observed changes in AMPK activation were paralleled by changes in AMPK protein levels (Figure 4.4) and were not associated with changes in the T172 phosphorylation of AMPK. This suggests that the increase in activity was due to chronically altered protein levels, rather than acute changes in cellular energy status. Finally, the time taken to extract muscle was not different between groups and therefore, any differences in AMPK activity are indicative of the treatment.

The improved metabolic action as a consequence of up-regulating AMPKα2 by Rosiglitazone may be due to an increase in fat oxidation, since increased β-oxidation in muscle cells has been shown to enhance insulin-stimulated glucose metabolism and protect against insulin resistance in the face of elevated intramyocellular lipid content (Perdomo et al. 2004). Future studies should determine whether the observed increase in skeletal muscle AMPK activity with chronic Rosiglitazone treatment is associated with a concomitant increase in fat oxidation in vivo. However, it is also possible that Rosiglitazone-mediated up-regulation of AMPKα2 may improve metabolic action via alternative mechanisms. Treatment with either Rosiglitazone (Brunmair et al. 2004) or AICAR (5-aminimidazole-4-carboxamide-1-beta-d-ribonucleoside) (Stoppani et al. 2002; Suwa et al. 2003) increases uncoupling protein expression in skeletal muscle. In addition, UCP3 over-expressing transgenic
mice display enhanced glucose tolerance and palmitate oxidation (Wang et al. 2003), a phenotype compatible with chronic TZD treatment. In the present investigation we found that UCP3 protein expression was up-regulated by chronic Rosiglitazone treatment, suggesting that this protein may account for part of its metabolic action in skeletal muscle.

Previously Musi et al. (2002) reported that the antidiabetic drug metformin (biguanide) increased AMPK<sub>α2</sub> activity in type 2 diabetic patients following a 10 wk treatment regime. A significant increase in Thr172 phosphorylation was observed but not a significant increase in AMPK<sub>α2</sub> levels in the biopsy samples. The increase in AMPK<sub>α2</sub> activity seen in their study was paralleled by decreases in ACC activity consistent with AMPK mediated phosphorylation. Previously, it has been suggested that metformin and Rosiglitazone activate AMPK by different pathways (Fryer et al. 2002), with Rosiglitazone causing an increase in AMP levels. Irrespective of the mechanism of activation, both drugs (metformin, human type 2 diabetics; Rosiglitazone, obese Zucker rats) enhance AMPK signalling via the α2 isoform and inactivate ACC.

In conclusion, the results from the present study demonstrate that chronic Rosiglitazone administration stimulated isoform-specific regulation of the AMPK signalling cascade in skeletal muscle from obese Zucker rats and in myotubes in vitro. The increased basal activity of AMPK<sub>α2</sub> paralleled changes in the phosphorylation state of its target protein, ACCβ, and occurred without detectable changes in cellular energy charge. These changes in AMPK<sub>α2</sub> seem likely to be an important component of the insulin sensitizing effect of Rosiglitazone that contribute to the improved metabolic action associated with chronic TZD drug administration. Future studies should determine the
mechanisms underlying altered transcriptional regulation and/or turnover of AMPKα2 protein in skeletal muscle from obese Zucker rats, and how this is reversed by TZD treatment.
CHAPTER FIVE: Exercise, but not Rosiglitazone reverses lipid-induced impairments in muscle glucose and lipid metabolism: Roles for AMPK and Akt/AS160

5.1 Introduction

The clinical onset of insulin resistance typically involves a combination of aberrant glucose and lipid metabolism. In skeletal muscle, an impaired ability to oxidise fatty acids (FA) and the accumulation of lipid species such as ceramide and diacylglycerol is associated with impaired insulin-stimulated glucose uptake, and whole-body insulin resistance (Bruce and Hawley 2004). Acute increases in lipid availability result in reduced muscle glucose uptake, oxidation and glycogen synthesis (Randle 1998). While these impairments to glucose metabolism following acute FA exposure have been largely attributed to impaired insulin signalling (Schmitz-Peiffer 2000), the effects of persistently elevated FA availability (i.e. obesity or high-fat feeding) in skeletal muscle are not known. Due to the progressive nature of insulin resistance, it is likely that its clinical onset involves co-ordinated changes in gene transcription and protein expression, in addition to acute signalling events. Furthermore, given the direct functional link between lipid and glucose homeostasis, it is plausible that chronic adaptations induced by increased FA supply involve pathways that are involved in the regulation of both lipid and glucose metabolism.

Akt has recently been identified as a regulator of skeletal muscle lipid metabolism in addition to its well-established role in the insulin-signalling pathway (Bouzakri et al. 2006). AMP-activated protein kinase (AMPK) also plays a dual role in the regulation of glucose uptake and storage, as well as FA turnover and utilization in skeletal muscle (Musi and Goodyear 2002) and may be involved in the etiology (Winder and Hardie 1999) and treatment (Musi and Goodyear 2002) of type 2 diabetes. Thus, determining the effects of chronically elevated lipid availability on the Akt and AMPK signalling pathways may provide
insight into the impairments in glucose and lipid metabolism that underpin the insulin resistant state. Furthermore, examining the biochemical and cellular mechanisms by which common treatments for insulin-resistance reverse impairments in both glucose and lipid metabolism may elucidate their specific modes of action.

Both pharmacological intervention (i.e. thiazolidinediones [TZD]), and lifestyle modification (i.e. exercise training) are clinically effective treatments for improving whole-body insulin sensitivity (Inzucchi 2002; Hawley 2004; Pedersen and Saltin 2006). However the mechanism(s) by which these therapies reverse lipid-induced insulin resistance in skeletal muscle is unclear. It has been suggested that peroxisome proliferator-activated receptor agonists, such as Rosiglitazone, improve insulin sensitivity in skeletal muscle by preventing the toxic accumulation of lipids in this tissue (Hockings et al. 2003; Jucker et al. 2003). However, “lipid steal” directly from skeletal muscle following chronic Rosiglitazone treatment has not been consistently observed. It has been suggested in chapter four and by Fryer et al. (2002) that Rosiglitazone activates AMPK in skeletal muscle, thereby enhancing glucose uptake and FA oxidation. Exercise training also has the ability to enhance AMPK signalling, glucose uptake and FA oxidation in muscle (Bruce and Hawley 2004), raising the possibility that combined treatment may produce additive effects in this tissue. Indeed, Hevener et al. (2000) demonstrated that the whole-body insulin-sensitising effects of the PPARγ agonist, Troglitazone and exercise training were additive in obese Zucker rats (Hevener et al. 2000). However, it is unclear whether these effects were attributable to improvements in skeletal muscle insulin sensitivity. Furthermore, it is not known whether the combined insulin-
sensitising effects of these treatments are associated with improvements in muscle lipid metabolism.

Accordingly, we used a model of lipid-induced insulin resistance, the high-fat fed rat, to examine the independent and interactive effects of Rosiglitazone treatment and exercise training on insulin-stimulated glucose transport, FA oxidation and lipid accumulation in skeletal muscle. In addition, the potential involvement of Akt and AMPK in these adaptations was examined because of their dual roles in lipid and glucose metabolism. It was hypothesised that a combination of exercise training and Rosiglitazone treatment would result in additive improvements in skeletal muscle insulin sensitivity and lipid metabolism compared to either treatment alone.

5.2 Materials and Methods

Animals

Male Sprague-Dawley rats (~4 wk of age) were given ad libitum access to a high-fat diet (HF; 58% kcal fat; Research Diets Inc., New Brunswick, NJ) and water for 4-wk to induce insulin-resistance. During the subsequent 4-wk experimental period, rats continued to eat a high-fat diet and were randomly allocated to one of the following groups (n=16/group): control (HF CON), exercise training (HF EX), Rosiglitazone treatment (HF RSG), or a combination of both exercise training and Rosiglitazone treatment (HF EX RSG). Exercise training consisted of treadmill running for 1 h/day, 5 day/wk on a 15% incline, at a speed that was gradually increased during the first week of training to 32 m/min. Rosiglitazone-treated rats received a diet containing 50 ppm Rosiglitazone (GlaxoSmith Kline, Stevenage, UK), which they consumed ad libitum at an average dose of 2.08 ± 0.06 mg/kg/day. A fifth group of rats (CF
CON; n=16) was fed a normal chow diet (17% kcal fat; Research Diets Inc.) for the duration of the study (8 wk), and acted as a control for HF CON. Following the experimental period, animals were fasted for 8-12 h prior to undergoing hindlimb perfusion for the measurement of insulin-stimulated 3-O-methylglucose transport (n=8/ treatment group), or \(^{14}\)C-palmitate uptake and oxidation (n=8/treatment group). EX animals underwent their last training bout 36-48 h prior to hindlimb perfusion.

**Hind limb perfusions**

Rats were anaesthetized with an intraperitoneal injection of sodium pentobarbital (6.5 mg/100 g body mass) and surgically prepared for hind limb perfusion as previously described (Herr et al. 2005). Briefly, cannulas were inserted into the abdominal aorta and vena cava, and the animals were killed via an intracardiac injection of pentobarbital as the hind limbs were washed out with 30 mL of Krebs-Henseleit buffer (KHB, pH 7.55; Sigma-Aldrich, Inc., MO, USA). The basic perfusate medium contained 30% washed time-expired human erythrocytes (Ogden Medical Center, Ogden, UT, USA) and was continuously gassed with a mixture of 95% \(\text{O}_2\) - 5% \(\text{CO}_2\) and warmed to 37°C. Perfusate flow rate was set at 7.5 mL/min during the stabilisation period and subsequent perfusion, during which rates of glucose transport or palmitate uptake were determined.

**Insulin-stimulated 3-O-methylglucose transport**

The cannulas were placed in line with a non-recirculating perfusion system and the hind limbs were allowed to stabilise during a 5 min washout period. Perfusions were performed in the presence of 500 µU/mL insulin (Eli Lilly Pty Ltd, IN, USA). The hind limbs were washed out with perfusate containing 1 mM glucose (Sigma-Aldrich, Inc., MO, USA) for 5 min in
preparation for the measurement of glucose transport. Glucose transport was measured over an 8 min period using a perfusate that contained 8 mM of the non-metabolisable glucose analogue 3-O-methylglucose (3-MG; 32 µCi 3-[3H0MG/mM, PerkinElmer Life Sciences, Boston, MA, USA) and 2 mM mannitol (60 µCi-[1-14C] mannitol/ mM, PerkinElmer Life Sciences) as an intracellular space marker. Immediately following the transport period, portions of the red quadriceps (RQ) 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 insulin stimulated skeletal muscle 3-MG transport were calculated as previously described (Yaspelkis et al. 2004).

14C-Palmitate uptake and oxidation rates

Rats were fasted for 8-12 h prior to being anaesthetized and having their right hind limb surgically prepared for perfusion. Prior to cannulation, the left iliac vessels were tied off and portions of the red quadriceps muscle were immediately excised from the left hind limb, freeze clamped in liquid N2 and stored for later analysis. This unperfused tissue was used for the analysis of AMP-activated protein kinase activity and phosphorylation. Skeletal muscle FA metabolism was assessed as previously described (Todd et al. 2005) by perfusing hind limbs (7.5 mL/min) with a recirculated perfusate containing 4% FA-free bovine serum albumin (Sigma-Aldrich, Inc., MO, USA), 500 µM albumin-bound palmitate (Sigma-Aldrich, Inc., MO, USA), and 5 µCi of albumin-bound [1-14C]palmitate (PerkinElmer) for 60 min (20 min equilibration period followed by a 40 min experimental period). Arterial and venous perfusate samples for the analysis of 14C palmitate were taken at 10, 20, 30 and 40 min of the experimental period. Arterial and venous samples for the analysis of 14CO2 were taken immediately following the equilibration period, and at 40 min.
Following the 40 min experimental perfusion period, muscle was excised and stored as described above. To determine plasma palmitate radioactivity, duplicate 100 µL aliquots of the perfusate plasma were mixed with liquid scintillation fluid and counted in a liquid scintillation analyser. The liberation and collection of $^{14}$CO$_2$ from perfusate samples was performed by injecting 2 mL of anaerobically collected perfusate into a sealed, 10 mL flask containing an equal volume of 1 M acetic acid (Sigma-Aldrich, Inc., MO, USA). The released $^{14}$CO$_2$ was trapped by an insert containing a strip of filter paper saturated with 500 µL of benzothonium hydroxide (Sigma-Aldrich, Inc., MO, USA). The flask was kept sealed overnight to allow complete absorption of the released CO$_2$ by benzothonium hydroxide, after which the insert was removed and placed into a scintillation vial where it was mixed with liquid scintillation fluid and counted in a liquid scintillation analyser.

**AMPK activity and AMPK subunit protein expression**

AMPK activity, pT172 phosphorylation and total protein content were analysed as previously described in chapter four. Briefly, ~5 mg of protein from RQ muscle lysates were incubated with AMPK$\alpha$1 or AMPK$\alpha$2 antibody-bound protein A sepharose beads (6MB, Amersham Biosciences, Uppsala, Sweden) for 2 h at 4°C. The AMPK activities in the immune complexes were measured in the presence of 200 mM AMP. Activities were calculated as picomoles of phosphate incorporated into the SAMS peptide per min, per milligram of protein subjected to immunoprecipitation (pmol/min/mg). Acetyl CoA carboxylase (ACC) was affinity purified by incubating the post-AMPK immunoprecipitation supernatants in Streptavidin Sepharose High Performance beads (Amersham Biosciences, Uppsala, Sweden) for 1 h at 4°C. Aliquots of the immunoprecipitated (AMPK), and affinity purified (ACC) proteins were
electrophoresed using 10%, or 7.5% SDS-PAGE, respectively and transferred to PVDF membranes. Site-specific phosphorylation was then detected by immunoblotting with anti-phospho-Thr172 (AMPK) or anti-phospho-Ser218 (ACC) antibodies. The membranes were stripped (Restore Western Blot Stripping Buffer, Pierce Biotechnology, Inc.), and total AMPK protein contents were determined using antibodies specific for the AMPK $\alpha_1$, and $\alpha_2$ isoforms. Total ACC protein was determined using a Streptavidin-HRP conjugate (Invitrogen Australia Pty. Ltd.).

**Western Blotting**

RQ muscle lysates (60 µg) were solubilized in Laemmli buffer, separated by SDS-PAGE, and transferred to PVDF membranes (BioRad Laboratories, CA, USA). The membranes were blocked, and incubated overnight at 4°C with antibodies specific for either add phospho-Akt ser473, Akt, Akt1, Akt2 (1:1000; Cell Signaling # 4058, 9272, 2967, 2962), AS160 (a gift from David James, Garvan Institute, Sydney), PGC-1 (Chemicon International, Inc. AB3242), or GLUT4 (Biogenes, UK 4670-1704). The immunoreactive proteins were detected with enhanced chemiluminescence (Amersham Biosciences) and quantified by densitometry. FAT/CD36 and FABPpm content were determined as previously described (Bonen et al. 1998).

**Analysis of intramuscular muscle lipids**

Skeletal muscle triacylglycerol (TAG), diacylglycerol (DAG) and ceramide contents were analysed as previously described in chapter three. Briefly, freeze-dried muscle was powdered, and cleaned of all visible connective tissue and blood under magnification. Skeletal muscle TAG (glycerol) content was determined fluorometrically, following Folch lipid extraction and saponification of
a portion of dry, powdered tissue. DAG and ceramide content were quantified using the DAG Kinase method on a separate aliquot of tissue.

**Blood Metabolite Measurements**

Blood was analysed for fasting concentrations of glucose, insulin, adiponectin and free fatty acids (FFA). Serum was obtained by centrifugation of whole blood collected at the time of euthanasia, and stored at –80°C until analysis. Serum glucose was determined using the MediSense2 Blood Glucose Testing system (MediSense Australia Pty., Ltd., Melbourne). Serum insulin and adiponectin levels were determined using enzyme immunoassay kits (Sensitive Rat Insulin RIA kit, Linco Research; Mouse/Rat Adiponectin ELISA kit, Otsuka Pharmaceutical Co., Ltd.). Serum FFA were determined using an enzymatic colorimetric method (NEFA C, Wako Chemicals, USA, Inc.).

### 5.2.1 Statistical Analysis

Results are presented as mean ± SEM. Differences arising from dietary interventions were determined using an unpaired t-test (CF CON vs. HF CON). Differences between HF treatment groups were determined using a one-way analysis of variance with a Student-Newman-Keuls post-hoc test (SigmaStat version 3.1.1.).

### 5.3 Results

Body mass was increased by high-fat feeding and RSG treatment, but was decreased by EX (Table 1, P<0.05). Changes in body mass were mirrored by changes in epididymal fat pat mass (Table 5.1). Serum adiponectin concentration was elevated in HF RSG (P=0.03 vs. HF CON), and decreased in HF EX (P=0.04 vs. HF CON). Serum free fatty acid (FFA) concentration was also decreased in both HF EX and HF EX RSG (P<0.02 vs. HF CON).
Table 5.1 Effect of chronic exercise, and/or RSG treatment on body mass, fat pad mass, and fasting serum composition in fat-fed rats.

<table>
<thead>
<tr>
<th></th>
<th>CF CON</th>
<th>HF CON</th>
<th>HF EX</th>
<th>HF RSG</th>
<th>HF EX RSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass (g)</td>
<td>434±6</td>
<td>464±8 *</td>
<td>417±5 †</td>
<td>470±8 ‡</td>
<td>404±7 §†</td>
</tr>
<tr>
<td>Epididymal fat pad mass (g)</td>
<td>6.2±0.3</td>
<td>10.7±0.6 *</td>
<td>6.8±0.4 †</td>
<td>10.3±0.5 ‡</td>
<td>6.6±0.3 §†</td>
</tr>
<tr>
<td>Serum:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td>14.9±1.2</td>
<td>13.9±0.9</td>
<td>13.7±1.0</td>
<td>13.35±1.3</td>
<td>10.0±1.1 †</td>
</tr>
<tr>
<td>Insulin (ng/mL)</td>
<td>1.23±0.25</td>
<td>1.21±0.24</td>
<td>0.84±0.16</td>
<td>1.25±0.21</td>
<td>0.88±0.20</td>
</tr>
<tr>
<td>Adiponectin (µg/mL)</td>
<td>2.09±0.30</td>
<td>3.46±0.80</td>
<td>1.58±0.26</td>
<td>5.83±0.65 ††</td>
<td>3.70±0.55 §</td>
</tr>
<tr>
<td>FFA (mM)</td>
<td>0.89±0.16</td>
<td>0.60±0.10</td>
<td>0.33±0.05 †</td>
<td>0.50±0.05</td>
<td>0.29±0.04 †</td>
</tr>
</tbody>
</table>

*P<0.05 vs. LN CON, †P<0.05 vs. HF CON, ‡P<0.05 vs. HF EX, §P<0.05 vs. HF RSG (n=8/group).

Glucose metabolism

Insulin-stimulated glucose transport in the red quadriceps muscle of HF controls (HF CON) was reduced by ~37% (P=0.005; Fig 5.1A) compared to chow-fed controls (CF CON) and was impaired a further ~32% (P=0.05) in HF RSG. Exercise training restored insulin-stimulated glucose transport in fat-fed and RSG-treated animals (P<0.05). Basal muscle glycogen content (Fig 5.3A) was increased in HF EX and HF EX RSG (P<0.05, vs. HF CON), and decreased with HF RSG (P<0.05, vs. HF CON).
Figure 5. 1  $^3$H-3-O-methylglucose and $^{14}$C-palmitate hindlimb perfusions. Muscle glucose transport (A) was measured in the red quadriceps muscle following hindlimb perfusion with $^3$H-3-O-methylglucose in the presence of insulin (500 µU/mL). Average $^{14}$C-palmitate uptake (B) and $^{14}$CO$_2$ production due to $^{14}$C-palmitate oxidation (C) were calculated using differences in arterial and venous perfusate concentrations during a 40 min hindlimb perfusion with 500 µM palmitate. Significant differences between groups (P<0.05; n=8/group) are indicated by the P values listed on the figure.
AMPK activity

AMPKα1 activity was unchanged by high-fat feeding, but increased ~30% in HF EX (P=0.01 vs. HF CON) and ~37% in HF EX RSG (P=0.006 vs. HF CON) [Fig 5.2A]. AMPK α2 activity was increased ~36% by high-fat feeding (P=0.03, HF CON vs. CF CON), but was not altered by EX or RSG (Fig 5.2B). Changes in both AMPK -α1, and -α2 activities were associated with similar changes in the total protein levels of these isoforms (P<0.05, Fig 5.2C). Phosphorylation of AMPK α1 at T172 was similar between HF CON and HF RSG, but tended to increase in HF EX, and HF EX RSG (P=0.1 vs. HF CON). HF-induced increases in activity and protein levels in the AMPKα2 isoform were associated with increased phosphorylation of the enzyme at T172 (CF CON vs. HF CON, P=0.01). We also examined the protein levels and phosphorylation status of the AMPK substrate, acetyl Co-A carboxylase (ACC). Although no changes in the total protein level of ACC were observed (Fig 5.2C) there was a significant increase in the level of phosphorylation of the enzyme at S218 with high-fat feeding (CF CON vs. HF CON, P=0.04).
Figure 5.2 Skeletal muscle AMP-activated protein kinase (AMPK) activity and protein expression.

AMPKα1 (A) and AMPKα2 (B) activities (pmol/min/mg) were measured using the SAMS peptide assay in protein isolated by immunoprecipitation of muscle lysates. Relative levels of isoform-specific AMPK T172 phosphorylation and total protein, as well as acetyl CoA carboxylase (ACC) S218 phosphorylation and total protein were quantified using Western blot analysis and densitometry (C). Significant differences between groups (P<0.05; n=8/group) are indicated by the P values listed on the figure.
Lipid Metabolism:

Muscle triacylglycerol (TAG; Fig 5.3B) and diacylglycerol (DAG; Fig 5.3D) content increased 20% and 13%, respectively in HF RSG (P<0.05 vs. HF CON), but were normalized by EX (HF RSG vs. HF EX RSG; P<0.05). Muscle ceramide (CER) content increased ~23% with high-fat feeding (CF CON vs. HF CON, P=0.01), but was unaffected by either EX or RSG treatment. Increased muscle lipid storage was associated with increased $^{14}$C-palmitate uptake in the perfused hindlimb (Fig 5.1B). Palmitate uptake was ~40% higher in HF CON compared with CF CON (P=0.04), and increased a further 40% in HF RSG (P=0.03). RSG-induced increases in palmitate uptake were associated with a ~50% increases in skeletal muscle FAT/CD36 protein content in both HF RSG and HF EX RSG (P<0.05 vs. HF CON; Fig 5.4A). No changes in the protein content of FABPpm were observed (Fig 5.4B), although there was a tendency for RSG treatment to reduce this putative FA transporter. Although $^{14}$C-palmitate uptake was not increased in HF EX (Fig 5.1B), a 2-fold increase in $^{14}$CO$_2$ production was observed (P=0.03 vs. HF CON; Fig 5.1C), suggesting that increased lipid oxidation may be a mechanism responsible for normalised lipid levels in HF EX RSG. Increased lipid oxidation with exercise training was associated with ~30% increase in the expression of PGC-1 (Fig 5.4C) in both HF EX (P=0.03 vs. HF CON) and HF EX RSG (P=0.07 vs. HF CON). In the liver, TAG was increased 36% by high-fat feeding (P=0.03), but was normalized in all three treatment groups (HF EX, HF RSG, HF EX RSG, P<0.001 vs. HF CON; Fig 5.3F).
Figure 5.3  Skeletal muscle carbohydrate and lipid storage.
Muscle glycogen (A), triacylglycerol (B), ceramide (C) and diacylglycerol (D) content were quantified in separate aliquots of red quadriceps (RQ) muscle. In order to determine whether muscle samples were contaminated with significant amounts of intermyocellular adipocytes, protein aliquots from both adipose tissue (15 µg), and muscle (80 µg) were separated by SDS-PAGE and probed for the adipose-specific proteins Perilipin A/B (E). Liver triacylglycerol was also measured (F). Significant differences between groups (P<0.05; n=8/group) are indicated by the P values listed on the figure.
Figure 5.4  Skeletal muscle lipid transporters and PGC-1 content.
Relative protein levels of FAT/CD36 (A), FABPpm (B) and PGC-1 (C) were quantified using Western blot analysis and densitometry. Significant differences between groups (P<0.05; n=8/group) are indicated by the P values listed on the figure.
Akt/AS160

Compared to chow-fed controls (CF CON), total Akt protein was reduced ~25% by high-fat feeding (HF CON; P=0.01) and was restored by EX (HF CON vs. HF EX; P=0.007; Fig 5.5A). The increase in total Akt was entirely attributable to an increase in the Akt1 isoform (Fig 5.5C), which was elevated ~50% by exercise training (HF CON vs. HF EX; P=0.04). RSG did not change Akt1 protein content, and appeared to blunt the EX-induced increase in Akt1 (HF EX vs. HF EX RSG; P=0.04). No changes in the Akt2 isoform (Fig 5.5D) were observed after either treatment. Phosphorylation of total Akt at the S473 residue (Fig 5.5B) was also altered by EX, with a 50% decrease in HF EX (P=0.01 vs. HF CON). AS160 protein was elevated ~15% in HF (CF CON vs. HF CON; P=0.004) and was normalized in both HF EX (P=0.04) and HF EX RSG (P=0.03; Fig 5.6A). GLUT4 protein content was decreased ~20% in HF CON (P=0.007), but was increased ~50% and ~40% in HF EX and HF EX RSG, respectively (P<0.001; Fig 5.6B)
Figure 5.5 Isoform-specific skeletal muscle Akt content.
Relative protein levels of total Akt 1/2 (A), Akt phosphorylation at S473 (B), as well as the isoform-specific content of Akt 1 (C), and Akt 2 (D) were quantified using Western blot analysis and densitometry. Significant differences between groups (P<0.05; n=8/group) are indicated by the P values listed on the figure.
5.4 Discussion

It is well-established that chronic Rosiglitazone treatment improves whole-body insulin sensitivity (Malinowski and Bolesta 2000). Less clear, however, is whether this enhanced insulin action is directly attributable to the effects of Rosiglitazone on skeletal muscle, or other insulin-sensitive tissues. In the present study, we observed that exercise training, but not Rosiglitazone
treatment reversed skeletal muscle insulin-resistance associated with a high-fat diet. Contrary to our hypothesis, Rosiglitazone treatment decreased insulin-stimulated glucose transport (P=0.05, Fig 5.1A) in the perfused hindlimb compared to HF CON. Hindlimb perfusion allows the calculation of insulin-stimulated glucose transport in specific skeletal muscle groups while minimizing interference from systemic factors or other insulin sensitive tissues (i.e. hepatic glucose output).

Few other investigations have determined the effect of chronic Rosiglitazone treatment directly on skeletal muscle glucose transport. Shen et al. (2004) recently reported that 2 wk of Rosiglitazone treatment increased insulin-stimulated glucose transport in the isolated EDL muscle of db/db mice (Shen et al. 2004). Unlike humans with insulin-resistance (Wojtaszewski et al. 2005), genetically-induced leptin receptor-deficient and leptin-deficient animal models of insulin resistance (i.e. fa/fa Zucker, ob/ob mouse) have well-documented impairments in basal skeletal muscle AMPK activity (Buhl et al. 2002; Yu et al. 2004; Sriwijitkamol et al. 2006). Impaired AMPK activity and insulin sensitivity in these models can be reversed by AICAR administration (Buhl et al. 2002; Yu et al. 2004), or Rosiglitazone treatment (as described in chapter four). The absence of basal AMPK deficiency and Rosiglitazone-induced changes in AMPK activity in the present study using a high-fat fed rats provides a likely mechanism for the absence of a Rosiglitazone-induced improvement in muscle insulin sensitivity. While the use of a leptin receptor-deficient model of insulin resistance (db/db mouse) by Shen et al. (2004) may provide some explanation for these inconsistent results, it should also be noted that the doses of Rosiglitazone (30 mg/kg) and insulin (10\(^4\) µU/mL) used in that
study were 15- and 20-fold higher, respectively, than those employed in the present study.

Studies examining the effect of Rosiglitazone on insulin-signalling in skeletal muscle have also produced variable results. Miyazaki et al. (2003) observed that chronic Rosiglitazone treatment (8 mg/day) for 16 wk resulted in increased skeletal muscle insulin-stimulated IRS-1 tyrosine phosphorylation and p85 association in patients with type 2 diabetes (Miyazaki et al. 2003). In contrast, others have observed no enhancement in the IRS-1/PI 3-kinase/Akt/AS160 signalling pathway after 26-wk of Rosiglitazone treatment (8 mg/day) in the skeletal muscle of patients with newly-diagnosed type 2 diabetes (Karlsson et al. 2005a). In the present study we saw no change in the total protein levels of Akt (Fig 5.5), AS160 or GLUT4 (Fig 5.6) following 4 wk of Rosiglitazone treatment. In contrast, exercise training normalized impairments to the Akt/AS160/GLUT4 signalling pathway caused by high-fat feeding. It has recently been observed that contraction (Bruss et al. 2005; Kramer et al. 2006), or a singlebout of endurance exercise (Deshmukh et al. 2006) can acutely increase AS160 phosphorylation in muscle. To our knowledge, the present investigation is the first to demonstrate changes in AS160 expression due to chronic high-fat feeding and exercise training. Our results suggest that high-fat feeding may impair insulin-stimulated glucose uptake by downregulating distal components of the insulin-receptor signalling cascade that lead to GLUT4 translocation and that these impairments are reversed by exercise training, but not Rosiglitazone treatment. In contrast, Hevener et al. (2000) observed additive effects of exercise training and Troglitazone treatment on GLUT4 content in the skeletal muscle of obese Zucker rats. Again, this apparent
discrepancy is likely attributable to the use of a leptin-receptor deficient model in Hevener et al. (2000) and a fat-fed model in the present investigation.

Although exercise training upregulated insulin-stimulated glucose transport as well as the expression of key components of the signalling pathway leading to GLUT4 translocation, a decrease in Akt phosphorylation at Ser473 was also observed in response to this intervention (Fig 5.5B). An increase in muscle insulin-stimulated glucose transport in the face of decreased Akt phosphorylation appears paradoxical, yet discrepancies between Akt activation and upstream insulin signalling (i.e. PI-3 Kinase activation) have previously been reported in several different models of insulin resistance (Kurowski et al. 1999; Song et al. 1999; Kim et al. 2000). Considering the complex regulation of Akt and its involvement in a range of signalling pathways (Taniguchi et al. 2006) it is likely that its phosphorylation state reflects the additive input of a number pathways.

In addition to exercise-induced changes in Ser473 phosphorylation, we observed increased expression of the Akt1 isoform after exercise training. It has been proposed that Akt2 plays the predominant role in insulin-stimulated glucose uptake (Bae et al. 2003; Brozinick et al. 2003), while Akt1 is involved in the regulation of protein synthesis and muscle hypertrophy (Nader 2005). However, Akt1 has recently been implicated in the regulation of FA uptake in skeletal muscle using isoform-specific siRNA based gene silencing (Bouzakri et al. 2006). More investigation into the specific function of Akt1 in exercise training adaptations in skeletal muscle is needed.

The effect of exercise training and Rosiglitazone treatment on AMPK signalling was also examined in the skeletal muscle of high fat-fed rats. High fat feeding resulted in a ~36% increase in AMPKα2 activity (Fig 5.2B), which was
not further augmented by Rosiglitazone treatment. This *in vivo* finding of increased AMPK activity with high-fat feeding is in agreement with previous work demonstrating that acutely increasing FA availability stimulates AMPK activity, ACC phosphorylation and palmitate oxidation in L6 myotubes (Fediuc et al. 2006; Watt et al. 2006). Furthermore, it was also observed that the FA-induced increase in AMPK activity was attributable to activation of the α2 isoform of AMPK which was associated with increased ACC phosphorylation, but not increased glucose transport. In contrast, AMPKα1 activity was not affected by diet, but was increased by exercise training, which was associated with improved insulin-stimulated glucose transport and glycogen storage. These observations are consistent with others reporting upregulation of AMPKα1 following exercise training in humans with type 2 diabetes (Wojtaszewski et al. 2005) and animal models of insulin resistance (Sriwijitkamol et al. 2006). The results of the present study point to the AMPK α subunit isoforms having distinct roles, with AMPKα2 activity being responsive to increased FA availability, and AMPKα1 activity being linked to exercise training-induced changes in glycogen storage and insulin-sensitivity.

The effect of Rosiglitazone treatment on skeletal muscle lipid storage is equivocal, as previous investigations have reported a decrease (Hockings et al. 2003; Jucker et al. 2003; Ye et al. 2003), an increase (Muurling et al. 2003; Todd et al. 2006), or no effect (Oakes et al. 1994; Oakes et al. 1997b; Mayerson et al. 2002; Coort et al. 2005) on muscle TAG content. In the present study, 4 wk rosiglitazone treatment resulted in a 20% increase in muscle TAG content (Fig 5.3B). We attribute this increase in lipid storage to an increase in lipid uptake (Fig 5.1B) resulting from a Rosiglitazone-induced upregulation of FAT/CD36 (Fig 5.4A). These findings are consistent with those of Wilmsen et
al. (2003), who found that 4-day treatment of skeletal muscle cells taken from type 2 diabetic subjects resulted in a 2.5-fold increase in FAT/CD36 (Wilmsen et al. 2003). Increased palmitate uptake without a concomitant increase in FA oxidation would be expected to increase the storage of lipids in skeletal muscle. Although a Rosiglitazone-induced increase in $^{14}$CO$_2$ production during hindlimb perfusion with $^{14}$C-palmitate was observed, this was likely due to increased uptake, rather than elevated oxidative capacity. In contrast, exercise training increased palmitate oxidation without increasing palmitate uptake, suggesting that the capacity to oxidize FA was upregulated in these animals. Increased palmitate oxidation was associated with elevated PGC-1 expression in both HF EX and HF EX RSG (Fig 5.4C). Increased capacity to oxidise FA provides a potential mechanism by which exercise prevents the increase in muscle lipid storage observed in animals treated with Rosiglitazone alone.

It has been suggested that Rosiglitazone acts to improve insulin sensitivity by increasing the uptake of FA into adipocytes, thereby reducing the availability of lipids for accumulation in other insulin-sensitive tissues, such as skeletal muscle (Hockings et al. 2003; Jucker et al. 2003). However, it has been previously described in chapter three of this thesis and by others (Muurling et al. 2003) that Rosiglitazone improves whole-body insulin sensitivity despite exacerbating the accumulation of lipids in skeletal muscle. The results of the present study corroborate these findings, and provide direct mechanistic evidence that chronic PPAR$_\gamma$ activation may promote lipid storage in muscle, as it does in adipose tissue, by upregulating lipid transport. Exercise training in combination with Rosiglitazone treatment prevented the Rosiglitazone-induced accumulation of lipids and decrease in insulin sensitivity despite increased lipid uptake and CD36 expression in this treatment group. Thus, it appears that
exercise training imparts a “metabolic flexibility” (Kelley 2002) allowing skeletal muscle to deal with increased lipid availability without compromising glucose metabolism.

Hevener et al. (2000) have previously demonstrated that combined TZD (Troglitazone) treatment and exercise training have additive beneficial effects on whole-body insulin sensitivity. Rosiglitazone did not improve skeletal muscle insulin sensitivity in the present study. However, the observation that lipid accumulation was reduced in the liver of Rosiglitazone-treated animals raises the possibility that this organ acts as a potential target for its insulin-sensitising actions. In support of this contention, Gastaldelli et al. (2006), have recently reported that Rosiglitazone treatment (8 mg/day for 12 weeks) decreased gluconeogenesis in patients with type 2 diabetes (Gastaldelli et al. 2006). Exercise-induced improvements in skeletal muscle insulin sensitivity, combined with the potential for Rosiglitazone to improve hepatic insulin action, provides a potential mechanism for the additive improvements in whole-body insulin action (following combined TZD treatment and exercise training) observed by Hevener et al (2000). Further investigation into the effects of Rosiglitazone treatment on lipid and glucose metabolism the liver, and other insulin-sensitive tissues are needed.

In conclusion, evidence has been provided that exercise training, but not Rosiglitazone treatment reverses skeletal muscle insulin resistance induced by lipid oversupply. Rosiglitazone is a potent whole-body insulin sensitiser, and exercise training reverses Rosiglitazone-induced impairments to skeletal muscle glucose transport. Therefore, the combined prescription of Rosiglitazone and exercise training is likely to be a more effective therapeutic option for individuals with insulin resistance than either treatment alone.
CHAPTER SIX: General Summary and Discussion
The primary aim of the studies undertaken for this thesis was to enhance our understanding of the mechanisms by which two common therapies for the treatment of insulin resistance, Rosiglitazone and exercise training, reverse aberrant lipid handling and improve insulin sensitivity in skeletal muscle. Several aspects of skeletal muscle lipid metabolism were examined with respect to their potential to improve glucose homeostasis in two distinct animal models of insulin resistance. The ability of Rosiglitazone and exercise training to alter the regulation of fatty acid transport, storage and utilisation was determined in three independent, but related investigations.

The first two studies (chapters three and four) used a genetically-induced model of insulin resistance, the obese Zucker rat, to determine the means by which Rosiglitazone alters skeletal muscle insulin sensitivity. Although “lipid steal” from skeletal muscle has been described as a potential mechanism by which PPARγ agonists improve insulin sensitivity, several studies have unveiled a disassociation between IMTG content and insulin sensitivity following Rosiglitazone treatment. This raises the possibility that rather than decreasing IMTG per se, Rosiglitazone may act to improve insulin sensitivity by reducing the accumulation of lipid intermediates that have a more direct mechanistic link to impairments in skeletal muscle insulin signal transduction, such as ceramide and diacylglycerol.

Contrary to this hypothesis, Rosiglitazone treatment increased the levels of ceramide and diacylglycerol in skeletal muscle despite improving whole-body insulin sensitivity. In addition, Rosiglitazone treatment decreased the activity of hormone-sensitive lipase and altered the composition of the fatty acid pool in skeletal muscle. These results were the first to suggest that Rosiglitazone may be a regulator of lipid metabolism in skeletal muscle, and that its lipid-altering
actions in this tissue may not simply be secondary to a reduction in plasma lipid availability. The observation that Rosiglitazone treatment improved glucose tolerance in the face of increased skeletal muscle lipid metabolites (TAG, DAG, ceramide) led to the conclusion that reduced skeletal muscle lipid accumulation is not the mechanism by which Rosiglitazone improves insulin sensitivity.

In light of this finding, it seemed likely that Rosiglitazone treatment improved skeletal muscle insulin sensitivity by an alternative and possibly, PPARγ-independent, mechanism. In this regard, it had been previously observed that Rosiglitazone could stimulate AMPK activity in vitro. However, the effects of chronic Rosiglitazone treatment on skeletal muscle AMPK in vivo were not known. Given the potential for AMPK to improve both glucose and lipid metabolism in skeletal muscle, AMPK activation is one mechanism by which Rosiglitazone could improve insulin sensitivity independent of skeletal muscle lipid accumulation. Accordingly, the study described in chapter four determined whether Rosiglitazone could induce chronic increases in AMPK activity in the skeletal muscle of obese Zucker rats.

The results of this study provided the first evidence that chronic Rosiglitazone treatment can increase skeletal muscle AMPK activity in vivo. The skeletal muscle of obese Zucker rats displayed decreased activity and expression of the AMPKα2 isoform, both of which were normalised to the level of lean controls following chronic Rosiglitazone treatment. ACC phosphorylation paralleled the changes in AMPKα2 activity, while no changes to the AMPKα1 isoform were observed. These results suggest that there may be an isoform-specific role for AMPKα2 in the regulation of skeletal muscle lipid metabolism.
The capacity to oxidise fatty acids is a better predictor of insulin sensitivity than intramuscular lipid accumulation. Therefore, it was hypothesised that Rosiglitazone-induced increases in AMPK activity may improve skeletal muscle insulin-sensitivity via AMPK-induced increases in lipid oxidation. Stimulation of skeletal muscle AMPK activity and increased capacity to oxidize fatty acids is a response typically observed after exercise training. In this regard, it is possible that Rosiglitazone treatment may exert an “exercise-like” effect in skeletal muscle. In order to test this hypothesis, the study described in chapter five determined the independent and interactive effects of Rosiglitazone treatment and exercise training on AMPK activation and a variety of direct and indirect measures of fatty acid oxidation. It was hypothesised that exercise training combined with Rosiglitazone treatment would result in additive improvements in skeletal muscle insulin sensitivity, AMPK activity and fatty acid oxidation compared to either treatment alone. These experiments were performed in a less severe model of insulin resistance (the high-fat fed rat), and in situ measurements of insulin stimulated glucose transport and fatty acid oxidation were undertaken using hindlimb perfusion, allowing for muscle-specific effects to be determined.

Insulin-stimulated glucose uptake was reduced by high-fat feeding, but insulin sensitivity was restored by exercise training. In contrast, Rosiglitazone treatment tended to exacerbate the insulin resistance associated with high-fat feeding, resulting in even lower insulin-stimulated glucose uptake. In hindlimb muscle perfused with palmitate rather than glucose the inverse relationship was observed: palmitate uptake was elevated by high-fat feeding and increased even further with Rosiglitazone treatment. Increased palmitate uptake in this tissue corresponded with increased palmitate oxidation in Rosiglitazone-treated
animals, whereas exercise trained animals displayed increased oxidation despite having levels of palmitate uptake similar to chow-fed control animals. Rosiglitazone-induced increases in palmitate uptake were associated with skeletal muscle lipid storage and increased expression of the putative FA transporter, CD36. The results from this study provide direct mechanistic evidence to show that chronic PPARγ activation may promote lipid storage in muscle, as it does in adipose tissue, by upregulating lipid transport.

The results from this experiment also provided new evidence for the distinct roles of the AMPK alpha subunit isoforms in glucose and lipid metabolism in skeletal muscle. AMPKα1 activity was increased by exercise training and was associated with increased insulin-stimulated glucose uptake, glycogen content and PGC-1 expression. In contrast, AMPKα2 activity was elevated by high-fat feeding and was associated with increased ACC phosphorylation. Importantly, no further increase in AMPKα2 activity was observed in response to Rosiglitazone treatment suggesting that this effect was specific to the leptin-receptor and AMPK deficient Zucker rat.

This study also provided novel information with respect to a potential mechanism by which glucose metabolism is downregulated by chronically elevated fatty acid availability. High-fat feeding increased skeletal muscle AS160 expression in conjunction with decreased GLUT4 expression, which would be expected to reduce insulin-stimulated glucose transport. Importantly, exercise training was shown to reverse these impairments, even in the face of persistently elevated fatty acid availability.

The results from this final study demonstrated that exercise training simultaneously increased the oxidation of lipids and the uptake and storage of glucose in the skeletal muscle of fat-fed rats. In contrast, Rosiglitazone
treatment resulted in increased fatty acid uptake, which appeared to further impair skeletal muscle glucose transport. Exercise training in combination with Rosiglitazone treatment prevented the Rosiglitazone-induced accumulation of lipids and decrease in insulin sensitivity despite increased lipid uptake and CD36 expression in this treatment group. Thus it appears that exercise training imparts a “metabolic flexibility” allowing skeletal muscle to deal with increased lipid availability without compromising glucose metabolism.

In summary, the results from the studies undertaken for this thesis provide novel information regarding the mechanisms of action of two insulin-sensitising therapies, exercise training and Rosiglitazone treatment, in skeletal muscle. Several questions remain that should be addressed by future work. For example, as Rosiglitazone did not improve skeletal muscle insulin sensitivity in a high-fat fed model (chapter five), it is not clear how it was effective in improving insulin sensitivity in other tissues. The observation that lipid accumulation was reduced in the liver of Rosiglitazone-treated animals raises the possibility that this organ acts as a potential target for its insulin-sensitising actions. Further investigation into the effects of Rosiglitazone treatment on lipid and glucose metabolism in this, and other insulin-sensitive tissues are needed. In addition, future research should determine the molecular mechanisms by which exercise training improves metabolic flexibility by allowing for the simultaneous upregulation of both glucose and lipid metabolism. Such information may provide clues for the development of more effective insulin sensitising agents that specifically target skeletal muscle. The results from this thesis suggest that chronic AMPKα1 activation may be involved in this process, but further investigation possibly involving skeletal muscle cell culture or isoform-specific AMPK knockout models are needed to confirm this hypothesis.
Continued discovery of the factors that lead to skeletal muscle insulin resistance, and the mechanisms by which currently available insulin-sensitising agents act to overcome these impairments may lead to the development of more effective treatments for this disease. Given the increasing prevalence of insulin resistance and type 2 diabetes world-wide, it seems unlikely that researchers in this field will run out of work in the near future.
CHAPTER SEVEN: References


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