SKELETAL MUSCLE NA^+K^+-ATPASE FUNCTION IN TYPE II DIABETES

A thesis submitted in fulfillment of
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Doctor of Philosophy

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ABSTRACT

Insulin resistance is a common factor in metabolic disorders, such as obesity and Type II diabetes mellitus. In skeletal muscle, insulin can induce the translocation of both glucose transporters and the α-subunit of the Na^+K^+-ATPase to the plasma membrane. Whilst the detrimental effects of insulin resistance upon insulin-mediated glucose uptake is well recognized, little is known about the effect on Na^+K^+-ATPase function and the potential benefits antidiabetic therapies, such as exercise training and Rosiglitazone can have on Na^+K^+-ATPase function in insulin resistant skeletal muscle. Therefore the primary aim of this dissertation was to determine the effect of insulin resistance on Na^+K^+-ATPase content and activity in rat skeletal muscle, and the possible beneficial effects of Rosiglitazone treatment and exercise training. Since Na^+K^+-ATPase is a membrane-bound enzyme and its function is dependant upon the membrane lipid milieu surrounding it, a second focus of this thesis was to examine a possible link between changes in enzyme activities and membrane phospholipids. The first study examined the effects of insulin resistance on maximal *in vitro* Na^+K^+-ATPase activity and content. The results demonstrated depression in Na^+K^+-ATPase activity and content with insulin resistance in rat skeletal muscle. This was shown in both, the genetically predisposed obese Zucker and high fat diet induced Sprague Dawley rat models of insulin resistance. The findings indicate compromised Na^+K^+-ATPase function, causing a reduced reserve for Na^+K^+-ATPase activation in insulin resistant

skeletal muscle. This may predispose diabetics to early muscle fatigue with associated impaired exercise tolerance and may further progress the development of type II diabetes and its associated complications. Membrane phospholipids influence the function and intrinsic activity of transmembrane proteins, such as the Na^+K^+-ATPase. Therefore, the second study investigated Na^+-K^+-ATPase function and membrane phospholipid composition in skeletal muscle of more mature obese Zucker rats. Insulin sensitising agents, Thiazolidinediones (TZD) have also been found to alter membrane phospholipids however their effect on Na^+-K^+-ATPase function has never been addressed. Hence, another purpose of the second study was to evaluate the effects of Rosiglitazone, a TZD derivative, on Na^+-K^+-ATPase function and membrane phospholipids in skeletal muscle. In this study, the Na^+K^+-ATPase activity, but not content, was found to be altered in the obese Zucker rats, which may have been due to the altered membrane phospholipid composition and desaturase activity found with this phenotype. Despite insulin sensitivity increasing through Rosiglitazone, Na^+K^+-ATPase activity was reduced in the lean rats only. Rosiglitazone had either no effect on Na^+K^+-ATPase in skeletal muscle of obese Zucker rats or, its effect was not seen because it was not additive to the decrease already observed with insulin resistance alone. The reduction in the lean group was not due to a decrease in enzyme content or alterations in membrane lipids but may possibly be due to elevated leptin levels or AMPK activation, leading to inhibition of the membrane bound Na^+K^+-ATPase. Therefore, treatment of insulin resistance with Rosiglitazone may increase insulin sensitivity but may also affect Na^+K^+-ATPase, which may limit skeletal muscle function. This apparent lack in pump capacity might lead to a decrease in specific muscle tension and a

reduced resistance to muscle fatigue during prolonged contractile activity. The final study for this dissertation investigated the effect of a more therapeutic dose of Rosiglitazone, and exercise training in high fat fed rats. The most important finding was the positive effect exercise training had on the reduced Na^+K^+-ATPase capacity found with insulin resistance. Exercise training significantly increased skeletal muscle Na^+K^+-ATPase content and its corresponding maximal activity but had a mixed effect on the membrane phospholipid profile. Rosiglitazone did not have the same desired effect, furthermore at more therapeutic doses, did not appear to have any effect. The other disappointing result from this study was that there was no correlation between the Na^+K^+-ATPase function and membrane phospholipids. In summary, the results of the studies undertaken for this thesis repeatedly demonstrated that insulin resistance has a detrimental effect on Na^+K^+-ATPase function in rat skeletal muscle. This may be due to changes in the phospholipid profile however this may depend on the insulin resistant animal model used. More therapeutic doses of the insulin sensitising drug, Rosiglitazone do not have an effect on Na^+K^+-ATPase in skeletal muscle and for the first time, it was shown that chronic exercise training increased Na^+K^+-ATPase activity and content, reversing the negative effect of insulin resistance on Na^+K^+-ATPase function.

DECLARATION

I, the candidate, Sonia Liana La Vita, certify that:

a) except where due acknowledgement has been make, 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 this thesis is the result of work which has been carried out since the official commencement date of the approved research program.

Sonia Liana La Vita

July 2007

RESEARCH OUTCOMES

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

LA VITA, S.L., LESSARD, S.J, TURNER, N., REID, J., FRASER, S.F. (2007) Rosiglitazone compromises Na^+K^+-ATPase function in skeletal muscle of lean Zucker rats. *In preparation.*

LA VITA, S.L., TURNER, N., FRASER, S.F. (2007) Chronic exercise training restores maximal Na^+K^+-ATPase function in insulin resistant skeletal muscle. *In preparation.*

LESSARD, S.J, **LO GIUDICE, S.L.**, LAU, W., REID, J., TURNER, N., FEBBRAIO, M.A., HAWLEY, J.A., WATT, M.J. (2004) Rosiglitazone enhances glucose tolerance by mechanisms other than reduction of fatty acid accumulation within skeletal muscle. *Endocrinology* **145**: 5565-5570.

LA VITA, S.L., TURNER, N., FRASER, S.F. (2005) Rosiglitazone compromises Na+,K+-ATPase function in skeletal muscle of Obese Zucker Rats. Poster Presentation given at Opening Symposium: The Na,K,Cl-Homeostasis and Na,K-Pumps of Muscle and Heart in Exercise and Disease, Denmark.

FRASER, S.F., **LA VITA, S.L.**, LESSARD, S.J, HAWLEY, J.A. (2007) Effect of Rosiglitazone and Exercise on Na^+K^+-ATPase function in insulin resistant rat skeletal muscle. Oral Presentation to be given at European Association for the Study of Diabetes Meeting, Amsterdam.

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ABBREVIATIONS

AMPK	AMP-activated protein kinase
ANOVA	one-way analysis of variance
AUC	area under the curve
BCA	bicinchoninic acid
CMC	carboxymethylcellulose
DAG-PKC	diacylglycerol-protein kinase C
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
DLF	digitalis-like factor
EPA	eicosapentaenoic acid
ER	endoplasmic reticulum
ERK	extracellular regulated kinases
FA	fatty acid
FFA	free fatty acid
GLUT4	glucose transporter 4
HF	high fat fed
HFIns	high fat fed & insulin stimulated
HFR	high fat fed & rosiglitazone treated
HFRIns	high fat fed, rosiglitazone treated & insulin stimulated
HFRX	high fat fed, rosiglitazone treated & exercise trained

HFRXIns	high fat fed, rosiglitazone treated, exercised trained & insulin stimulated
HFX	high fat fed & exercised trained
HFXIns	high fat fed, exercised trained & insulin stimulated
IMTGs	intramuscular triglycerides
IRS	insulin receptor substrates
ISE	ion-selective electrode
KHB	Krebs-Henseleit buffer
LZ	lean Zucker
LZR	Rosiglitazone treated lean Zucker
MEK	MAP ERK kinase
n-3	omega-3
n-6	omega-6
NC	chow fed normal control
NCIns	chow fed & insulin stimulated
OGTT	oral glucose tolerance test
OZ	obese Zucker
OZR	Rosiglitazone treated obese Zucker
PC	phosphatidylcholine
PI 3-kinase	phosphatidylinositol-3-kinase
PKC	protein kinase C
PKG	cGMP-dependant protein kinase
PPAR-γ	Peroxisome proliferator-activated receptor-γ

PUFA	Polyunsaturated fatty acid
RG	red gastrocnemius
SE	standard error
SR	sarcoplasmic reticulum
STZ	streptozotocin
T_3	thiiodothyronine
TG	triglycerides
TVS	Tris-Vanadate-Sucrose
TZDs	Thiazolidinediones
VO_{2max}	maximal oxygen consumption
WG	white gastrocnemius
Δ^5desaturase	delta(sup 5)-desaturase
Δ^6desaturase	delta(sup 6)-desaturase
Δ^9desaturase	delta(sup 9)-desaturase
3-MG	3-*O*-methylglucose
3-OMF	3-*O*-methlylfluorescein
3-OMFP	3-*O*-methlylfluorescein phosphate
3-OMFPase	3-*O*-methlylfluorescein phosphatase

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Chapter 1

INTRODUCTION

The Na^+K^+-ATPase is an enzyme located in the cytoplasmic membrane of all excitable animal cells and is responsible for restoring Na^+ and K^+ gradients, maintaining the membrane potential and consequently the excitability of a cell (Clausen *et al.*, 1998). Skeletal muscles contain the majority of Na^+K^+-ATPase in the body and the ability of a muscle to contract resides in the excitability of the cell (Clausen & Everts, 1989). Na^+K^+-ATPase is responsible for re-accumulating the K^+ lost, back into the muscle cell whilst removing Na^+ (Nielsen & Clausen, 2000).

The activity and concentration of the Na^+K^+-ATPase enzyme is subject to regulation by a variety of hormonal and non-hormonal factors. The major physiological stimuli for the Na^+K^+-ATPase are excitation and hormonal activators. Excitation is indisputably the most effective stimulus (Nielsen & Harrison, 1998), whereby repetitive high-frequency stimulation often induces a net loss of K^+, and a net gain of Na^+, that exceeds the maximum capacity for K^+ influx and Na^+ efflux via the Na^+K^+-ATPase (Lindinger *et al.*, 1995; Hallen, 1996). The membrane potential of skeletal muscle must be restored for the ability of subsequent action potentials to occur. Under these conditions, hormonal induced stimulation of the enzyme can help to restore membrane excitability and can also lead to temporary hyperpolarisation of the membrane (Overgaard *et al.*, 1997). A number of hormones can influence the Na^+K^+-ATPase in an

acute or chronic manner, such as insulin, adrenaline, aldosterone and thyroid hormone (Ewart & Klip, 1995) but important control of Na^+K^+-ATPase rate is achieved by its sensitivity to insulin. Two determinants of insulin sensitivity are physical activity and lipid metabolism.

In addition to insulin's important function of regulating blood glucose concentrations, the hormone also plays a central role in Na^+ and K^+ homeostasis. Insulin has been shown to act as a regulator of Na^+K^+-ATPase in a variety of tissues (Deachapunya *et al.*, 1999; Feraille *et al.*, 1999; Sweeney & Klip, 2001; Al-Khalili *et al.*, 2003; McKenna *et al.*, 2003), skeletal muscle being the main target tissue. Independent of other hormones, insulin has been shown to promote the uptake of K^+ into skeletal muscle cells primarily through stimulation of the Na^+K^+-ATPase (Brodal *et al.*, 1974), countering the hyperkalemia induced by exercise (Kanbe & Kitasato, 1986). However the mechanisms by which insulin exerts its control and the signalling pathways involved remain unclear.

Insulin resistance is the lack of tissue response to insulin and has long been known to be a precursor of type II diabetes and is associated with obesity (Cleland *et al.*, 1998). Several skeletal muscle defects have been shown to occur with insulin resistance and their effect on Na^+K^+-ATPase and K^+ homeostasis has been investigated. Several studies have found a decrease in the Na^+K^+-ATPase activity and expression with diabetes (Kjeldsen *et al.*, 1987; Ng *et al.*, 1993; Takeda *et al.*, 1996; Kuwahara *et al.*, 1997; Djurhuus *et al.*, 2001). Diabetes can cause serious health complications including heart

disease, blindness and kidney failure. Impairment of the Na^+K^+-ATPase through insulin resistance may contribute to these diabetic complications.

The intrinsic activity of Na^+-K^+-ATPase, is dependant upon the membrane lipid milieu surrounding it. Irregular Na^+ ion transport has been seen in disease states with abnormal lipid metabolism such as atherosclerosis, obesity and diabetes (Hughes & York, 1983; Chen *et al.*, 1995; Makar *et al.*, 1995). Therefore, the effect of the membrane lipid composition on Na^+K^+-ATPase function may offer an explanation for this detrimental effect.

Often treatment for type II diabetes is hindered by the persistence of obesity and a pharmacological approach is commonly used to reinstate normoglycemia. Rosiglitazone is an oral antidiabetic drug, belonging to a class of insulin sensitizers called Thiazolidinediones (TZD) (Cuzzocrea *et al.*, 2004). Studies have shown Rosiglitazone improves hyperglycaemia and hyperinsulinemia in insulin resistant and Type II diabetic patients (Lebovitz *et al.*, 2001; Hung *et al.*, 2005). However TZDs have been shown to cause excessive weight gain, fluid retention and repress the gene expression of stearoyl-CoA desaturase 1, an enzyme which catalyses the desaturation of membrane phospholipids (Kurebayashi *et al.*, 1997). This can cause alterations in the membrane structure and function including fluidity, permeability, enzyme activity, ion channels and transport (Komers & Vrana, 1998). TZDs are also known to increase the risk of heart failure (Delea *et al.*, 2003).

It has been well documented that with regular exercise emerge physiological and biochemical benefits. Exercise training has been shown to decrease insulin resistance and increase Na^+K^+-ATPase concentrations in skeletal muscle suggesting regular physical activity could be an important therapeutic strategy in the treatment of type II diabetes (McKenna, 1995; McKenna *et al.*, 1996; Green *et al.*, 1999; Juel *et al.*, 2000).

However, very few studies have investigated the effects of insulin resistance on the concentration and function of Na^+K^+-ATPase and none have explored the effects of Rosiglitazone or exercise on Na^+K^+-ATPase in insulin resistant skeletal muscle. Type II diabetes is a major health concern and prevalence is expected to double in the next 25 years (Dunstan *et al.*, 2002; Agarwal *et al.*, 2005). Therefore, research that can help elucidate mechanisms that contribute to the aetiology as well as the treatments options for this disease are vital.

Chapter 2

LITERATURE REVIEW

2.1 Introduction

The Na^+K^+-pump, also referred to as Na^+K^+-ATPase, was first identified in 1957 by a Danish researcher, Jens Christian Skou, who isolated an energy-dependant enzyme from peripheral nerves which could be activated by the concentration of Na^+ and K^+ (Skou, 1957). Four decades later Skou's role in demonstrating the molecular basis for active transport of Na^+ and K^+ across the membrane was recognised with the 1997 Danish Nobel price in Chemistry (Clausen & Persson, 1998). Since then, the Na^+K^+-ATPase enzyme has become an area of intense research interest to a diverse group of scientists. The critical role of Na^+K^+-ATPase in specialised tissues has drawn the attention of a broad spectrum of disciplines who are intrigued by the electrochemical gradient generated while molecular genetics techniques have revealed the primary structure and different isoforms of the enzyme (Lingrel, 1992).

Na^+K^+-ATPase is recognised as one of the most important enzymes of a cell. It plays a vital role in maintaining K^+ homeostasis, the cell membrane potential and consequently the excitability of the cell. The Na^+K^+-ATPase also provides the energy to drive essential Na^+ coupled transport of various nutrients into the cell and is involved in controlling cellular pH, osmotic balance and cell volume (Skou & Esmann, 1992).

2.2 Na^+K^+-ATPase in Skeletal Muscle

The Na^+K^+-ATPase is a ubiquitous protein found in all excitable tissue, especially nerve and muscle cells. Skeletal muscle contains the largest pool of K^+ and a large portion of Na^+K^+-ATPase in the body (Clausen & Everts, 1989) giving this organ the capacity for rapid Na^+ and K^+ exchange and effectively regulate extracellular K^+ concentrations. By transporting Na^+ and K^+ across the sarcolemma, the Na^+K^+-ATPase plays a key role in the prevention of fatigue in skeletal muscle by maintaining membrane excitability during muscle contraction (Nielsen & Overgaard, 1996).

2.2.1 Na^+K^+-ATPase Structure

The Na^+K^+-ATPase exists as a functional heterodimer, composed of 2 polypeptide chains, a catalytic alpha (∝)-subunit and a heavily glycosylated beta (β)-subunit (McDonough & Farley, 1993). Additional regulatory proteins belonging to a FXYD family of proteins have also been associated with the Na^+K^+-ATPase (Reis *et al.*, 2005). A gamma subunit (FXYD2) has been identified, although it's expression is essentially restricted to the kidney (Blanco & Mercer, 1998; Therien *et al.*, 1999) and phospholemman (FXYD1) which has been identified in heart, skeletal muscle, and neuronal tissues and is associated with the ∝-subunit (Feschenko *et al.*, 2003; Reis *et al.*, 2005). The ∝-subunit conveys the channel properties of the enzyme through 10 transmembrane segments which contain the intracellular binding sites for Na^+ and ATP and Pi, and extracellular binding sites for K^+ and specific cardiac glycosidic inhibitors (Hasler *et al.*, 1998). The β-subunit does not participate in the ion transportation but is required for the normal functioning of the enzyme (Blanco & Mercer, 1998).

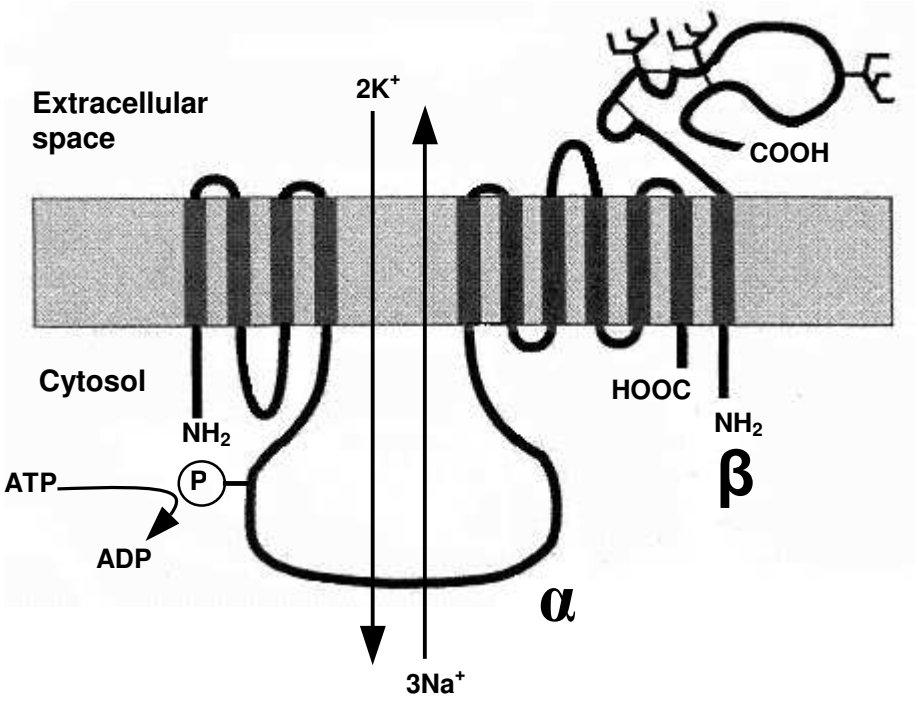

Figure 2.1 Model of the Na^+K^+-ATPase structure. P denotes phosphorylation. (Fambrough *et al.*, 1994)

Unaccompanied, the ∝-subunit promptly degrades in or near the endoplasmic reticulum (ER) hence, the primary function of the β-subunit is to act as a chaperone by stabilising the correct folding of the ∝-subunit and delivering it from the ER to the plasma membrane (Hasler *et al.*, 2001). This protects the ∝-subunit from proteolysis and cellular degradation. The β-subunit also influences the cation sensitivity of Na^+K^+-ATPase, which produces different affinities for Na^+ and K^+ (Jaisser *et al.*, 1992). The β-subunit is comprised of a hydrophobic transmembrane segment, a short sarcoplasmic tail and an extracellular portion containing 3 disulfide bridges and polysaccharide chains attached. Studies have suggested that the ∝-subunit interacts with the β-subunit through all 3 domains (Hasler *et al.*, 2001).

The tertiary structure of Na^+K^+-ATPase is based upon other P-type pumps. The Na^+K^+-ATPase ∝-subunit is thought to transverse the cytoplasmic membrane 10 times according to a homology model based on the Ca^{2+}-ATPase crystal structure (Toyoshima *et al.*, 2000; Tal *et al.*, 2001), refer to Figure 2.2. The transmembrane helices are woven into the membrane to form a pore/channel.

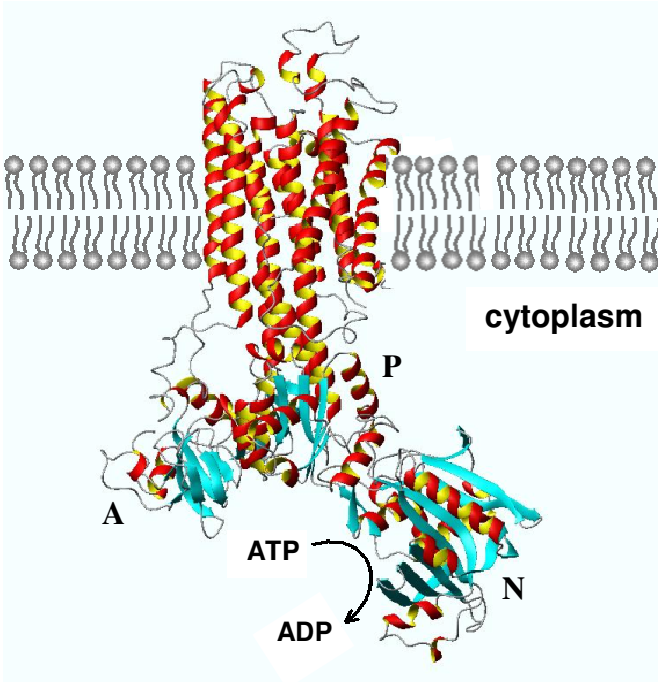

Figure 2.2 Crystallographic structure of the rabbit skeletal muscle Ca^{2+}-ATPase (Toyoshima *et al.*, 2000). The head region is composed of three cytoplasmic domains: the P-domain has the phosphorylation site, the N-domain contains the ATP binding site and the A-domain, or actuator domain. The structure was obtained in the presence of two Ca^{2+} ions, bound in the transmembrane domain.

2.2.2 Na^+K^+-ATPase Function

Na^+K^+-ATPase translocates positively charged ions across the sarcolemma against their concentration gradients. Specifically, it imports 2 K^+ ions whilst simultaneously extruding 3 Na^+ ions for each ATP molecule hydrolysed. Steep concentration gradients of the two ions are formed and maintained across the sarcolemma. A high Na^+ concentration is maintained outside the muscle, 130-145mM in serum (Worth, 1985), whilst the concentration remains between 5 and 20mM inside muscle (Sjogaard, 1983). On the contrary, approximately 75% of total K^+ is located in the intracellular space of human skeletal muscle cells, ~160mM, whilst plasma concentration is maintained between 3.5 and 5.3mM (Sejersted & Sjogaard, 2000). Extremely high and low plasma K^+ concentrations can contribute to muscle fatigue or even lead to possible paralysis and cardiac arrest, therefore maintenance of K^+ homeostasis is crucial for survival.

Furthermore the enzyme is electrogenic, generating an electrical gradient by transferring disproportional numbers of ions across the cytoplasmic membrane. One net positive charge is associated with the third Na^+ pumped into the sarcoplasm for each cycle, causing positive charges to accumulate outside the cell, and hence creating an electrical current (Sturmer *et al.*, 1991; Domaszewicz & Apell, 1999). Both the concentration gradients and the electrical gradient constitute the membrane potential which at rest is approximately -75mV across the sarcolemma.

The main function of Na^+K^+-ATPase is to restore Na^+ and K^+ gradients following an action potential so that subsequent action potentials can occur (Schwartz *et al.*, 1975).

During an action potential in the working skeletal muscle, ion channels are opened and there is an influx of Na^+ during depolarization and efflux K^+ during repolarization. Extracellular K^+ levels can increase to 8mM in plasma and 10mM in interstitial space within minutes of intense exercise, dissipating the K^+ gradient which could induce action potential block (Vyskocil *et al.*, 1983; Klitgaard & Clausen, 1989). Therefore, Na^+K^+-ATPase are essential for re-accumulating the K^+ lost and help maintain excitability so that maximal activation of Ca^{2+}-releasing channels in the sarcoplasmic reticulum and myosin contractile filaments can occur (Green, 1998; Ortenblad *et al.*, 2000). Na^+K^+-ATPase not only restores ionic concentration gradients but also contributes to repolarization of the membrane due to its 3:2 ion exchange ratio.

In addition, the Na^+ concentration gradient maintained by the Na^+K^+-ATPase is a central mechanism for controlling multiple cellular processes. The Na^+ gradient provides the energy required for Na^+-coupled transport of essential substrates into the cytoplasm (Lingrel, 1992). Plasma membrane transporters work by coupling the downhill movement of Na^+ to the uphill transport of substrates such as glucose, amino acids and vitamins into the cell. The Na^+ gradient also moves ions such as H^+, Ca^{2+} and Cl^- across the sarcolemma which help maintain cellular pH, osmotic balance and cell volume (Rosic *et al.*, 1985). These mechanisms also modulate cell growth, differentiation and protein synthesis. Na^+ is also involved in the recycling of neurotransmitters released after neuronal activity. Transmitters are moved back into the cytoplasm by cotransport with Na^+ (Rudnick, 1998).

2.2.3 Mechanism of Action

As the name suggests, ATP energises the Na^+K^+-ATPase and several studies have shown that the enzyme preferentially uses glycolytically derived ATP (Okamoto *et al.*, 2001; Dutka & Lamb, 2007). Phosphorylation of the enzyme causes a change in conformation and its affinity for Na^+ and K^+ ions. The kinetic mechanism of Na^+K^+-ATPase involves a number of steps based on a simplified version of the Albers-Post transport cycle (Apell *et al.*, 1998). Prior to phosphorylation the binding site of the enzyme is exposed to the sarcoplasm and has a greater affinity for Na^+ [E_1•ATP]. The binding of the third Na^+ ion at the inner membrane surface activates the enzyme ATPase [$(Na_3)E_1$•ATP] and in the presence of Mg^{2+} ions, ATP is hydrolysed, releasing sufficient energy to power the pump. The phosphate group that binds to the membrane protein [$(Na_3)E_1$-P] causes the protein to change shape, placing the Na^+ binding sites at the outer membrane surface [P-$E_2(Na_3)$]. The K^+ binding sites are immediately revealed and as the enzyme's affinity for K^+ is now greater than Na^+, the Na^+ ions are expelled [P-E_2]. The binding of K^+ ions from the interstitial fluid [P-$E_2(K_2)$] causes the release of the phosphate [$E_2(K_2)$] and this dephosphorylation causes K^+ occlusion. ATP binds to the enzyme which returns to its original conformation [$(K_2)E_1$•ATP], carrying the K^+ ions to the inner membrane surface and releasing them into the cell [E_1•ATP]. Refer to figure 2.3 for schematic representation of transport cycle reactions.

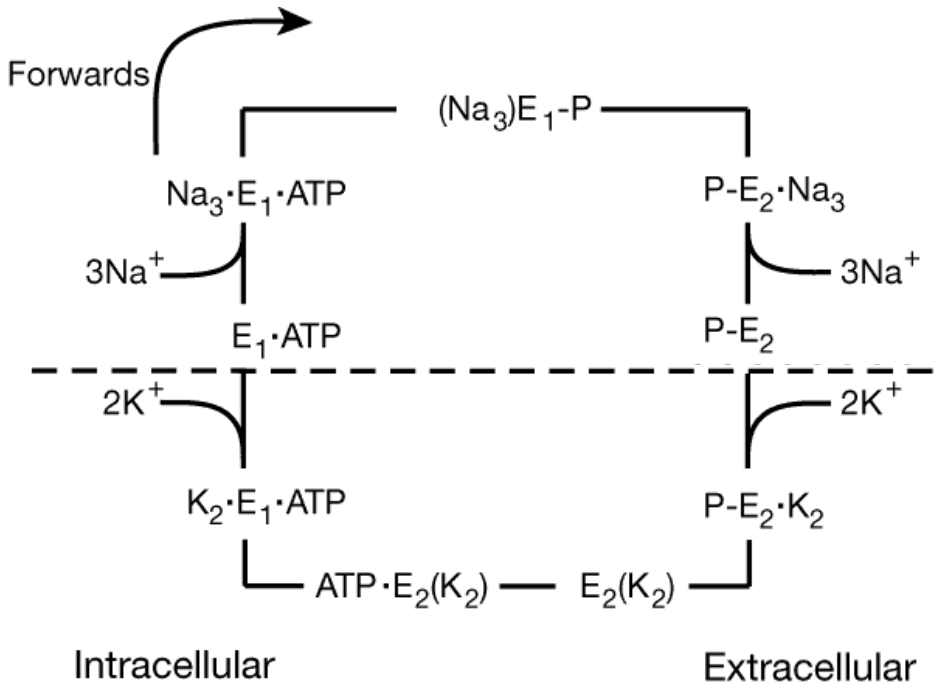

Figure 2.3 Simplified Albers-Post transport cycle. The dashed (horizontal) line separates Na^+- and K^+-translocation pathways (Holmgren *et al.*, 2000).

The cations must traverse the lipid bilayer of the membrane. Three amino acids within the transmembrane region of the enzyme, which play a major role in ion transport, are Ser775, Asp804 and Asp808 (Lingrel *et al.*, 1998). The 3 residues are involved with the K^+ binding site but it is also possible that other amino acids influence Na^+ and K^+ binding.

2.2.4 Subunit Isoforms and Expression

The Na^+K^+-ATPase ∝-subunit is known to exist in 4 different isoforms $\propto_1$, $\propto_2$, $\propto_3$ and $\propto_4$, and is approximately 112-kDa in size, while the smaller β-subunit has only 3 known variants, β_1, β_2 and β_3, and varies in size between 40-60-kDa depending on the degree of

glycosylation (Blanco & Mercer, 1998). Since $\propto_1$ isoform is found in almost every tissue, it is considered to be of a house-keeping nature while the other 3 $\propto$ isoforms are expressed in particular tissues, for instance $\propto_2$ is mostly confined to skeletal, smooth and cardiac muscle and brain glial cells (He *et al.*, 2001). In mammalian skeletal muscle, all subunit isoforms are expressed with the exception of $\propto_4$ (Murphy *et al.*, 2006). $\propto_3$ and β_3 are present in trivial amounts, therefore Na^+K^+-ATPase isozymes predominantly consist of $\propto_1$, $\propto_2$, β_1 and β_2 (Fowles *et al.*, 2004).

2.2.4.1 Tissue and Developmental Specificity

The subunit isoforms are expressed in a tissue- and developmental-specific manner and convey different kinetic characteristics of Na^+K^+-ATPase isozymes. In rat skeletal muscle, mRNA and protein expression of the $\propto_1$ isoform is low but constant throughout development whilst $\propto_2$ dominates in adult life (Lingrel, 1992). The β-subunits are of distinct but not exclusive tissue distribution with β_1 found predominately in slow-twitch oxidative muscle fibres and β_2 specifically expressed in glycolytic fast-twitch muscle (Hundal *et al.*, 1993). In the muscle membranes of red oxidative fibres, the abundance of $\propto_2$ was found to always exceed $\propto_1$ (Lavoie *et al.*, 1997), therefore the prevalent isoform complement in the red portions of the gastrocnemius is $\propto_2\beta_1$, while $\propto_1\beta_2$ and $\propto_2\beta_2$ are predominate in the white portion.

2.2.4.2 Subunit Functional Properties

Since the establishment of molecular biology techniques, the functional properties of distinct isoforms have become clearer. The diversity of the $\propto$ isoform enables the

Na^+K^+-ATPase to demonstrate varying affinity for ions (Zahler *et al.*, 1997; Blanco & Mercer, 1998) and cardiac glycosidic inhibitors (Repke *et al.*, 1995). In rodents, the $\propto_1$-isoform displays ouabain affinity 100-fold lower than $\propto_2$ and is considered to be ouabain resistant, however no such difference has been reported in humans. In addition, the associated β-subunit has been shown to influence the interaction of the enzyme with Na^+ and K^+ (Eakle *et al.*, 1994; Eakle *et al.*, 1995; Blanco & Mercer, 1998). Isoforms have also been shown to display differing functional roles in muscle contractility, with a reduction in $\propto_2$- and $\propto_1$-subunits causing an increase and decrease, respectively, in isometric force (He *et al.*, 2001; Lingrel *et al.*, 2003). However the mechanisms by which the subunits demonstrate these variations are unknown.

2.2.5 Na^+K^+-ATPase Localisation

The surface of a skeletal muscle fibre is composed of 2 distinct domains, the plasma membrane called the sarcolemma and a network of internal tubules called T-tubules. The T-tubular system represents a large extension of the sarcolemma forming up to 85% of the total sarcolemma membrane area in mammalian skeletal muscle (Dulhunty *et al.*, 1984). Na^+K^+-ATPase are found in the sarcolemma as well as in the T-tubules with particular subunit localisation. The $\propto_1$-isoform is mainly located in the sarcolemma, whereas $\propto_2$-isoform can be found in both the sarcolemma and dispersed within the muscle fibres, concentrated along the T-tubules (Williams *et al.*, 2001). The T-tubules only contain a minor proportion of the enzyme and due to its narrow structure, it has limited diffusional space for the K^+ release during muscle contraction (Sejersted & Sjogaard, 2000). This predisposes the site to action potential blockage in which the T-

tubules fail to propagate an action potential and thus contribute to cell inexcitability. The need for local glycolytically produced ATP is also likely to be important in the maintance of T-tubule membrane polarisation and excitability by Na^+K^+-ATPase (Dutka & Lamb, 2007).

It has also been suggested that an intracellular pool exists for the 2 subunits and that they can translocate to the plasma membrane upon stimulation. Hundal et al reported an increase in the concentration of $\propto_2$-subunits in the sarcolemma that corresponded with a decrease in intracellular stores (Hundal *et al.*, 1992). The Na^+K^+-ATPase subunit stores were later found to be different from the GLUT4 intracellular vesicles (Aledo & Hundal, 1995).

2.3 Regulation of the Na^+K^+-ATPase

The Na^+K^+-ATPase enzyme demonstrates a high degree of plasticity with up- or down-regulation depending on the stimulus. It is known that the Na^+K^+-ATPase enzyme is regulated by a variety of hormonal and non-hormonal factors, however the molecular mechanism by which these stimuli exert their effects remain poorly understood. Despite this, it is known that excitation elicits the largest activation response of the pump, whilst endocrine control through insulin, adrenaline and thyroid hormones are also important (Clausen, 1996). In particular, insulin plays a key role in acute and chronic regulation of Na^+K^+-ATPase activity and concentration (Sweeney & Klip, 1998).

Strict regulation of plasma K^+ concentration is essential as hyperkalemic conditions interfere with muscle contraction and in extreme cases can cause paralysis or cardiac arrest. The kidneys are responsible for the long-term control of plasma K^+ concentration, but skeletal muscles can carry out immediate Na^+ and K^+ exchange, and consequently are responsible for the acute ongoing plasma K^+ adjustments (Clausen & Everts, 1989). The function of Na^+K^+-ATPase in skeletal muscle is inturn controlled by short and long-term regulation. The mechanisms shown to be involved include changes in enzyme activity, subcellular distribution or subunit expression (Sweeney & Klip, 1998).

2.3.1 Acute Control

Acute activation of the Na^+K^+-ATPase enzyme can occur within minutes and the three major stimuli are excitation, catecholamines and insulin (Clausen & Everts, 1989). These activators are essential for reducing exercise-induced hyperkalemia, ensuring prompt recovery of excitability. Na^+, insulin, adrenaline and amylin have all been shown to increase lactate production in skeletal muscle as a result of stimulated Na^+K^+-ATPase activity and increased glycolytic ATP consumption (James *et al.*, 1999b; McCarter *et al.*, 2001; Okamoto *et al.*, 2001). This suggests that ATP consumption by the Na^+K^+-ATPase could possibly regulate the rates of aerobic glycolysis and glycogen breakdown.

2.3.1.1 Excitation

Skeletal muscle uses less than 10% of Na^+K^+-ATPase maximal activity to uphold ionic concentration gradients at rest (James *et al.*, 1999a). During muscle contraction, a pronounced decrease in transmembrane Na^+ and K^+ gradients occur due to these ions

leaking across the sarcolemma with the passing of an action potential. Plasma K^+ levels can rise above 8mmol/L (Klitgaard & Clausen, 1989; Medbo & Sejersted, 1990; Vollestad *et al.*, 1994) while interstitial K^+ concentrations can increase up to 10mmol/L (Vyskocil *et al.*, 1983). When the ion gradient dissipation is unopposed, there is loss of membrane excitability (Macdonald *et al.*, 2007). This creates the urgent need for Na^+K^+-ATPase activation to restore the ion gradients so subsequent action potentials can occur. Electrical stimulation has been repeatedly shown to rapidly induce Na^+-K^+-ATPase activity in skeletal muscle. Electrical stimulation can induce Na^+K^+-ATPase activity after 1 second of stimulation, evident by an increase in $^{86}Rb^+$ uptake that can be abolished by ouabain (Buchanan *et al.*, 2002). The effect was also seen with low frequency stimulation (2Hz) for 2 minutes (Buchanan *et al.*, 2002). The cause of excitation-induced activation of Na^+K^+-ATPase can be partly due to an increase in intracellular Na^+ concentrations, however Na^+K^+-ATPase activity has been shown to persist beyond normal resting levels, causing an undershoot in intracellular Na^+ concentrations (Juel, 1986; Nielsen & Clausen, 1997). Therefore excitation is believed to activate Na^+K^+-ATPase by increasing the apparent affinity of Na^+K^+-ATPase for intracellular Na^+. Another possible explanation for the excitation-induced increase in ion transport could involve the translocation of Na^+K^+-ATPase subunits from an intracellular pool to the sarcolemma. With added functional pumps in the membrane, the capacity for ion transport increases (Clausen & Hansen, 1977).

2.3.1.2 Hormonal Control

Hormones contribute only 5% of the increase in enzyme activity caused by electrical stimulation of the muscle (Sweeney & Klip, 1998). Repetitive high-frequency

stimulation often induces a net loss of K^+, and a net gain of Na^+, that exceeds the maximum capacity of the Na^+K^+-ATPase, concomitantly there is a progressive decline in contractile force (Clausen & Everts, 1988; Ortenblad *et al.*, 2000). In this state, Na^+K^+-ATPase activity is dependant on hormonal control (Clausen *et al.*, 1993; Overgaard *et al.*, 1997).

2.3.1.3 Insulin

Insulin is an anabolic hormone synthesised in the pancreas by the β endocrine cells. The main function of insulin is to reduce the blood level of glucose by promoting its entry into the skeletal muscle. In addition to insulin's important function of regulating blood glucose concentration, the hormone also plays a central role in K^+ homeostasis by promoting K^+ entry into skeletal muscle (Sweeney & Klip, 1998). There is a negative feedback relationship between plasma K^+ concentrations and insulin secretion (Clausen & Everts, 1989). Elevated plasma K^+ concentration stimulates insulin secretion, and insulin in turn enhances K^+ uptake by Na^+K^+-ATPase, therefore counter-balancing hyperkalemia.

Insulin has been shown to act as a regulator of the Na^+K^+-ATPase in a variety of tissues. However, skeletal muscle is the main peripheral insulin target tissue, since up to 85% of all glucose up-take occurs here (Galuska *et al.*, 1998), and it is the main storage tissue for glucose and K^+ in the body .

The underlying mechanisms resulting in the acute insulin-induced activation of Na^+K^+-ATPase differ with respect to the type of tissue involved. In some tissues, insulin activates the Na^+K^+-ATPase secondary to an insulin-induced elevation of intracellular

Na^+. For example through the Na^+/H^+ exchanger and the $Na^+/K^+/2Cl$- cotransporter. However, previous studies have shown that this is evident in adipocytes and hepatocytes, but not for skeletal muscle (Weil *et al.*, 1991; Therien & Blostein, 2000). In rat adipocytes and kidney cells, insulin has been shown to upregulate enzyme activity by increasing the affinity of the ∝-subunit for Na^+ by lowering $k_{0.5}$ for Na^+ (ie. the intracellular Na^+ concentrations that stimulates the pump to 50% of its maximum pump rate) (McGill & Guidotti, 1991; Feraille *et al.*, 1995). However, insulin stimulation of Na^+K^+-ATPase in skeletal muscle has been mainly attributed to the translocation of $\propto_1$-, $\propto_2$- and β_1- subunits to the plasma membrane from an intracellular storage compartment (Hundal *et al.*, 1992; Al-Khalili *et al.*, 2003). It has been proposed that phosphorylation of the ∝-subunit activates the otherwise latent and inactive Na^+K^+-ATPase molecule (Al-Khalili *et al.*, 2004).

It has also been suggested that insulin may act directly upon a Na^+ dependent inhibitor of the pump (McGill & Guidotti, 1991). Insulin would increase Na^+ affinity of the inhibitor and consequently alleviate inhibition of the enzyme. However, this theory has not had further support and the inhibitor remains unknown.

2.3.1.4 Adrenaline

Catecholamines have been shown to influence Na^+K^+-ATPase activity. One of those most studied is adrenaline. Adrenaline is known to stimulate Na^+K^+-ATPase activity in skeletal muscle, primarily after exercise-induced hyperkalemia (Overgaard *et al.*, 1997; James *et al.*, 1999). Na^+K^+-ATPase activation occurs by the hormone binding to β_2-adrenoreceptors and raising cyclic AMP production (Clausen & Flatman, 1980).

Administration of β_2-adrenoreceptor agonists, such as Salbutamol has also been shown to alleviate hyperkalemia (Overgaard *et al.*, 1997). Signalling pathways seems to be tissue-specific and implicate both PKC- and AMPK-mediated pathways but the mode of regulation still remains controversial.

2.3.2 Chronic Control

The long-term regulation of the Na^+K^+-ATPase in skeletal muscle involves the cellular distribution of the enzyme and changes in membrane concentration. While adrenal steroids such as aldosterone and dexamethasone can cause changes in pump concentrations, basal levels of these steroids seem to be of minor importance in skeletal muscle (Dorup & Clausen, 1997). The major endocrine factor stimulating the synthesis of Na^+K^+-ATPase is thyroid hormone (Clausen & Everts, 1989; Azuma *et al.*, 1993). K^+ intake has also been shown to modulate Na^+K^+-ATPase concentrations in skeletal muscle (Norgaard *et al.*, 1981).

2.3.2.1 Thyroid Hormone

The thyroid hormone, Thiiodothyronine (T_3) serves as chronic pump regulator, which does not alter catalytic properties of the enzyme but regulates the mRNA and protein levels of Na^+K^+-ATPase $\propto_2$ and β_2 isoforms (Azuma *et al.*, 1993). A significant correlation exists between thyroid function and the concentration of Na^+K^+-ATPase, whereby hyperthyroidism (increased T_3 level) and hypothyroidism (reduced T_3 level) causes the number of ^{3}H-ouabain binding sites to increase and decrease, respectively (Kjeldsen *et al.*, 1984c). Changes in enzyme concentration are fully reversed when thyroid status is normalised by therapy (Kjeldsen *et al.*, 1984c). Uncontrolled diabetes

and starvation are 2 conditions which display hypothyroidism (Clausen, 2003). The fact that both conditions exhibit a down-regulation of the Na^+K^+-ATPase concentration in skeletal muscle may indicate the reduction of the T_3 level partly accounts for the observed effects. The decline in $\propto_2$ protein levels is more profound than mRNA, indicating a translational or post-translational regulatory effect. T_3 treatment for hypothyroidism increases mRNA and protein abundance of Na^+K^+-ATPase $\propto_2$ and β_2 isoforms in skeletal muscle (Azuma *et al.*, 1993).

Thyroid hormone has also been shown to increase the passive permeability of Na^+ and K^+ in the skeletal muscle membrane (Ismail-Beigi *et al.*, 1986). This may be the result of T_3 induced changes in the fatty acid and phospholipid composition of the sarcolemma, increasing its fluidity and permeability (Pilarska *et al.*, 1991; Bangur *et al.*, 1995). This would suggest that the thyroid-induced elevation in Na^+K^+-ATPase content is an adaptation to counter-balance the surplus in Na^+ and K^+ leaks.

2.3.2.2 Insulin

There is less research on the effects of prolonged insulin action compared with an acute response, however it is important to understand the potential actions of the hormone in hyperinsulinemic states such as obesity and type II diabetes. Insulin has been shown to alter gene expression of Na^+K^+-ATPase subunits. One study showed long term exposure of 3T3-LI fibroblasts to insulin increased $\propto_2$ mRNA but decreased β_1 mRNA (Russo & Sweadner, 1993) This was again shown by another study which demonstrated the same $\propto_2$ mRNA increase in vascular smooth muscle (Russo & Sweadner, 1993; Tirupattur *et al.*, 1993). The changes could be a result of increased transcription, mRNA

stabilisation and/or protein translation and stabilisation. More recently Deachapunya et al, showed long-term exposure to insulin for 4 days resulted in enhanced Na^+ absorption with a further increase in Na^+K^+-ATPase transport activity in porcine glandular endometrial epithelial cells grown in primary culture (Deachapunya *et al.*, 1999).

It is also known that insulin acts as a long term regulator by promoting muscular K^+ clearance on the basis of K^+ ingested. Bundgaard and Kjeldsen showed rats on a 2 week K^+ rich diet had increased Na^+K^+-ATPase concentrations while rats on a K^+ depleted diet had decreased concentrations (Bundgaard & Kjeldsen, 2003).

2.3.2.3 Exercise Training

Many longitudinal studies with animals and humans have reported an up-regulation in Na^+K^+-ATPase concentration in skeletal muscle as a result of exercise training. Various types of training, including strength, sprint and endurance training have caused an increase in the number of ^{3}H-ouabain binding sites in human skeletal muscle (Klitgaard & Clausen, 1989; McKenna *et al.*, 1993; Madsen *et al.*, 1994). The molecular events responsible for increasing Na^+K^+-ATPase content are currently unknown. Furthermore, exercise training alters a wide range of hormones, including adrenaline, insulin and aldosterone, which are implicated with Na^+K^+-ATPase expression regulation, making signalling mechanisms as a result of exercise training unclear. Given that the effect of training is confined to the working muscle, the signalling mechanism must be of a local nature. Hyperkalemic stress imposed on the muscle may be an

important factor in exercise-induced elevation of Na^+K^+-ATPase content and improved K^+ uptake in skeletal muscle.

2.3.3 Role of Insulin

By binding to the insulin receptor, insulin can activate a variety of intracellular signalling processes and modulate cell function. Principally, insulin's role in both glucose and K^+ cellular uptake is essential, however control of K^+ homeostasis is an important yet often underestimated action of insulin. Independent of other hormones, insulin has been shown to promote the uptake of K^+ into the skeletal muscle cell primarily through stimulation of the Na^+K^+-ATPase, counter-balancing the hyperkalemia induced by exercise (Kanbe & Kitasato, 1986). Insulin favorably activates subunits demonstrating ouabain sensitivity. $\propto_2$ and β_1 subunits are preferentially involved in insulin responses (Hundal *et al.*, 1992).

2.3.3.1 Insulin Signalling

The signalling pathway of insulin generally requires a sequence of events that begins with insulin binding to its cell surface receptor protein and in turn activates the intrinsic tyrosine kinase activity of the receptor (Saltiel & Kahn, 2001). The activated receptor kinase then phosphorylates tyrosine residues on protein substrates called insulin receptor substrates (IRS) inside the cell which act as "docking" sites, binding other cellular signalling molecules, eg: phosphatidylinositol-3-kinase (PI 3-kinase), to form complexes of intracellular signalling proteins (Kahn, 1998). The mechanism and the signalling pathway involved are still poorly understood and clearly warrant further investigation. Various cellular processes are stimulated through the signalling cascade

such as glucose and lipid metabolism as well as cell growth and Na^+K^+-ATPase activation. Defective insulin signalling is a chief characteristic of obesity and type II diabetes and leads to a number of associated complications.

2.3.4 Phosphorylation Signalling Pathways

Hormones play a significant role in modulating Na^+K^+-ATPase activity and most regulate through signalling mechanisms that modulate the activities of protein kinases. Hormone action directly interacts with protein kinase A, C, G, or protein phosphatase 2B and will inhibit or stimulate Na^+K^+-ATPase activity depending on tissue type. Extracellular regulated kinases (ERK) 1 and 2, and upstream kinase MAP ERK kinase (MEK) 1 and 2 have also been implicated in the signalling pathway of Na^+K^+-ATPase phosphorylation (Al-Khalili *et al.*, 2004).

As the name suggests, AMP-activated protein kinase (AMPK), is activated by the build up of intracellular AMP. AMPK is not only tissue specific but also species dependant. AMPK has been shown to activate Na^+K^+-ATPase activity in rat (Li & Sperelakis, 1993) and frog (Venosa, 2005) but suppress activity in squirrel (MacDonald & Storey, 1999) skeletal muscle. Very few studies have investigated the mechanism by which AMPK alters Na^+K^+-ATPase activity, however it is thought to be via direct phosphorylation of the pump at site Ser-934 (NB numbering of amino acids used includes the post-translationally cleaved NH2-terminal of 5 amino acids) (Fisone *et al.*, 1994).

The signal transduction of insulin on the Na^+K^+-ATPase involves an insulin receptor, protein kinases and the ∝-subunit of the Na^+K^+-ATPase. Studies have shown that

insulin's effect on Na^+K^+-ATPase is not additive to protein kinase C (PKC) and that insulin exerts its control via a PKC-dependent pathway (Sampson *et al.*, 1994). Chibalin et al, also found an insulin receptor tyrosine kinase inhibitor completely abolished insulin mediated phosphorylation of the ∝-subunit (Chibalin *et al.*, 2001). PI 3-kinase inhibitor, wortmannin also blocked insulin stimulated ∝-subunit translocation to the sarcolemma (Al-Khalili *et al.*, 2003). Lipid products of PI 3-kinase are known to been implicated in the insulin signalling cascade and activate PKC (Sweeney *et al.*, 2001). Therefore insulin regulation of Na^+K^+-ATPase follows a tyrosine kinase, PI 3-kinase and PKC activation pathway, refer to figure 2.4.

Recently, a study by Al-Khalili and collaborators found ERK1/2 MAP kinase to be involved in insulin stimulated Na^+K^+-ATPase activity, as an inhibitor of upstream kinase MEK1/2 stopped insulin mediated ∝-subunit phosphorylation and translocation to the membrane (Al-Khalili *et al.*, 2004). In addition, a PKC inhibitor completely blocked insulin stimulated ERK1/2 phosphorylation, indicating the kinase is most likely downstream from PKC, refer to figure 2.4.

Regulation of Na^+K^+-ATPase by PKC may be dependant on cytosolic Ca^{2+} concentration but this is controversial. PKC, like AMPK can regulate the pump via direct phosphorylation of the ∝ subunit at site Ser-23, which in turn causes endocytosis and inhibition of the pump (Chibalin *et al.*, 1998). However this is not the mechanism seen in skeletal muscle as PKC has been shown to activate Na^+K^+-ATPase activity in frog (Venosa, 2005) and rat (Li & Sperelakis, 1993; Sampson *et al.*, 1994).

cGMP-dependant protein kinase G (PKG), is activated by intracellular cGMP. In skeletal muscle, cGMP/PKG actions are antagonistic to those of cAMP/AMPK, with raised cGMP inhibiting Na^+K^+-ATPase activity (Li & Sperelakis, 1993). At present, it is unknown whether PKG regulation of Na^+K^+-ATPase activity is by direct phosphorylation or through secondary modulators (Therien & Blostein, 2000).

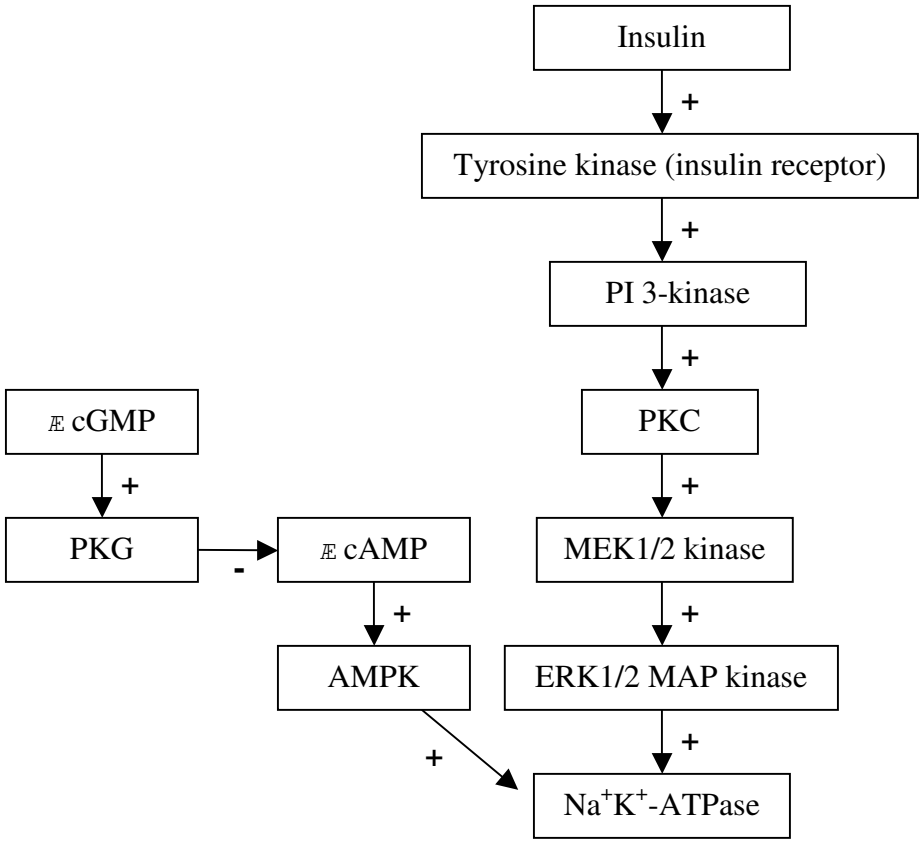

Figure 2.4 Proposed Na^+K^+-ATPase signalling pathway diagram.

Protein phosphatases 2B or PPB2 is a major participant in protein phosphatase-dependant regulation of Na^+K^+-ATPase. Protein phosphatases can actively reverse the effects of protein kinases on Na^+K^+-ATPase in various tissues, including skeletal muscle (MacDonald & Storey, 1999).

2.3.5 Inhibition

Principal inhibitors of the Na^+K^+-ATPase enzyme are the digitalis glycosides, digoxin and ouabain. These compounds have been repeatedly associated with a severe rise in plasma K^+ and are extensively used for treatment of cardiac insufficiency. The drugs are effective by binding to and blocking the K^+ binding site, repressing K^+ influx and Na^+ extrusion. Inhibition of cardiac Na^+K^+-ATPase causes a rise in intracellular Na^+, which effectively reduces Ca^{2+} efflux via the Na^+/Ca^{2+} Exchanger (Clausen, 1998). Elevated levels of cytoplasmic Ca^{2+} are accumulated in the sarcoplasmic reticulum (SR), leading to a greater Ca^{2+} release from the SR with each electric pulsation of the cardiac muscle.

K^+ deficiency induced by diet or diuretics also reduces Na^+K^+-ATPase content in skeletal muscle. The concentration of ^{3}H-ouabain binding sites has been positively correlated with tissue concentration of K^+. K^+ deficiency leads to a fall in plasma K^+ levels, which the body rectifies by releasing stored intracellular K^+ from skeletal muscle (Thompson & McDonough, 1996). The hypokalemic state is corrected but there is an associated decrease in active Na^+K^+-ATPase numbers. Treatment to reinstate normal K^+ content in skeletal muscle will also restore Na^+K^+-ATPase concentrations in skeletal muscle (Kjeldsen *et al.*, 1984b).

Another well known inhibitory regulator of Na^+K^+-ATPase is dopamine. This catecholamine is a natriuretic factor, known to inhibit Na^+K^+-ATPase activity in a variety of tissues, but particular regions of the kidney (Aperia *et al.*, 1987).

Reduced physical activity as a result of aging or disease causes a reduction in the content of Na^+K^+-ATPase in skeletal muscle (Norgaard *et al.*, 1990; Ng *et al.*, 2003). Immobilisation studies in animals and humans have also shown decreased concentrations of ^{3}H-ouabain binding sites in skeletal muscle (Kjeldsen *et al.*, 1986; Leivseth & Reikeras, 1994). The reduced Na^+K^+-ATPase capacity caused by inactivity may exacerbate exercise-induced hyperkalemia and fatigue often seen with disease states such as type II diabetes (Schneider *et al.*, 1984; Regensteiner *et al.*, 1995; Baldi *et al.*, 2003). However, Na^+K^+-ATPase content will adjust to meet with functional demands as seen in training or remobilising studies (Leivseth *et al.*, 1992; Jebens *et al.*, 1995) which suggests exercise as a potential treatment for diabetes.

2.4 Membrane Composition and Na^+K^+-ATPase

Since the Na^+K^+-ATPase is a membrane-embedded protein, the surrounding membrane phospholipids are important for the intrinsic function of the enzyme. The cell membrane is an important barrier that regulates the flow of fluid and molecules in and out of the cell. The phospholipid composition of membranes can influence the function of various proteins embedded within. However it is unclear whether the lipid-protein interactions are effective via direct contact and binding or indirectly by means of

order and fluidity. Species-crossover experiments have suggested that the molecular activity of Na^+K^+-ATPase is predominately modulated by the physical properties of its immediate lipid milieu (Wu *et al.*, 2001).

2.4.1 Membrane Phospholipids

Membranes are made up of amphiphilic molecules call phospholipids. The phospholipid structure consists of a 3-carbon glycerol backbone molecule to which 2 fatty acyl chains are attached and a phosphate group esterified to the third carbon of the glycerol.

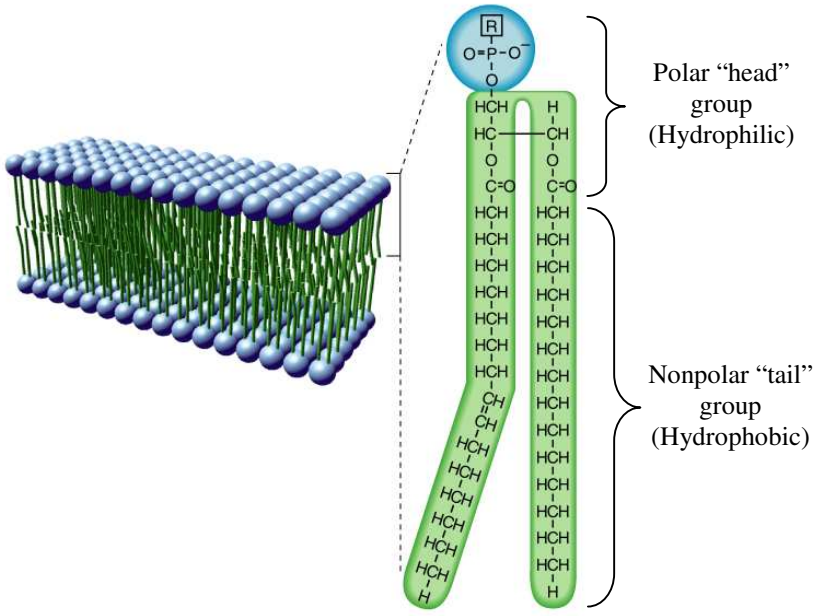

Figure 2.5 Bilayer membrane and Phospholipid structure. Modified from

Since the acyl chains are made up of carbon and hydrogen and share their electrons evenly, they have no charge and constitute the hydrophobic portion of the phospholipid, whereas electrons in phosphate head group are not shared equally, giving

this end of the molecule a hydrophilic nature (Mathews & van Holde, 1990). Different classes of phospholipids are characterised by the head-group esterified to the phosphate group and molecular species within the different classes differ by the position, length and extent of unsaturation of the acyl chains (Mathews & van Holde, 1990).

Phospholipids are held together only by weak hydrogen bonds between the heads and the even weaker interactions between the hydrophobic lipid molecules in the tails but these thousands of non-covalent interactions between the lipids make the membrane bilayer a sturdy enclosing structure (Mathews & van Holde, 1990). On the other hand, it is a well known phenomenon that the bilayer system is extremely dynamic. Phospholipid molecules can travel sideways along the membrane or flip from one side to the other, making the cell membrane flexible and continually moving and flowing (Mathews & van Holde, 1990).

2.4.2 Membrane Proteins

Essential components of the lipid membrane are membrane proteins. Membrane proteins contain both hydrophilic and hydrophobic areas and can penetrate completely through or partially into the phospholipid bilayer through an ∝ helix. The ∝-subunit of Na^+K^+-ATPase is thought to contain 10 transmembrane segments, spanning the membrane through ∝ helices (Tal *et al.*, 2001). The membrane is virtually impermeable to polar solutes such as glucose, minerals and ions because it is a costly process energetically. Therefore regulation of the translocation of solutes in and out of the cell is a significant function of membrane transport proteins with Na^+K^+-ATPase regulating Na^+ and K^+ counter transport.

2.4.3 Membrane Characteristics

Increasing research into the phospholipid composition of cell membranes has indicated that they are important for the function of transmembrane proteins (de Lima Santos *et al.*, 2005). Alterations in membrane fatty acyl chains and phospholipid compositions have been shown to influence membrane permeability, ion channels and transport, and cause a lowering of membrane-bound enzyme activities (Spector & Yorek, 1985). Past experiments have demonstrated that function and intrinsic activity of the membrane ion transporter, Na^+-K^+-ATPase is influenced by the lipid milieu in which it exists (Abeywardena *et al.*, 1983; Storch & Schachter, 1984). Modifications that can affect protein function are alteration of the phospholipid head group, acyl chain length and unsaturation, and insertion of cholesterol (Cantor, 1999). The effect of membrane lipid composition on Na^+K^+-ATPase function may explain irregular Na^+ ion transport observed in disease states with abnormal lipid metabolism such as atherosclerosis, obesity and diabetes (Hughes & York, 1983; Chen *et al.*, 1995; Makar *et al.*, 1995).

2.4.3.1 Fluidity and Bilayer Thickness

Fluidity is considered an important property of the membrane because of its effect on transmembrane protein conformation, transport processes and membrane permeability (Pilch *et al.*, 1980; Lynch *et al.*, 1987; Lande *et al.*, 1995). The degree of unsaturation and length of acyl chains in adjacent phospholipids affects lateral movement and conformational change of an integral protein (Wu *et al.*, 2001). While the length of acyl chains simply increases bilayer thickness, the double bonds in unsaturated acyl chains inhibit freedom of rotation about the bond and prevent phospholipids from compacting themselves. This creates a higher degree of conformational flexibility of a protein in the

membrane, improving efficiency of ligand binding and activity of membrane-bound enzymes (Hollan, 1996). A study using hepatocyte membranes reported an increase in lipid fluidity was accompanied by an increase in Na^+K^+-ATPase specific activity (Storch & Schachter, 1984).

However, there is growing evidence that the bilayer thickness and lipid acyl chain order rather than fluidity, are the primary factors determining enzyme activity (Cornelius, 2001). One study found Na^+K^+-ATPase activity measured by labelled Rubidium (K^+ analogue) uptake was inhibited when short acyl chain phosphatidylcholine (PC) was incorporated into erythrocytes compared with longer acyl chain PC (Dwight & Hendry, 1995), while another showed that increasing the fatty acid chain length in PC-liposomes increased the percentage of Na^+K^+-ATPase incorporated (de Lima Santos *et al.*, 2005) supporting the notion that bilayer thickness is an important factor for integral protein function.

The type of phospholipid head-group has also been associated with modulating membrane protein function. Negatively charged lipids such as phosphatidylserine and phosphatidylglycerol have been found to enhance bilayer thickness and fluidity, and increase Na^+K^+-ATPase activity (Kimelberg & Papahadjopoulos, 1972; Cornelius & Skou, 1984).

2.4.3.2 Membrane Permeability and Cholesterol

Membrane permeability is another important function of the membrane and can be seriously altered by the lipid interior. Excessive fluidity causes non-selective cation leakage while closely packed lipids and rigidness make translocation of solutes across the

membrane difficult (Armstrong *et al.*, 2003). Integrated cholesterol is known to promote membrane rigidness and bilayer thickness by increasing acyl chain order. Cholesterol restricts the bending action of neighbouring phospholipid acyl chains, forcing the chain to be elongated (Cornelius, 2001). A deficiency in cholesterol causes excessive fluidity and non-selective cation permeability (Grunze & Deuticke, 1974). Polyunsaturated fatty acids (PUFAs) allow a higher incorporation of cholesterol (rigidness) in the membranes to balance their fluidity and provide satisfactory permeability (Colin *et al.*, 2003). By increasing bilayer thickness, cholesterol may function to compensate for short acyl chains in membranes. A study by Cornelius, found that in the absence of cholesterol, the phospholipid which sustained optimal Na^+K^+-ATPase activity was C22:1 PC, yet in the presence of cholesterol, the phospholipid was substituted with a saturated PC of shorter acyl chain length, 18:0 (Cornelius, 2001). Optimal Na^+K^+-ATPase activity was reported as being dependent on the hydrophobic thickness of the bilayer, however the class of phospholipids chosen for the study was not optimal for Na^+K^+-ATPase activity.

Obesity and type II diabetes are also associated with changes in membrane phospholipid composition with studies suggesting decreased fluidity contributes to the development of insulin resistance (Manco *et al.*, 2004). PUFAs may prevent the pathogenesis of insulin resistance by alterations in cell membrane fluidity which alters the expression and/or presentation of a wide range of receptors, including the GLUT-4 and insulin receptors (Das, 2005). PUFAs enhance insulin action by increasing the number of

insulin receptors and the affinity of insulin to its receptors (Hainault *et al.*, 1993; Mori *et al.*, 1997).

2.4.3.3 Omega Fatty Acids

Membrane fluidity is increased by phospholipids composed of PUFAs, in particular omega-3 PUFAs. Specifically, docosahexaenoic acid (DHA) contains 22 carbons and 6 double bonds and is the longest and most unsaturated fatty acid commonly present in biological membranes. Mammals must synthesise DHA by elongation of ∝-linolenic acid through a series of enzymatic reactions involving elongases and desaturases (Petrache *et al.*, 2001). DHA plays an important structural role in the cytoplasmic membrane of cells and has been shown to increase the unsaturation index, disordering and fluidity (Hashimoto *et al.*, 1999; Petrache *et al.*, 2001). Omega-6 PUFAs have been associated with inhibiting the absorbance of omega-3 PUFAs, in a competitive nature (Iritani & Fujikawa, 1982). The net result is an increase in omega-6/omega-3 ratio, which reflects reduced membrane fluidity (Farkas *et al.*, 2002). There is also evidence to suggest a high omega-6/omega-3 ratio is deleterious on insulin action in skeletal muscle (Storlien *et al.*, 1996). The significance of omega-3 is widely recognised, with deficient diets associated with numerous diseases, including insulin resistance in humans and rats (Wu *et al.*, 2001) while lard diets high in DHA and eicosapentaenoic acid (EPA) prevent insulin resistance and obesity (Hainault *et al.*, 1993; Mori *et al.*, 1997; Taouis *et al.*, 2002). However the mechanisms sustaining such a protective effect of omega-3 PUFA remain unclear.

2.5 Obesity and Type II Diabetes

Diabetes is characterised by excessively high blood glucose and occurs in 2 forms. In Type I diabetes previously known as Insulin Dependent Diabetes Mellitus, the pancreatic β-cells produce little or no insulin. Type II diabetes is characterised by varying degrees of insulin resistance and relative insulin deficiency. There are currently 150 million people suffering diabetes worldwide, and this number is expected to rise to 300 million by 2025 (Zimmet *et al.*, 2001). In Australia, the AusDiab study reported 7.4% of the population with diabetes, 90% of which have type II diabetes (Dunstan *et al.*, 2002), and many others are pre-diabetic or undiagnosed. Research has shown that for every person diagnosed with diabetes there is another who has it but has not yet been diagnosed (Colagiuri, 2002). The reason for this is the progressive nature of the disease. It involves a slow and late onset due to a gradual build up of glucose in the blood stream as a consequence of insulin resistance. Most are not diagnosed until after the age of 40 years. The evidence is overwhelming that adverse lifestyle factors are the cause of type II diabetes, which is becoming an ever-increasing health problem. There is general agreement that obesity is closely associated with type II diabetes with 80% of people with type II diabetes being overweight or obese.

2.5.1 Pathophysiology and Complications

The pathophysiology of type II diabetes appears to involve defects in both insulin action (insulin resistance) and secretion (insulin deficiency). Depending on the severity, the disease does not necessarily involve insulin injections, however it is a dynamic disease in which untreated individuals often become more insulin deficient with time.

The healthcare problem of type II diabetes is further exacerbated by its association with obesity and cardiovascular risk factors, particularly dyslipidaemia and hypertension (Stolar & Chilton, 2003). Complications associated with type II diabetes often set in prior to diagnosis as hyperglycemia is often not severe enough for the individual to notice symptoms. The disease-associated complications involve microvascular complications such as retinopathy (blindness), neuropathy (decreased nerve activity) and nephropathy (kidney disease), and macrovascular complications including cardiovascular disease, stroke and premature atherosclerotic vascular disease (Stolar & Chilton, 2003). Not all diabetics develop all the complications nor to the same degree. However, cardiovascular disease is undoubtedly the leading cause of mortality and morbidity in type II diabetes, even the pre-diabetic state of hyperinsulinemia and impaired glucose tolerance poses a considerable cardiovascular risk (Zimmet *et al.*, 2001).

2.5.2 Insulin resistance

Insulin resistance is defined as a decreased biological response of peripheral target tissues to normal concentrations of circulating insulin. β-cells in the pancreas compensate by releasing more insulin to maintain euglycemia, but overtime are unable to maintain high rates of insulin secretion and hyperglycemia results (Goldstein, 2002), refer to figure 2.6. The condition is found in both individuals suffering obesity and type II diabetes and also in normal individuals as it is the primary defect, preceding type II diabetes by up to 20 year. Insulin resistance is closely associated with a variety of cardiovascular risk factors including glucose intolerance, visceral adiposity, dyslipidemia and hypertension.

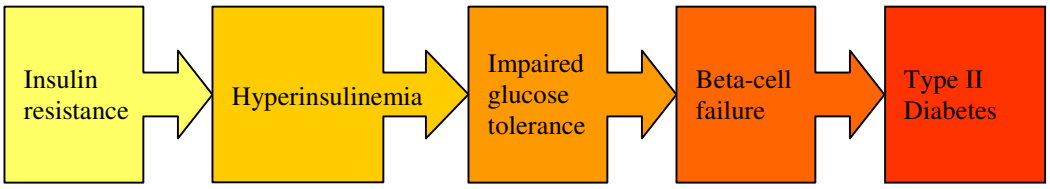

Figure 2.6 Progression to Type II diabetes. Modified from (Rao *et al.*, 2004).

2.5.2.1 Skeletal Muscle

Skeletal muscle is the primary tissue affected by insulin resistance as it is the main insulin target tissue and performs most glucose up-take via glucose transporter 4 (GLUT4) (Zierath *et al.*, 2000). Impaired glucose uptake in skeletal muscle is not due to abnormal concentrations of GLUT4 in insulin resistant skeletal muscles but presumably caused by a reduction in insulin stimulated translocation of GLUT4 from intracellular vesicles to the membrane. The defect in insulin action is at the cellular level though the faulty signalling pathway is still unclear. Insulin resistance also reduces fat oxidation capacity in skeletal muscle and therefore increases circulating free fatty acid (FFA) levels and lipid storage including triglycerides dispersed within muscle (Kelley & Simoneau, 1994). Several studies have shown insulin resistance to have a tighter correlation with the amount of intramuscular triglycerides (IMTGs) rather than other common markers, such as body mass index, waist-to-hip ratio or total body fat (McGarry, 2002).

2.5.2.2 Adipose Tissue

Insulin resistance also affects adipose tissue which fails to respond to insulin action causing altered fatty acid (F.A) metabolism. Obesity is strongly associated with insulin resistance. Additional visceral adipose tissue causes increased lipolysis and the secretion of excessive FFA into circulation (Bjorntorp, 1990; Nielsen *et al.*, 2004). Increased

plasma FFA compete with glucose for utilisation in skeletal muscle, resulting in increased blood glucose concentration which provides the stimulus for further increased insulin secretion (Karpe & Tan, 2005). Excessive F.A circulation can also cause dyslipidemia and vascular dysfunction (Ginsberg, 2000) and interfere with insulin signalling directly and impair hepatic insulin removal, exaggerating the degree of hyperinsulinemia (Bergman, 2000; Goodpaster & Kelley, 2002).

2.5.2.3 Hypertension

Insulin resistance is frequently associated with hypertension. Non-obese and non-diabetic patients with essential hypertension (hypertension without a known cause) have been shown to exhibit greater insulin resistance and elevated insulin levels when compared to normal controls. However the mechanism by which insulin exerts its effect is not fully understood (Shen *et al.*, 1988; Pollare *et al.*, 1990). Serum digitalis-like factor (DLF) is a Na^+K^+-ATPase inhibitor and functions to increase Na^+ removal by decreasing renal Na^+ reabsorption and increase vascular resistance by increasing cardiac output and therefore systolic blood pressure. Hyperinsulinemia has been shown to elevate circulating DLF in healthy controls (Carroll *et al.*, 2001). This may explain a possible mechanism by which chronically elevated insulin levels may cause hypertension, however not all individuals with insulin resistance display hypertension (Levy *et al.*, 1994; Reaven, 2003).

2.5.2.4 Na^+K^+-ATPase dysfunction

Type II diabetes and insulin resistance is frequently accompanied by altered regulation of the Na^+-K^+-ATPase enzyme. Modification in Na^+K^+-ATPase activity may be the

result of structural changes of binding sites or altered content in the cell membrane. Na^+K^+-ATPase concentration can vary through altered transcriptional, translational or post-translation degradation. However, disagreement exists over the effect of insulin resistance on Na^+-K^+-ATPase function with numerous studies showing conflicting or no change with insulin resistance in various tissue types of both humans and rats. Na^+K^+-ATPase activity has been shown to be decreased in human erythrocytes (Baldini *et al.*, 1989; Kiziltunc *et al.*, 1997), nerve tissue (Scarpini *et al.*, 1993), platelets (Rabini *et al.*, 1998) and retinal cells (Ottlecz *et al.*, 1993) from type II diabetics patients. While other studies have reported no change in human erythrocytes (Jannot *et al.*, 2002) and rat skeletal muscle (Banyasz & Kovacs, 1996; Ferrer-Martinez *et al.*, 1996).

Na^+K^+-ATPase concentration in human skeletal muscle has previously been reported to be higher with type II diabetes (Schmidt *et al.*, 1994). However the author also pointed out that the mean concentration for the normal muscle was less than previously reported ranges. If a mean value for normal muscle from previous studies by this group was used then this elevated concentration would not have been reported. Djurhuus and colleagues (2001) conducted a study involving identical twins discordant for type II diabetes, where the diabetic twin group demonstrated an approximate 20% reduction in muscle [^{3}H]-ouabain binding capacity compared to the healthy twin group. The ^{3}H-ouabain binding capacity difference within twin pairs was negatively correlated with the corresponding difference in waist/hip ratio, indicating the development of abdominal fat in type II diabetics is associated with reduced muscle Na^+K^+-ATPase concentration.

As previously mentioned the Na^+K^+-ATPase has been repeatedly shown to preferentially use glycolytically derived ATP in a variety of tissues, including skeletal muscle (Okamoto *et al.*, 2001; Dutka & Lamb, 2007), whereby an increase in Na^+K^+-ATPase activity is associated with an increase in aerobic glycolysis. This suggests that when glycolysis is inhibited, Na^+K^+-ATPase activity may be impaired. This may offer a possible explanation for the compromised Na+K+-ATPase activity seen with insulin resistance.

Obesity and type II diabetes are also associated with changes in membrane phospholipid composition which may explain the different effects seen with Na^+K^+-ATPase function (Borkman *et al.*, 1993; Pan *et al.*, 1994). Studies of insulin deficiency have demonstrated a relationship between insulin and F.A desaturase activity, the enzyme responsible for inserting double bonds (Tilvis & Miettinen, 1985; el Boustani *et al.*, 1989), whilst others have shown relationships exist between the F.A composition of phospholipids in skeletal muscle and the action of insulin (Vessby *et al.*, 1994; Clore *et al.*, 1998). Reducing desaturase activity may contribute to impaired insulin action by decreasing the amounts of PUFA in the membrane. This may then influence the action of insulin by modulating the function of membrane proteins that mediate insulin action such as insulin receptors and glucose transporters. It may alter Na^+K^+-ATPase function through direct effects on the physical properties of the surrounding lipid environment.

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2.5.2.5 Treatments

Diabetes has reached epidemic proportions in many populations and the growing prevalence is posing a massive health problem worldwide. Although type II diabetes is known to be a lifestyle-related condition, healthcare resources are largely spent on treatment with little emphasis on preventive care. Treatment of the disease is usually with dietary and exercise advice as well as anti diabetic medications, which are generally needed for the rest of the patient's life and often in increasing doses (Rosenberg *et al.*, 2005). Drug medications effective in lowering blood glucose levels include sulfonylureas, meglitinides, biguanides (metformin), alpha-glucosidase inhibitors and thiazolidinediones, however none are without side-effects and consequences of long term use are not clear (Luna & Feinglos, 2001; Mayerson & Inzucchi, 2002). Current evidence strongly suggests that environmental and behavioural factors are major determinants of the increasing rates of diabetes (Zimmet *et al.*, 2001). Obesity and physical inactivity are the two main risk factors for the development of insulin resistance and ultimately type II diabetes (Franz *et al.*, 2003; Sullivan *et al.*, 2005). With around 56% of the Australian adult population being either overweight or obese (Baur, 2001), focusing on these factors offers an opportunity for preventing diabetes. Two major intervention studies, the American and the Finnish prevention study, used moderate exercise and modified diet to demonstrate a similar 58% decline in the incidence of type II diabetes (Tuomilehto *et al.*, 2001; Knowler *et al.*, 2002).

2.6 Thiazolidinediones

Obesity-related insulin resistance is a characteristic feature of type II diabetes and often the persistence of obesity interferes with conventional therapy such as diet and exercise. In these cases, a pharmacological approach is commonly used to reinstate normoglycemia. Thiazolidinediones (TZDs) are a class of compounds that function as insulin sensitizers and are expected to be used by an estimated 50 000 diabetics as an oral antidiabetic drug (Burton, 2003; Ferre, 2004). Many studies have shown administration of TZDs improves hyperglycaemia and hyperinsulinemia in insulin resistant rodent models and Type II diabetic patients (Saltiel & Olefsky, 1996; Olefsky, 2000). TZDs have additional positive effects on F.A metabolism by decreasing TG, FFA and LDL-cholesterol levels, whilst increasing plasma HDL-cholesterol concentrations (Komers & Vrana, 1998; Wilmsen *et al.*, 2003). Sensitised skeletal muscle might increase the known insulin-induced Na^+-K^+-ATPase function (Al-Khalili *et al.*, 2003), however, TZDs have also been shown to cause undesirable side effects including altered membrane phospholipid composition which is integral to normal Na^+-K^+-ATPase function (Komers & Vrana, 1998).

2.6.1 Mechanisms of Action

TZDs function via agonistic action on the nuclear peroxisome proliferator-activated receptor-γ (PPAR-γ) (Spiegelman, 1998). These receptors are expressed on intra-nuclear transcription factors in insulin target tissues; skeletal muscle, adipose tissue and liver and are activated by natural ligands, F.As and their derivatives (Ricote *et al.*, 1998; Wahli, 2002). Once TZD bind to the transcription factor, the activated PPAR-γ

complex regulates the transcription of genes involving lipids (refer to figure 2.7), whereby stimulation or inhibition is dependant on the specific gene and tissue concerned (Lebovitz, 2002).

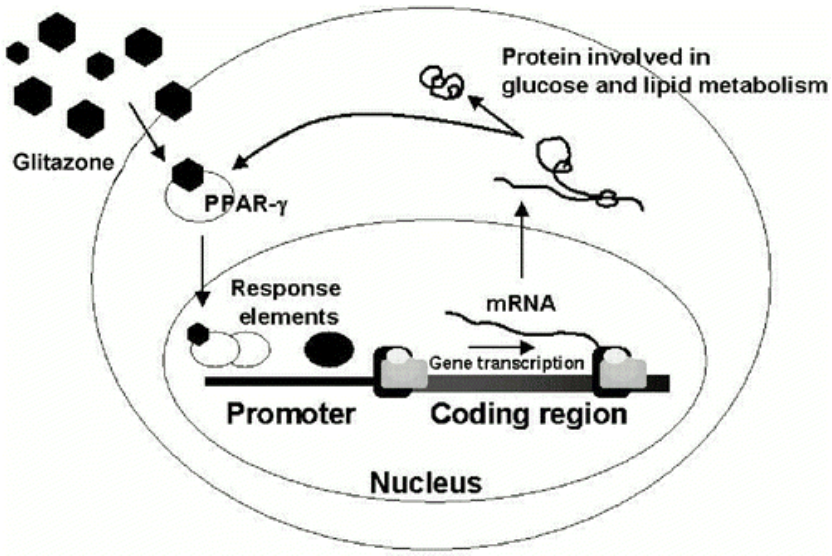

Figure 2.7 Activation of PPAR-γ nuclear receptor (Mudaliar & Henry, 2001)

TZDs ameliorate insulin resistance by regulating a series of genes involved in altering lipid metabolism, adipose tissue differentiation, and insulin action (Lebovitz, 2002). Many of the specific gene changes elicited by TZD are at present still unknown, however studies have linked PPAR-γ gene allele variations to obesity and type II diabetes (Argmann *et al.*, 2005; Ghoussaini *et al.*, 2005).

Although PPAR-γ is highly expressed in white and brown adipose tissue, the insulin sensitising effect of TZDs is largely seen in skeletal muscle (Vidal-Puig *et al.*, 1997). There are 3 proposed explanations for this phenomenon (Kliewer *et al.*, 2001). 1. PPAR-γ may be responsible for activating the release of signalling molecules from

adipocytes which in turn affect glucose metabolism in other tissues, such as skeletal muscle. 2. PPAR-γ reduces serum lipid concentrations and this has a secondary effect on glucose utilisation in skeletal muscle as high circulating FFA and TG are known to inhibit glucose utilisation. 3. PPAR-γ may have direct effect on glucose metabolism in skeletal muscle which is why they are expressed at low levels. Most data supports the hypothesis that insulin sensitising effects of TZDs are secondary to altered adipose tissue metabolism. However the mechanism underlying the pharmaceutical action of these drugs remains poorly understood (Kahn *et al.*, 2000).

2.6.2 Rosiglitazone

Rosiglitazone is a TZD derivative and a PPAR-γ agonist that is better known as Avandia on the market (Cuzzocrea *et al.*, 2004). It differs from other members of the TZDs class, such as troglitazone and pioglitazone, by carrying a different side chain (see figure 2.8) (Mudaliar & Henry, 2001) and displaying a higher affinity for PPAR-γ (Young *et al.*, 1998).

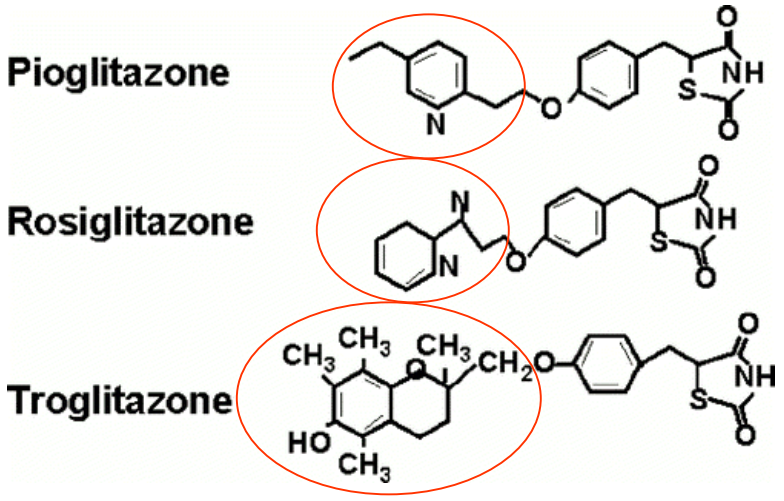

Figure 2.8 Structures of TZD agents. Modified from (Mudaliar & Henry, 2001)

The pharmacokinetic profile of rosiglitazone is not altered by food intake and the compound is fully metabolized with no unchanged drug detected in urine (Mudaliar & Henry, 2001). Diabetic patients commence rosiglitazone treatment with a 4 mg oral dose daily but may increase to 8 mg daily if an inadequate response is seen after 12 weeks of treatment (Mudaliar & Henry, 2001). Rosiglitazone increases insulin sensitivity by enhancing glucose metabolism in muscle and decreasing glucose biosynthesis in the liver. Like the other TZDs, rosiglitazone is thought to ameliorate insulin resistance by altering lipid metabolism (Miyazaki *et al.*, 2001). Some of these changes include decreased hepatic secretions of lipids into plasma, induced de novo F.A synthesis and lipid uptake in muscle (Watkins *et al.*, 2002; Ferrannini, 2005). A fall in circulating FFA reduces the competition with glucose for utilisation in skeletal muscle, resulting in decreased blood glucose concentrations (Ferrannini, 2005).

2.6.2.1 Benefits

The major effect of rosiglitazone treatment is the improvement in insulin action, resulting in decreased insulin resistance. Rosiglitazone has been reported to restore insulin's ability to suppress hepatic gluconeogenesis and improve peripheral glucose uptake, without stimulating insulin production (Miyazaki *et al.*, 2001; Carey *et al.*, 2002; Wagstaff & Goa, 2002). In a study by Miyazaki and colleagues, rosiglitazone treatment (8 mg/day) for 12 weeks significantly decreased fasting plasma glucose levels and glycated haemoglobin 1c without changes in plasma insulin concentrations (Miyazaki *et al.*, 2001). They also showed a decrease in basal endogenous glucose production and an increase in whole body glucose clearance rate.

Rosiglitazone has also been shown to reduce circulating FFA and TG (Mayerson *et al.*, 2002). Rosiglitazone stimulates the differentiation of stem cells within adipose tissue into mature adipocytes, promoting FFA and TG uptake and storage. This action in humans is specific to stem cells in peripheral subcutaneous adipose, rosiglitazone has little or no effect on stem cells in visceral adipose tissue (Lebovitz, 2002). Therefore redistributed fat in a favourable direction and lessening visceral adiposity, a major driver of type II diabetes (Pedersen *et al.*, 1993). Although it seems rosiglitazone, at least partly exerts its insulin-sensitising effect via PPAR-γ action, it may also involve a PPAR-independent mechanism, involving AMPK via the AMP/ATP-dependent pathway (Fryer *et al.*, 2002; Feinstein *et al.*, 2005; Lessard *et al.*, 2006).

In addition to its beneficial effects on glucose and lipid metabolism, rosiglitazone has been associated with other advantageous affects. Bakris et al found that rosiglitazone at 4 mg twice daily significantly reduced systolic blood pressure and diastolic blood pressure in type II diabetic patients (Bakris *et al.*, 2003).

2.6.2.2 Side effects

In general, TZDs have been shown to cause excessive weight gain and fluid retention (and oedema) increasing the risk of heart failure. Other reported adverse effects include reduced hematocrit and white blood cell count, altered liver function and hepatotoxicity (Sood *et al.*, 2000).

Haemoglobin and hematocrit counts are reduced in patients with rosiglitazone treatment in a dose-related fashion (Mudaliar & Henry, 2001). Fluid retention is the most common

drug-related side effect of rosiglitazone in clinical practice and may be the cause of decreased hemoglobin/hematocrit via hemodilution (Wagstaff & Goa, 2002). However, available data is conflicting and does not completely support this concept. Other researchers have suggested fluid retention and tissue oedema seem to be part of a vascular 'leak' syndrome (Scheen, 2004). A large clinical trial with rosiglitazone reported peripheral oedema occurred in 4.8% of patients receiving rosiglitazone alone, with a higher incidence (14.7%) occurring with combined insulin therapy compared to 1.3% receiving placebo (Hollenberg, 2003). Patients treated with rosiglitazone have also shown slight decreases in white blood cell counts (Haffner *et al.*, 2002).

Fluid retention by glitazones can reveal or aggravate congestive heart failure, an issue of particular concern in patients with type II diabetes who are insulin-treated or have pre-existing heart failure. This may limit the therapeutic application of rosiglitazone in the prevention of cardiovascular disease.

Rosiglitazone treatment for 12-52 weeks has been reported to increase mean body weight by 1 to 3 kg (Hollenberg, 2003). The increase in body weight with rosiglitazone is due to increased peripheral subcutaneous and not visceral fat masses (Carey *et al.*, 2002). Even though the fat is redistributed in a favourable direction, no long-term follow-up is yet available and weight gain can only exacerbate the problem of pre-existing obesity, furthering the struggle to be physically active (Ferre, 2004). The mechanism of weight gain remains unclear but probably involves a combination of fluid retention and fat accumulation.

Other TZDs, such as troglitazone and pioglitazone have been shown to repress the gene expression of stearoyl-CoA desaturase 1, reducing the biosynthesis of PUFA from saturated F.As (Kurebayashi *et al.*, 1997). Modification of membrane phospholipids can alter membrane properties such as fluidity, permeability and membrane ion transport (Komers & Vrana, 1998). The intrinsic activity of Na^+-K^+-ATPase, is dependant upon the membrane lipid milieu surrounding it.

With potential links to congestive heart failure and liver failure, little research has been done on the side effects and chronic effects of rosiglitazone. It is a research area which very much requires attention to determine the safe use of these new insulin sensitisers in patients with type II diabetes.

2.7 Exercise

With the prevalence of type II diabetes expected to double in the next 25 years (Dunstan *et al.*, 2002; Agarwal *et al.*, 2005), healthcare resources need to be spent on preventive care. Focusing on environmental and behavioural factors such as a sedentary lifestyle and obesity, the major determinants of the increasing rates of diabetes, offers an opportunity for preventing diabetes (Zimmet *et al.*, 2001). When thinking prevention, there is always a tendency to concentrate on diet. Whilst this is important, particularly in reducing dietary fats, the overwhelming evidence is that the main cause of positive energy balance in weight gain is due to inactivity (Wei *et al.*, 2000; Tuomilehto *et al.*, 2001; Knowler *et al.*, 2002; Troiano, 2002).

It has been well documented that with regular exercise emerge physiological and biochemical benefits. Studies have shown exercise training improves insulin sensitivity and increases insulin-stimulated skeletal muscle glucose transport in insulin resistant animal models and type II diabetics (Ivy *et al.*, 1989; Perseghin *et al.*, 1996). Acute bouts of exercise only provide temporary improvements in insulin sensitivity with benefits lasting up to 48 hours (Ivy *et al.*, 1983; Etgen *et al.*, 1993), however increasing evidence shows chronic exercise training induces metabolic adaptations that cause improved insulin action in insulin resistant skeletal muscle (Hawley, 2004).

Several studies have also demonstrated an increase in Na^+K^+-ATPase concentration with exercise in skeletal muscle of lean animal models (Kjeldsen *et al.*, 1986; Tsakiridis *et al.*, 1996) and healthy individuals (Green *et al.*, 1993; McKenna *et al.*, 1993; Madsen *et al.*, 1994). However, no one has investigated the potential benefits of exercise on Na^+K^+-ATPase in skeletal muscle of insulin resistant animals or Type II diabetics.

2.7.1 Acute Response

Na^+K^+-ATPase is rapidly activated within seconds following excitation and contraction in skeletal muscle through a combination of electrical, ionic and hormonal factors. Previous studies have shown the up-regulation of Na^+K^+-ATPase activity however the mechanism is not clear. Studies using electrical stimulation at 60 or 120 Hz for 10 seconds in rat soleus muscle found an increase in Na^+K^+-ATPase activity by 12 to 22-fold (Everts & Clausen, 1994; Nielsen & Clausen, 1997). After electrical stimulation, there is an initial rise in intracellular [Na^+] due to leakage but this is then followed by an 'undershoot' below the resting level. This may indicate that Na^+K^+-ATPase activation is

elicited by a rise in it's affinity for intracellular Na^+ (Juel, 1986; Buchanan *et al.*, 2002). Some studies have claimed the increase in activity is due to acute subunit translocation to the membrane from an unidentified intracellular location (Tsakiridis *et al.*, 1996; Juel *et al.*, 2000; Juel *et al.*, 2001). In humans, a short high intensity bout of exercise significantly increased the plasma membrane content of the $\propto_2$-, total $\propto$ and β_1-subunits (Juel *et al.*, 2000). Similar findings have been found in animal studies (Juel *et al.*, 2001). Murphy and colleagues showed a single bout of prolonged submaximal exercise increased mRNA expression of $\propto_1$, $\propto_3$ and β_2 isoforms, and protein abundance of the $\propto_3$ (Murphy *et al.*, 2006). One study showed 1 hour of treadmill running in rats caused a significant increase in the plasma membrane content of $\propto_1$- and $\propto_2$-subunits and elevation of the $\propto_1$- and β_2-subunit mRNAs, however no difference in subunit content was found in the internal membrane fraction or total membranes (Tsakiridis *et al.*, 1996). However, more recently, McKenna and colleagues have challenged this theory, reporting conflicting results that indicate subunit translocation to the membrane does not take place (McKenna *et al.*, 2003).

2.7.2 Long term Effect

Na^+K^+-ATPase is regulated in a physical activity dependant manner, in which exercise training causes upregulation and inactivity leads to down-regulation of Na^+K^+-ATPase concentration (Clausen, 1998). Chronic exercise training has been shown to increase Na^+K^+-ATPase content in skeletal muscle whereby a relatively intense level of physical activity is required (Madsen *et al.*, 1994; Evertsen *et al.*, 1997). Elevated sarcolemma Na^+K^+-ATPase content would increase K^+ uptake and help alleviate exercise induced

hyperkalemia, therefore enhance muscle performance and delay fatigue. A study by Harmer et al, showed improved K^+ regulation with exercise training, whereby hyperkalemia was reduced during intense exercise after sprint training (Harmer *et al.*, 2000) and other studies have reported a 16% increase in the concentration of Na^+K^+-ATPase in human skeletal muscle with sprint training (McKenna *et al.*, 1993) and approximately 15% with endurance training (Green *et al.*, 1993; Madsen *et al.*, 1994; Fraser *et al.*, 2002).

However the effect of exercise training can be reversed as inactivity causes down-regulation of Na^+K^+-ATPase content. In a rat study by Kjeldsen et al, detraining after 6 weeks of endurance training caused a decrease in Na^+K^+-ATPase content to pre-training levels in skeletal muscle (Kjeldsen *et al.*, 1986). The study also reported a 20% reduction in the [^{3}H]-ouabain binding site content in rat muscle when inactivity was induced by limb immobilization (Kjeldsen *et al.*, 1986). In humans, a 26.6% decrease was reported in the Na^+K^+-ATPase content of the deltoid muscle from patients with shoulder impingement (Leivseth & Reikeras, 1994).

2.7.3 Exercise and Diabetes

The effect of insufficient Na^+K^+-ATPase activity and raised extracellular [K^+] was well demonstrated by Clausen and colleagues in isolated rat skeletal muscle, when extracellular [K^+] was raised to 12.5mM and there was a 95% loss in contractile force (Clausen *et al.*, 1993). Early muscular fatigue can compromise exercise capacity and is likely to impact on the ability to perform activities of daily living. Type II diabetics have been known to have reduced exercise capacity, with impaired maximal oxygen

consumption (VO_{2max}) and increased submaximal VO_2 during exercise compared to healthy controls (Schneider *et al.*, 1984; Regensteiner *et al.*, 1995; Baldi *et al.*, 2003).

The mechanism by which exercise improves skeletal muscle insulin action with respect to glucose metabolism are fairly well described, including increases in GLUT4 protein (Houmard *et al.*, 1993).

2.8 Animal Models of Insulin Resistance

The progressive nature of insulin resistance and its associated chronic metabolic changes have been made easier to study with the use of suitable animal models of insulin resistance. The appropriate experimental animal model is required to depict the inevitable progression of increased weight (obesity) and insulin resistance, impaired glucose tolerance and on to frank diabetes with associated cardiovascular risk factors. Given that dietary factors are important in the aetiology of both insulin resistance and obesity in humans, an over-eating or high-fat fed rat model can be used to simulate the disease in humans.

2.8.1 Obese Zucker Rat

The obese Zucker rat is a well established experimental model of insulin resistance and morbid obesity that increases the likelihood of Type II diabetes. The autosomal homozygous recessive genotype (fa/fa) of the obese Zucker causes the rat to develop faulty leptin receptors (Phillips *et al.*, 1996; Tirabassi *et al.*, 2004). Leptin is an adipose-specific hormone that contributes to appetite regulation. Obese Zucker rats acquire hyperphagia, hyperinsulinemia, insulin resistance, glucose intolerance and ultimately type

II diabetes (Shafrir, 1992). The rats also develop other associated features of type II diabetes such as hyperlipidemia and hypertension. Defects in insulin receptor signalling have been described at both receptor and post-receptor sites (Ahmad & Goldstein, 1995).

2.8.2 High Fat Fed Rats

Rats that are fed high fat diets, are generally well accepted as a valid rodent model for insulin resistance, obesity and dyslipidemia. High levels of some dietary fats can cause insulin resistance in rats in as little as 4 weeks. Storlien et al, compared the effect of different high-fat diets on insulin sensitivity in rats and found that diets high in saturated, monounsaturated and omega-6 PUFAs induced severe insulin resistance, whilst a diet including long chained omega-3 PUFAs normalised insulin action (Storlien *et al.*, 1991). Another study also demonstrated the importance of omega-3 by reporting a diet deficient in omega-3 fed to rats induced insulin resistance, hypertension and obesity (Barnard *et al.*, 1998). High-fat feeding in rats displays skeletal muscle defects in insulin signalling and disregulated metabolism that are seen in insulin resistant humans (Kraegen *et al.*, 1991). Limited research has been done involving Na^+K^+-ATPase and diet induced models of diabetes, however, Na^+K^+-ATPase activity has been found to be compromised in skeletal muscle and liver from lard-fed rats (Takeuchi *et al.*, 1995). The growing prevalence of this disease and its strong association with obesity indicate that lifestyle factors make an important contribution to its onset and thus, the high fat fed rat represents a well suited model for studying insulin resistance.

2.8.3 Other Insulin Resistant Rat models

Sugar-enriched diets also produce hepatic insulin resistance in rats. Chronic feeding of sucrose or fructose to normal rats causes impaired glucose tolerance, loss of tissue sensitivity to insulin, hyperinsulinemia and hypertension independently of obesity (Thirunavukkarasu *et al.*, 2004). Fructose infusion in humans has been shown to result in impaired insulin regulation of glucose production (Wei & Pagliassotti, 2004).

An injection of streptozotocin (STZ) (80-90 mg/kg body weight) into neonatal rats causes glucose intolerance when these animals reach adulthood (Portha *et al.*, 1989). However, a faster alternative and easier approach to the study of type II diabetes is the administration of a single sub-diabetogenic amount of streptozotocin (40 mg/kg body weight) to the adult rat (Rasschaert & Malaisse, 1993; Ramesh & Pugalendi, 2006). All the same, both models represent an unnatural mode of acquiring type II diabetes and do not simulate the natural progression of insulin resistance and hyperinsulinemia in the human population. Therefore the drug induced model was not chosen for use in this project.

Glucocorticoid treatment causing insulin resistance to cellular glucose uptake is a well known phenomenon. Recently, a study showed Dexamethasone, a synthetic glucocorticoid provoked insulin resistance to cellular uptake of K^+ in rat skeletal muscle despite a significant increase in Na^+K^+-ATPase $\propto_2$ isoform levels (Rhee *et al.*, 2004). Nevertheless, acquiring obesity related insulin resistance through a pharmacological approach is not physiological in many respects.

2.9 Aims and Hypotheses

2.9.1 General Aims

This thesis investigated the effect of insulin resistance on Na^+K^+-ATPase function in skeletal muscle, and the effect of rosiglitazone and/or exercise training on Na^+K^+-ATPase function in insulin resistant skeletal muscle. Biochemistry techniques used to measure Na^+K^+-ATPase activity and content were maximal K^+-stimulated 3-*O*-MFPase activity in crude muscle homogenates and vanadate facilitated [^{3}H]-ouabain binding site using whole tissue, respectively. Since insulin is a major regulator of Na^+-K^+-ATPase function, the first study of the thesis investigated the effect of insulin resistance on

Na^+K^+-ATPase function in skeletal muscle using 2 animal models of insulin resistance and morbid obesity, the obese zucker rat and high fat fed Sprague Dawley (Chapter 4). Rosiglitazone is an oral antidiabetic drug that improves hyperglycaemia and hyperinsulinemia in insulin resistant and type II diabetic patients via insulin sensitising mechanisms (Lebovitz *et al.*, 2001; Hung *et al.*, 2005) but may also have a detrimental effect on membrane lipids which is integral to the functioning of Na^+-K^+-ATPase. Therefore the second study (Chapter 5) investigated the effects of rosiglitazone on Na^+-K^+-ATPase function in obese zucker skeletal muscle. Chronic exercise training has been shown to decrease skeletal muscle insulin resistance in insulin resistant animal models (Ivy *et al.*, 1989) and diabetics (Perseghin *et al.*, 1996) as well as upregulate Na^+-K^+-ATPase content in lean skeletal muscle (Green *et al.*, 1993; Evertsen *et al.*, 1997). However no study has investigated the effect of exercise on Na^+-K^+-ATPase in insulin resistant skeletal muscle. The third study (Chapter 6) investigated the effect of

rosiglitazone treatment and exercise training on Na^+-K^+-ATPase function in insulin resistant skeletal muscle of high fat fed Sprague Dawley rats. This study aimed to determine whether exercise or rosiglitazone would alleviate the detrimental effect of insulin resistance on Na^+-K^+-ATPase function and since exercise and rosiglitazone treatment improve insulin sensitivity via different mechanisms, whether the two effects of the therapeutic treatments would be additive.

2.9.2 Hypotheses

The hypotheses tested in this thesis were:

1. Na^+K^+-ATPase activity and concentration assessed by maximal *in vitro* 3-*O*-MFPase activity and [^{3}H]-ouabain binding site content, respectively would be depressed with insulin resistance in rat skeletal muscle (Chapter 4).
2. Rosiglitazone will increase maximal *in vitro* Na^+K^+-ATPase activity and membrane content in healthy and insulin resistant rat skeletal muscle via its insulin sensitising effect (Chapter 5).
3. Exercise training will alleviate depressed maximal *in vitro* Na^+K^+-ATPase activity and membrane content with insulin resistance in rat skeletal muscle (Chapter 6).
4. Rosiglitazone and exercise will have an additive affect on Na^+K^+-ATPase activity and content in insulin resistant rat skeletal muscle (Chapter 6).

Chapter 3

GENERAL METHODOLOGY

3.1 Study Design

This thesis investigated Na^+K^+-ATPase function in insulin resistant skeletal muscle from 2 experimental animal models. Genetically predisposed obese Zucker rats with their age matched lean controls and high fat fed Sprague Dawley rats with their standard chow fed controls. Chapter 4 compares the Na^+K^+-ATPase function investigated in skeletal muscle from 15 week old obese Zucker rats and in basal and insulin stimulated muscle of high-fat fed 17 week old Sprague Dawley rats. In the second study (Chapter 5), the effect of insulin resistance on Na^+K^+-ATPase function is further examined with more insulin resistant animals, 25 week old obese Zucker rats. This study also explored the effects of the anti-diabetic drug, Rosiglitazone, on Na^+K^+-ATPase function in skeletal muscle. The third study (Chapter 6) examined the effects of rosiglitazone and exercise on Na^+K^+-ATPase function in skeletal muscle using high-fat fed rats.

3.2 Experimental Animals

Two different animal models were used to complete this dissertation. The availability of obese Zucker rats became critically low in the early stages of the final research study and proposed a severe delay in the completion of this research. This problem offered the opportunity to investigate Na^+K^+-ATPase function in another insulin resistant animal

model. The high-fat fed model was chosen as it represents a significant dietary change that has occurred in Western society, an unhealthy fast food culture. This study was undertaken at the California State University of Northridge (CSUN) in the United States.

3.2.1 Lean & Obese Zucker Rats

All obese (fa/fa) and age matched lean (fa/?) Zucker rats were obtained from Monash Animal Laboratory (Melbourne, Australia). On arrival, rats were housed 4 per plastic cage in an environmentally controlled room, at a temperature of 22±1°C and relative humidity of 50±2%, on a 12-hour fixed light-dark cycle and were allowed ad libitum access to water and rat chow (67.5% carbohydrate, 11.7% fat, 20.8% protein; Barastock Ltd. Victoria, Australia). Care and treatment of these animals was in accordance with procedures outlined by the Australian National Health and Medical Research Council guidelines and all surgical and experimental procedures performed were approved by the Animal Experimentation Ethics Committee of RMIT University.

3.2.2 Sprague Dawley Rats

All Sprague Dawley rats were obtained from Harlan Sprague Dawley, Inc., (Indianapolis, IN). On arrival, rats were housed 3 per cage in a temperature controlled animal room (21±1°C) maintained on a 12-hour fixed light-dark cycle. Rats were allowed ad libitum access to water and standard rat chow (73% carbohydrate, 11% fat, 16% protein, D12328; Research Diets Inc., New Brunswick, NJ). Care and treatment of these animals complied with guidelines for laboratory animal use as published by the U.S Department of Health and Human Resources and all surgical and experimental

procedures performed were approved by the Institutional Animal Care and Use Committee at CSUN.

3.3 Muscle Analysis

The focal tissue of interest for this thesis was skeletal muscle as it is the major tissue for insulin-stimulated glucose uptake and disposal and is therefore considered the major site of insulin resistance. Skeletal muscle also contains the majority of Na^+K^+-ATPase in the body. The gastrocnemius was the muscle of choice as it contains both red oxidative and white glycolytic fibres which can be easily separated based on visible colour differences. However this thesis concentrated on red oxidative muscle as these fibres respond to endurance exercise training and contain a larger percentage of Na^+K^+-ATPase $\propto_2$-subunits than white glycolytic muscle (Bray *et al.*, 1977). This subunit is ouabain sensitive (Repke *et al.*, 1995) and can be quantitatively measured by radioactive labelled [^{3}H]-ouabain, and displays a higher degree of plasticity making it more susceptible to insulin induced changes (Al-Khalili *et al.*, 2003).

3.3.1 Animal Sacrifice

In brief, for study 1, lean and obese Zucker rats at 15 weeks of age were anaesthetised with pentobarbital (6.5 mg/100g body wt) before being sacrificed by cardiac injection of pentobarbital. For study 2, Zucker rats were sacrificed by asphyxiation with CO2 inhalation followed by decapitation. All Sprague Dawley rats were anaesthetised with pentobarbital (6.5 mg/100g body wt) before preparation for single hind limb perfusion

(described in detail in Chapter 4) and then finally rats were sacrificed via intracardiac injection of pentobarbital.

3.3.2 Muscle Collection

The hind limb muscle, gastrocnemius was rapidly excised and immediately divided into red and white portions to represent red gastrocnemius (RG) and white gastrocnemius (WG) respectively. Muscle groups were snap frozen in liquid nitrogen, and stored at -80°C until further preparation and/or analysis.

3.3.3 Muscle Homogenisation

Muscle samples weighing 20±2 mg were homogenised for Na^+-K^+-ATPase activity analysis using the 3-OMFPase assay (Fraser & McKenna, 1998). They were homogenised in the appropriate volume of homogenate buffer (5% w/v), containing 250 mM sucrose, 2 mM EDTA and 10 mM Tris (pH 7.40). Muscle was homogenised on ice for 4°C for 2 x 20 sec at 20,000 rpm using an Omni 1000 (Omni International, Marietta, GA). Muscle homogenates were immediately frozen in liquid nitrogen, and stored at -80°C until 3-OMFPase activity analysis.

3.3.4 3-OMFPase Assay

The rate of Na^+K^+-ATPase enzyme activity can be determined using a synthetic substrate called 3-*O*-methlylfluorescein phosphate (3-OMFP). The 3-OMFP can be hydrolysed by Na^+K^+-ATPase in the presence of K^+ to yield a phosphate group and a fluorescent compound, 3-*O*-methlylfluorescein (3-OMF) (Huang & Askari, 1975). This compound can be measured to quantify Na^+K^+-ATPase activity. Fluorescence was

measured by a Jasco FP-750 spectrofluorometer containing a temperature controlled chamber with a fluid injection port.

The Na^+-K^+-ATPase activity was measured using maximal K^+ stimulated in vitro 3-*O*-methlylfluorescein phosphatase (3-*O*-MFPase) activity (Fraser & McKenna, 1998). This technique was optimised for human skeletal muscle however these condition also gave optimal conditions for rat skeletal muscle (Fraser & La Vita unpublished observations). Muscle samples were made permeable by four freeze-thaw cycles, exposing the binding sites for Na^+, K^+, and ATP. Samples were then diluted 1/5 in cold homogenate buffer and placed on ice waiting analysis. 30µl of chilled sample was added to 2.5ml of pre-heated assay medium containing 5 mM $MgCl_2$, 1.25 mM EDTA and 100 M Tris buffer and 0.04 µM 3-OMF (pH 7.40) and incubated to 37°C. 40µl of 10 mM 3-OMFP was added through the injection port to initiate the reaction. After 80 seconds, 10µl of 2.58 mM KCl (final concentration 10 mM) was added through the injection port to stimulate K^+-dependent phosphatase activity and fluorescence was measured for another 80 seconds. The assay solution in the cuvette was continuously stirred throughout the reaction time. The maximal K^+-stimulated 3-*O*MFPase activity was determined by the increase in activity after the addition of KCl. 3-*O*-MFPase activity was resolved by the difference in slope gradient before and after the addition of KCl and was also based on the average of 3-4 successive trials. Na^+-K^+-ATPase activity was first determined as nmol• min^{-1}•g^{-1} wet weight but later expressed as pmol• min^{-1}• mg^{-1} muscle protein to correct for any connective tissue present in rat skeletal muscle that could not be removed during muscle dissection.

3.3.5 Muscle Protein Analysis

Muscle protein content of the homogenate was determined spectrophotometrically by the micro bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). A water-soluble purple colored reaction product is formed by the interaction of two molecules of BCA with one Cu^{1+} ion that exhibits a strong absorbance at 562 nm and is linear with increasing protein concentrations. Hence, the amount of reduction is proportional to the protein present. The BCA assay was selected to measure protein content because it is faster, easier and more sensitive than the Lowry method (Stoscheck, 1990) and has a larger linear working range than the Bradford assay (Zor & Selinger, 1996). The BCA assay was applied to the microtiter plate method using bovine serum albumin (0-2000 μg/ml) for the standard curve.

3.3.6 Vanadate Facilitated ^{3}H-ouabain Binding Method

Ouabain is a cardiac glycoside which can be used at low concentrations to treat congestive heart failure via partial inhibition of cardiac muscle Na^+K^+-ATPase (Schoner, 2001). However, it is a complete inhibitor if given in high enough concentrations. Due to ouabain's ability to specifically bind at a one to one ratio with Na^+K^+-ATPase, radioactive labelled [^{3}H]-ouabain is commonly used to quantify the content of Na^+K^+-ATPase in skeletal muscle. In rat skeletal muscle, the number of [^{3}H]-ouabain binding sites depends on many factors but at a mature age of 16 weeks, is approximately 300-400 pmol per gram of wet tissue weight and slightly higher in slow-twitch muscles compared with fast-twitch muscles (Fowles *et al.*, 2004).

Pump content in muscle tissue samples was quantified using the [^{3}H]-ouabain binding method. The standard ouabain binding assay (using 1000 nM [ouabain]) is known to measure the content of α_2 isoforms in rat skeletal muscle solely because the α_1 isoform is highly ouabain resistant (Kjeldsen *et al.*, 1986). For this assay muscle samples were cut into triplicate pieces weighing approximately 5 mg each. Muscle pieces were pre-washed twice for 10 min in a Tris-Vanadate-Sucrose (TVS) buffer containing 10 mM Tris-HCl, 3 mM MgSO4, 1 mM $NaVO_3$ and 250 mM sucrose (pH 7.3) at 37°C before being incubated in TVS buffer containing a saturating concentration of [^{3}H]-ouabain (1000 nM, 0.6 μCi/ml) for 120 min at 37°C. Vanadate facilitates the binding of [^{3}H]-ouabain and allows quantification of the Na^+K^+-ATPase content in skeletal muscle (Hansen, 1982). Unbound ouabain was removed by 4 x 30 min washouts using TVS buffer at 0°C. Muscle pieces were blotted, weighed and placed in 0.5 ml 5% trichloroacetic acid with 0.1 mM ouabain solution, overnight at 4°C. Samples were then counted for ^{3}H radioactivity in a scintillation mixture (Opti-Fluor, Packard). The content of [^{3}H]ouabain binding sites was determined and expressed as picomoles per gram wet weight (for details, see Clausen & Hansen, 1977).

3.3.7 Membrane Phospholipid Analysis

Membranes phospholipid analysis was completed in a collaborative study Wollongong University / Garvan Institute of Medical Research (NSW). 15-30mg portions of the RG was prepared for analysis and sent to Wollongong University for determination of the phospholipid fatty acid (FA) profile by gas-liquid chromatography performed by Dr Nigel Turner.

All solvents used in the lipid analysis were of ultra-pure grade and were from Merck Pty (Kilsyth, Victoria, Australia). Analytical grade butylated hydroxytoluene was from Sigma Aldrich (Castle Hill, NSW, Australia). Skeletal muscle lipids were extracted by standard methods (Folch *et al.*, 1957) using chloroform-methanol (2:1 vol/vol) containing butylated hydroxytoluene (0.01% wt/vol) as an antioxidant. Phospholipids were separated by solid-phase extraction on Strata SI-2 silica cartridges (Phenomenex, Pennant Hills, NSW, Australia). FA analysis of the phospholipid fraction was determined, as described in detail previously (Pan & Storlien, 1993). Briefly, phospholipid fractions were transmethylated with 14% (wt/vol) boron trifluoride in methanol, and FA methyl esters were separated by gas-liquid chromatography on a Shimadzu GC 17A gas chromatograph (Shimadzu Oceania, Sydney, Australia) with FAMEWAX column.

Individual FAs were identified by comparing each peak's retention time to those of external standards. The relative amount of each FA (percentage of total FA) was determined by integrating the area under the peak and dividing the result by the total area for all FAs. The sum of saturates, monounsaturates, and polyunsaturates was calculated, and total proportions of n-6 and n-3 FAs were also determined. Unsaturation index, which represents the average number of double bonds per 100 FA molecules, was calculated by summing the products of the proportion (mol%) of each unsaturated FA multiplied by its number of double bonds. The product-precursor ratio of several FAs was used to gain an indication of enzyme activity. The estimated enzyme activities included those of elongase, calculated as the stearic acid (18:0)-to-palmitic acid

(16:0) ratio; Δ5 desaturase, calculated as the arachidonic acid [20:4(n-6)]-to-di-homo--linolenic acid [20:3(n-6)] ratio; Δ6 desaturase, calculated as the di-homo--linolenic acid [20:3(n-6)]-to-linoleic acid [18:2(n-6)] ratio (assuming that Δ6 desaturase and not elongase is rate limiting); and Δ9 desaturase, calculated as the oleic acid [18:1(n-9)]-to-stearic acid (18:0) ratio.

3.4 Blood Analysis

Blood samples were collected in each study. Collection and analysis varied between studies however where possible consistency was maintained.

3.4.1 Blood Collection

For study 1, fasting blood was collected via cardiac puncture into a serum separating tube. Blood was immediately centrifuged and serum frozen for later analysis of glucose, ions and lipids. For study 2, mixed arteriovenous blood was collected in heparinised tubes for analysis of risk factors, plasma insulin and lipids, and blood glucose. For study 3, fasting blood was collected via cardiac puncture in an eppendorf tube, immediately centrifuged and plasma separated and frozen.

3.4.2 Blood Analysis

For study 1, I performed all blood analysis on a Modular Analytic SWA P module (Roche Diagnostics, Mannheim, Germany). Serum total cholesterol was analysed with the enzymatic colorimetric CHOD-PAP method on the Modular system. A homogenous enzymatic colorimetric test was used for the determination of serum

HDL cholesterol on the Modular system. Serum triglycerides were measured applying the colorimetric enzymatic GPO-PAP assay at the Modular system. LDL cholesterol was calculated utilising the Friedewald equation (Friedewald *et al.*, 1972):

LDL-Cholesterol = Total Cholesterol – (HDL-Cholesterol + Triglycerides/5).

Non-fasting serum glucose was measured on the Modular system with the glucose/hexokinase method. Creatinine was measured using a colorimetric enzymatic assay on the Modular system while Sodium, Potassium and Chloride were measured by an ion-selective electrode (ISE) (Roche Diagnostics).

Our collaborators performed the blood analysis in study 2, where plasma insulin was measured by enzyme immunoassay kits (Ultra-sensitive Rat Insulin Elisa, Mercodia AB, Uppsala, Sweden). Plasma levels of total cholesterol and triglycerides were measured using enzymatic assays CHOD-PAP and GPO-PAP (Roche, Basel, Switzerland), respectively. Blood glucose was measured using a MediSense® glucometer (MediSense, Australia).

All Sprague Dawley blood was analysed as fasting serum by a colleague. Insulin was measured by enzyme radioimmunoassay kits (Sensitive Rat Insulin RIA kit, Linco Research). Free fatty acid concentration was determined using an enzymatic colorimetric method (NEFA C test kit; Wako Chemicals, USA, Inc) and serum glucose was measured using an automated analyser (2300 Stat Plus Glucose and L-Lactate Analyzer; Yellow Springs Instruments, Yellow Springs, OH).

3.5 Statistical Anlaysis

Statistical analysis was performed on SPSS version 11.5 software (SPSS Inc, 2002). Descriptive statistics included means and standard error (SE). Significance was accepted at $p<0.05$.

Independent T test was used to determine statistical significance between non paired data, (ie. lean vs obese Zucker rats Chapter 4) while one-way analysis of variance (ANOVA) was used to determine statistical significance between groups when more than 2 existed, followed by Tukey's honestly significant difference test.

3.6 Limitations

There were several limitations to the thesis as a whole, in addition to the limitations of the individual studies. The main factor causing disruption to the flow of the thesis was supply shortage of the male obese Zucker rat after research had commenced. This limitation arose as a result of breeding problems within the Zucker colony at Monash Animal Laboratory (Melbourne, Australia) and since there had been no prior warning or knowledge of this occurrence, it was unavoidable.

3.6.1 Study 1

The supply of the male old obese Zucker rat became critically low during the first study which prevented the sequence of studies to be completed using one animal model of the same sex. Consequently, another insulin resistant animal model had to be chosen and the high fat fed rat model was preferred. Although the housing and treatment of the two animal models were identical, they could not be compared as the research involving

the two different animal groups were taken place at different times and in different places. Therefore the two groups were investigated separately. The shortage of male obese Zucker rats also prevented the investigation of insulin stimulated muscle in this group. The insulin concentration used in insulin stimulation of the Sprague Dawley rat hindlimbs did not produced the expected results in Na^+K^+-ATPase activity and content. This may have been because a low physiological dose of insulin was used.

3.6.2 Study 2

Study 2 was a collaborative study, which presented the opportunity to investigate the effect of rosiglitazone on skeletal muscle of a diabetic rat model. However the choice to use female experimental animals was decided by the collaborators and therefore presented the main limitation of study 2. These older female Zucker rats could not be compared to the younger male rats from the previous study and hence an aging effect could not also be examined. The mixed arterial venous blood collection was decided by the collaborators introducing another variation between study 1 and 2.

3.6.3 Study 3

In study 3, the high fat fed model was used to determine the effects of rosiglitazone at a more therapeutic dose and investigate the effects of exercise training on Na^+K^+-ATPase in insulin resistant skeletal muscle. In view of the fact that the study using high fat fed Sprague Dawley rats was so large, labour was reduced by sharing rat training and laboratory work. This it self became a limitation as the entire study was not under our constant supervision. Any discrepancies were reported and therefore this limitation was minimised. The second limitation of this study was that rats treated with rosiglitazone,

obtained the drug through their intake of food. The food was weighed to monitor the ingested amount however four rats shared a cage and therefore, each rat's intake of rosiglitazone was averaged and could not be strictly controlled.

Chapter 4

STUDY 1: INSULIN RESISTANCE DEPRESSES Na^+K^+-ATPASE FUNCTION IN RAT SKELETAL MUSCLE

4.1 Introduction

Insulin resistance is clinically defined as the body's relative inability to increase glucose uptake and utilisation by a known quantity of insulin that would otherwise occur in a normal individual (Lebovitz, 2001). The body compensates for this deficiency by increasing insulin secretion from the pancreas. However when the body fails to respond to the elevated insulin levels, high plasma glucose levels persist and type II diabetes prevails. Type II diabetes is also associated with decreased exercise capacity and hence can predispose sufferers to early onset of fatigue (Schneider *et al.*, 1984; Regensteiner *et al.*, 1995; Baldi *et al.*, 2003). Muscle fatigue is a highly complex and multifactorial event, occurring via a variety of mechanisms at numerous sites within the cell (Fitts, 1994; Allman & Rice, 2002). One mechanism contributing to fatigue during intense exercise is the impairment of sarcolemma excitability, induced by diminishing transmembrane Na^+ and K^+ gradients as a result of inadequate Na^+K^+-ATPase activity (Nielsen & Overgaard, 1996; Overgaard *et al.*, 1997; Fraser *et al.*, 2002).

At rest, skeletal muscles use less than 10% of Na^+K^+-ATPase maximal activity to maintain ionic concentration gradients (James *et al.*, 1999a). During muscle contraction, the passing of an action potential causes Na^+ and K^+ leakage across the sarcolemma and

a pronounced decrease in transmembrane Na^+ and K^+ gradients. Intense exercise can cause extracellular K^+ concentrations to rise up to 10mmol/L (Vyskocil *et al.*, 1983).

If the ion gradients are not reinstated, there is loss of membrane excitability (Nielsen & Overgaard, 1996). Insufficient Na^+K^+-ATPase activity has also been suggested to play a role in the development of diabetic complications such as neuropathy, nephropathy and retinopathy (Winegrad, 1987; Raccah *et al.*, 1992). In addition, there is also evidence to suggest altered Na^+K^+-ATPase function is also involved in the development of diabetic myopathy (Kjeldsen *et al.*, 1987).

Since insulin is a known activator of Na^+K^+-ATPase, it would be important to know the effects of insulin resistance and elevated levels of circulating insulin on Na^+K^+-ATPase function. Previous studies have investigated the effects of type II diabetes on Na^+K^+-ATPase, however disagreement exists with numerous studies showing conflicting or no change in various tissue types of both diabetic humans and rats (refer to Table 4.1). Compromised Na^+K^+-ATPase activity with type II diabetes has been reported in human erythrocytes (Baldini *et al.*, 1989; Kiziltunc *et al.*, 1997), platelets (Rabini *et al.*, 1998) and nerve tissue (Scarpini *et al.*, 1993; Sima *et al.*, 2000), and also in rat nerve tissue (Wada *et al.*, 1999; Kitahara *et al.*, 2002) and retinal cells (Ottlecz *et al.*, 1993). However other studies have reported no change in rat (Levy *et al.*, 1990; Zemel *et al.*, 1990) or human erythrocytes (De La Tour *et al.*, 1998; Jannot *et al.*, 2002), and rat skeletal muscle (Banyasz & Kovacs, 1996; Ferrer-Martinez *et al.*, 1996).

Table 4.1 Previous Na^+K^+-ATPase studies with insulin resistance or diabetes.

Tissue	Na^+K^+-ATPase activity (change, % of control)	Na^+K^+-ATPase content (change, % of control)	Reference
Skeletal Muscle			
Rat	0	-	Ferrer-Martinez et al., 1996
Rat	-	0	Banyasz et al., 1996
Human	-	30% decrease	Djurhuus et al., 2001
Platelets			
Human	33% decrease	-	Rabini et al., 1998
Retinal cells			
Rat	14 % decreased	-	Ottlecz et al., 1993
Erythrocytes			
Rat	0	-	Zemel et al., 1990
Rat	0	-	Levy et al., 1990
Human	15 % decrease	-	Kiziltunc et al., 1997
Human	62 % decrease	70 % decrease	Baldini et al, 1989
Human	0	-	Jannot et al., 2002
Human	0	-	De La Tour et al., 1998
Nerve tissue			
Rat	80 % decrease	-	Kitahara et al., 2002
Rat	20 % decrease	-	Wada et al., 1999
Human	35 % decrease	-	Sima et al., 2000
Human	59% decrease	-	Scarpini et al., 1993

A study involving identical twins discordant for type II diabetes found the diabetic twin group demonstrated an approximate 20% reduction in muscle [^{3}H]-ouabain binding

capacity compared to the healthy twin group (Djurhuus *et al.*, 2001). An inverse correlation was found between the differences in [^{3}H]-ouabain binding capacity between the twin pairs and corresponding differences in waist/hip ratio. This indicated that reduced muscle Na^+K^+-ATPase concentration was associated with the development of abdominal fat in type II diabetics.

This chapter investigated Na^+K^+-ATPase function in skeletal muscle of 15 week old lean and obese Zucker rats, and in basal and insulin stimulated skeletal muscle of high-fat fed and chow fed Sprague Dawley rats aged 14 weeks. The hypotheses tested were (1) Insulin resistant skeletal muscle from obese Zucker rats will have depressed Na^+K^+-ATPase function and (2) Insulin resistant skeletal muscle from high fat fed Sprague Dawley rats will have depressed Na^+K^+-ATPase function and not respond to insulin stimulation.

4.2 Methods

Two different animal models of insulin resistance were used in this study, genetically predisposed obese Zucker (OZ) rats with their age matched lean Zucker (LZ) controls and high fat fed Sprague Dawley rats with their standard chow fed controls. All animal handling and experimental procedures for muscle extraction and blood sampling and analysis have been fully described earlier (Chapter 3).

4.2.1 Obese Zucker Rats

Red oxidative gastrocnemius muscle was analysed for 3-*O*-MFPase activity and [^{3}H]-ouabain binding site content. Blood samples were analysed for glucose, lipids and ions. The availability of obese Zucker rats became critically low in the early stages the final research study and proposed a severe delay in the completion of this research. This problem offered the opportunity to investigate Na^+K^+-ATPase function in another insulin resistant or diabetic animal model. This time round, a high-fat fed rodent model was chosen. Given that dietary factors are important in the etiology of both insulin resistance and obesity in humans and high-fat feeding in rats displays skeletal muscle defects in insulin signalling and disregulated metabolism that are seen in insulin resistant humans (Kraegen *et al.*, 1991), a high-fat fed rat model was considered an appropriate substitute.

4.2.2 High Fat Fed Sprague Dawley Rats

At California State University of Northridge (CSUN), United States, 80 male Sprague Dawley rats were ordered at 4 weeks of age, 32 of which will be discussed in this chapter, the remaining 48 will be discussed in Chapter 6. The 32 rats were acclimatised for 2 weeks, allowed ad libitum access to water and standard rat chow (73% carbohydrate, 11% fat, 16% protein, D12328; Research Diets Inc., New Brunswick, NJ) before being randomly assigned to either a very high fat diet containing hydrogenated coconut oil (58% of calories as fat and 26% as carbohydrate, D12330N; Research Diets) or normal chow for an 8 week period. The caloric density of the chow diet was 4.1 kcal/g and that of the high fat was 5.6 kcal/g. This resulted in lower daily food consumption in grams for the rats fed the high fat diet however there was no difference

in caloric intake (244.3 ± 4.4 versus 261.0 ± 9.2 kcal/kg of body mass per day) between chow and high fat diet fed rats. At the end of their respective diets, animals were fasted for 8-12 h prior to undergoing hind limb perfusion for the measurement of insulin-stimulated 3-*O*-methylglucose transport, whereby 8 rats received perfusate without insulin and 8 rats received perfusate containing insulin.

4.2.2.1 Hind limb Perfusion preparation

Rats were anaesthetised with pentobarbital (6.5 mg/100g body wt) and body weight recorded. All rat surgery for hindlimb perfusion was performed by Dr Ben Yaspelkis. Left and right epididymal fat pad were quickly dissected out before preparing rats for single hind limb perfusion as previously described (Herr *et al.*, 2005). Briefly, cannulas were inserted into the abdominal aorta and vena cava, blood was collected 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). 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% O_2- 5% 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 uptake were determined.

4.2.2.2 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. After the washout period, 8 minutes of hind limb perfusion was performed with a perfusate containing 8

mM of the non-metabolised glucose analogue 3-*O*-methylglucose (3-MG; 32 μCi 3-[^{3}H-*O*-MG/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 glucose transport perfusion, portions of the red oxidative gastrocnemius were excised from both hind limbs, blotted on gauze dampened with cold KHB, freeze clamped in liquid N_2 and stored at -80°C for later transport to RMIT for further analysis. Rates of insulin stimulated skeletal muscle 3-MG transport were calculated as previously described (Yaspelkis *et al.*, 2004).

RG extracted from 8 chow fed, (normal control NC) and 8 high fat fed (HF) were classed as basal tissue. Insulin stimulated RG was extracted from another 8 chow fed (NCIns) and 8 high fat fed (HFIns) rats following hindlimb perfusion with the same perfusate but containing a physiological concentration of insulin at 500 μU/ml. Basal and insulin stimulated muscle were analysed for 3-*O*-MFPase activity and [^{3}H]-ouabain binding site content of Na^+K^+-ATPase. Blood samples were immediately centrifuged and serum analysed for insulin, glucose and FFA.

4.3 Results – Lean v's Obese Zucker Rats

Despite not all rats having their weights measured and recorded due to methological issues, at aged 15 weeks, the male OZ were 34% heavier ($P < 0.001$) than LZ (OZ 415 ± 7 grams, n=6; LZ 309 ± 9 grams, n=5).

4.3.1 Blood Analysis

Data from the blood analysis is shown in Table 4.2. Blood could not be collected from 1 OZ and 1 LZ, hence reducing n to 7. OZ were hyperglycaemic, displaying elevated glucose levels (OZ 19.9 ± 3.1 mmol/L, $P < 0.01$ compared with LZ). OZ also suffered hyperlipidemia with their total cholesterol and triglycerides significantly higher than LZ ($P < 0.001$). The HDL:LDL ratio was significantly lower in the OZ compared with the LZ. Electrolytes were in the normal ranges for both groups with no differences between groups.

Table 4.2 Blood analysis in lean and obese Zucker rats.

	LZ	**OZ**
Glucose (mM)	6.9 ± 0.9	19.9 ± 3.1[#]
Total cholesterol (mM)	1.29 ± 0.04	3.57 ± 0.19[†]
Triglycerides (mM)	0.46 ± 0.06	3.74 ± 0.23[†]
HDL:LDL ratio	3.96 ± 0.49	1.69 ± 0.22[†]
Serum Potassium (mM)	5.8 ± 0.7	5.6 ± 0.6
Serum Sodium (mM)	140 ± 1	141 ± 1
Urea (mM)	7.8 ± 0.5	8.6 ± 0.3
Creatinine (mM)	0.034 ± 0.003	0.039 ± 0.004

Values are means ± SE; n=7; Significance for LZ v's OZ is # at $p<0.001$ and † at $p<0.001$.

4.3.2 3-O-MFPase activity

To determine the effects of insulin sensitivity on Na^+K^+-ATPase activity in skeletal muscle, we measured maximal 3-*O*-MFPase activity in red oxidative gastrocnemius. There appeared to be a decrease in 3-*O*-MFPase activity in OZ compared to LZ in RG. This difference became significant (29%) when activity was expressed relative to muscle protein content (LZ 7212 ± 497, OZ 5110 ± 404 $pmol.min^{-1}.mg^{-1}$ protein, $P < 0.01$). Results are shown in Figure 4.1.

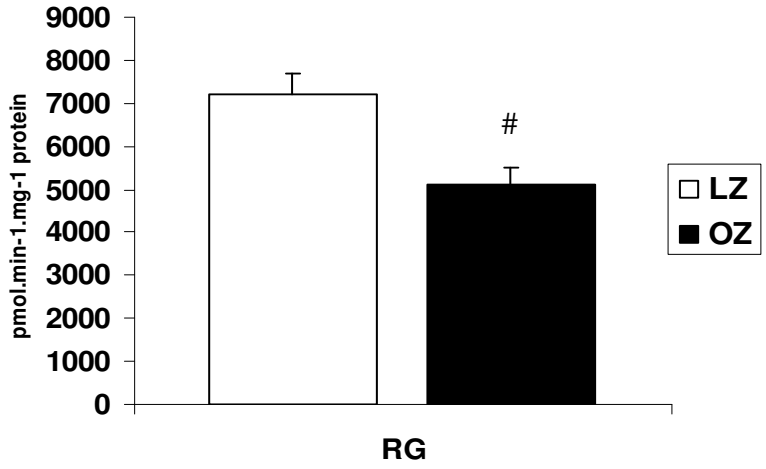

Figure 4.1 Na^+K^+-ATPase activity measured by 3-*O*-MFPase assay in RG from LZ and OZ rats. Data are means ± SE for 8 rats per group. # $p<0.01$.

4.3.3 Na^+K^+-ATPase content

The content of Na^+K^+-ATPase were determined using vanadate facilitated [^{3}H]-ouabain binding method on whole muscle samples. In red gastrocnemius, there was a 22% ($P < 0.001$) decrease in the number of Na^+K^+-ATPase in OZ when compared with LZ rats. Results are displayed in Figure 4.2.

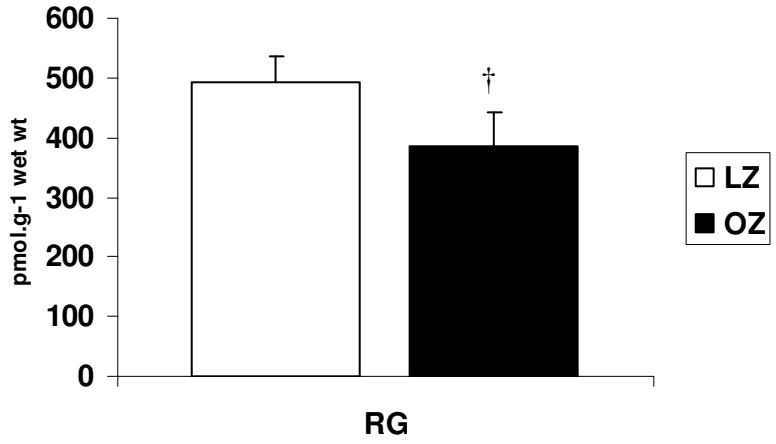

Figure 4.2 Na^+K^+-ATPase content measured by Vanadate facilitated [^{3}H]-ouabain binding assay in RG of LZ and OZ rats. Data are means ± SE for 8 rats per group. † Significance for OZ<LZ at p<0.001.

4.3.4 Relationships between Na^+K^+-ATPase content and activity

It was anticipated that Na^+K^+-ATPase content in OZ and LZ would be related to maximal Na^+K^+-ATPase activity. A significant correlation was found however it held a moderate correlation value (r=0.5, P <0.05).

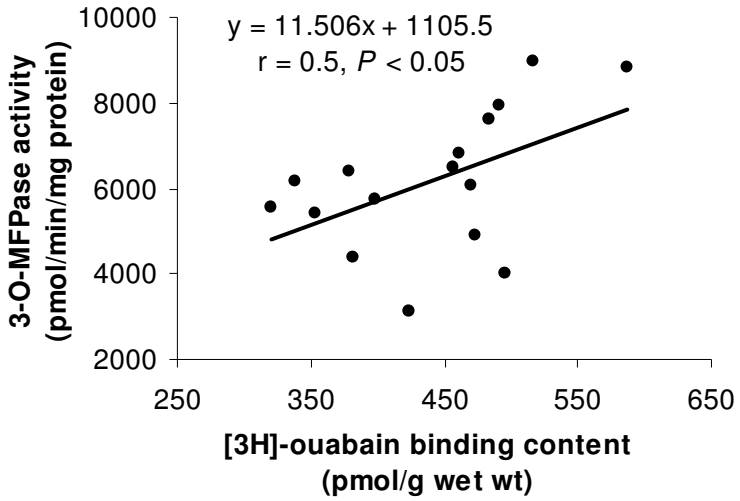

Figure 4.3 The relationship between Na^+K^+-ATPase content and maximal activity in RG. n =16

4.3.5 Relationships between Na^+K^+-ATPase content and serum diabetic markers

To determine whether Na^+K^+-ATPase content in rat skeletal muscle was influenced by diabetic status, the relationship between ouabain binding sites and diabetic markers, serum glucose, triglycerides, total cholesterol and HDL:LDL ratio was examined. Na^+K^+-ATPase content was found to be negatively correlated with serum glucose levels (Figure 4.4a, $P < 0.01$), triglycerides (Figure 4.4b, $P < 0.01$) and total cholesterol (Figure 4.4c, $P < 0.01$), while positively correlated with HDL:LDL ratio (Figure 4.4d, $P < 0.01$).

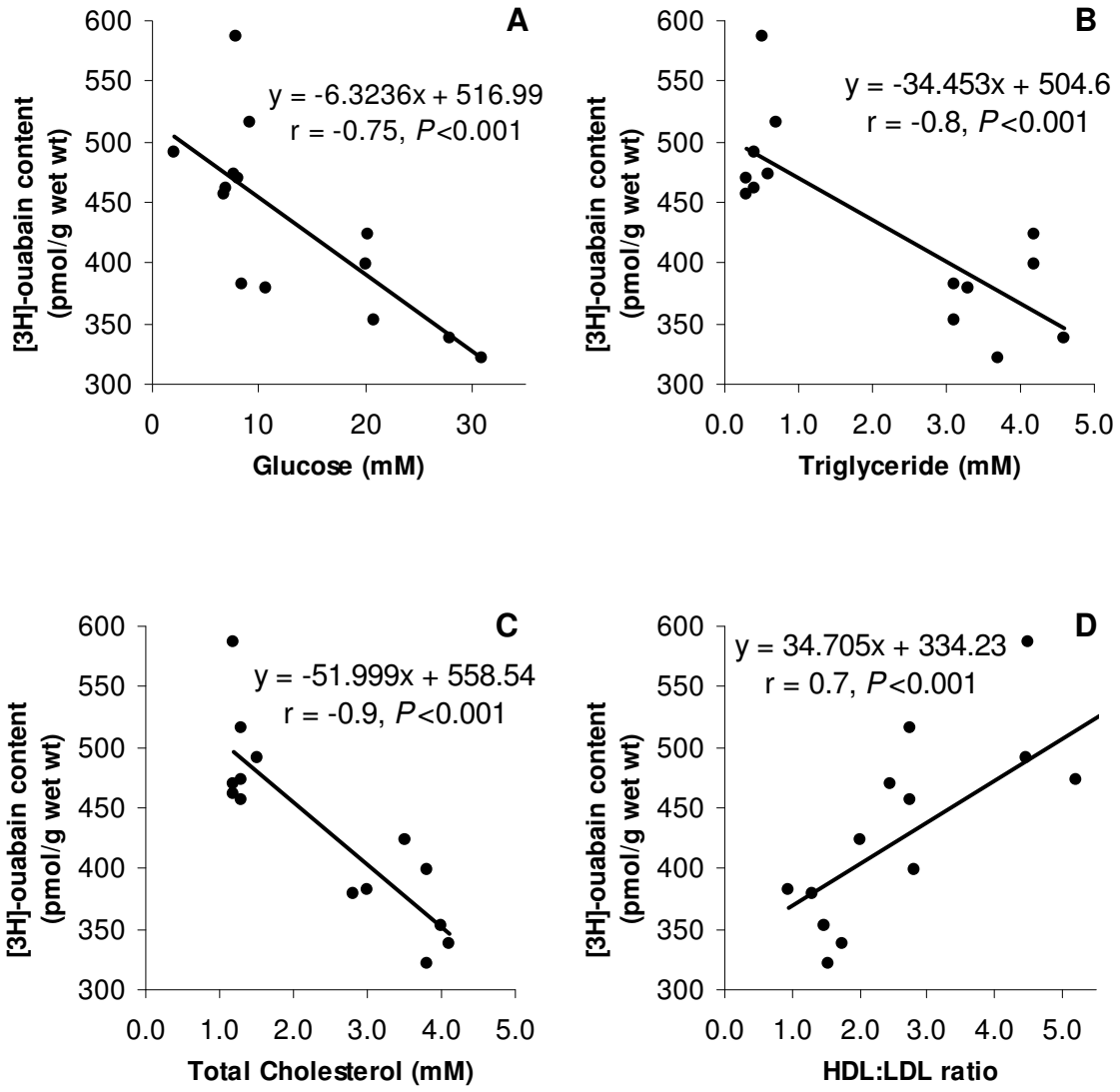

Figure 4.4 Rat Na^+K^+-ATPase content in RG as a function of serum glucose (A), triglycerides (B), total cholesterol (C) and HDL:LDL ratio (D). n =14 (NB not all bloods were obtained)

4.3.6 Rat Na^+K^+-ATPase activity and content as a function of body weight

To determine whether the reduction in Na^+K^+-ATPase activity and content in OZ may be related to characteristic features of insulin resistance, we investigated the association

between 3-*O*-MFPase activity or [^{3}H]-ouabain binding content with body weight. There was a significant inverse relationship between the activity and content of Na^+K^+-ATPase in skeletal muscle and rat body weight (Figure 4.5a and 4.5b, $P < 0.01$).

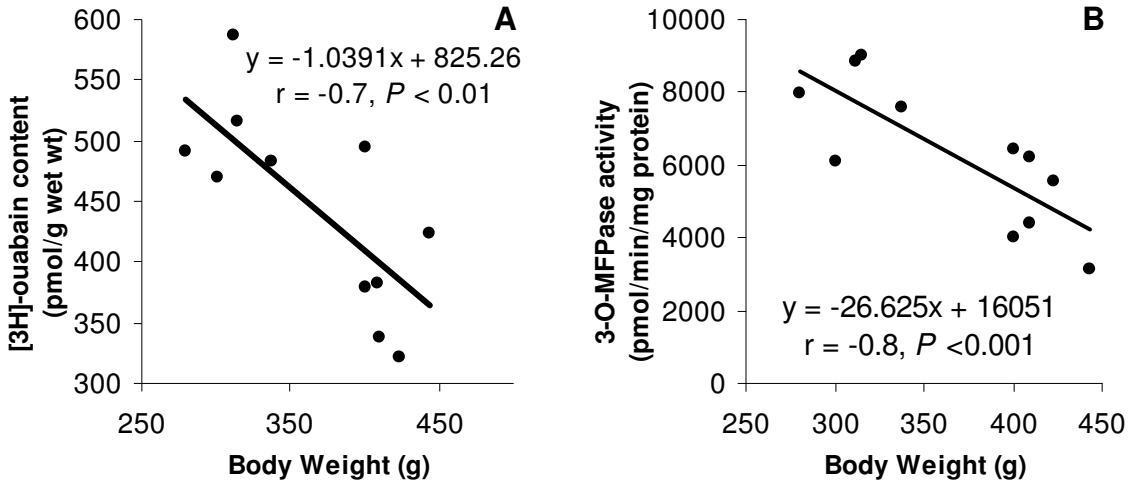

Figure 4.5 The relationship between Na^+K^+-ATPase content (a) and activity (b) in RG and rat body weight. n =11 (NB not all rat body weights were obtained)

4.4 Results – Chow Fed v's High Fat Fed Sprague Dawley Rats

4.4.1 General and metabolic characteristics

Clinical and metabolic characteristics for the two groups are shown in Table 4.3. At 14 weeks, the male HF rats did not appear to gain appreciable amounts of weight, however they were 7% heavier than NC (HF 460 ± 10, n=16; NC 430 ± 6 grams, n=15, $P < 0.05$). Epididymal fat pad weight increased by 1.9 fold in HF rats (HF 10.6 ± 0.8, NC 5.7 ± 0.2 grams, n=16 $P < 0.001$). The epididymal fat pads were used as an indicator of body fat percentage as epididymal fat pad weight correlate well with total body fat in rodents (Eisen & Leatherwood, 1981; Levy *et al.*, 2000).

Blood could not be collected from 1 HF rat, hence reducing n to 7. Serum glucose concentrations were elevated due to haemolysis and along with insulin and FFA were not different between rat groups.

Table 4.3 General and metabolic characteristics in chow fed controls and high fat fed Sprague Dawley rats.

	NC	**HF**
Body Mass (g)	430 ± 6	460 ± 10*
Epididymal Fat Pad mass (g)	5.7 ± 0.2	10.6 ± 0.8†
Blood Glucose (mM)	17.5 ± 1.5	16.4 ± 1.0
Plasma Insulin (ng/ml)	1.23 ± 0.25	1.21 ± 0.24
Free Fatty Acids (mM)	0.89 ± 0.16	0.60 ± 0.10

Values are means ± SE; Significance for NC v's HF is at *p<0.05, † p<0.001.

4.4.2 Glucose Uptake

Glucose uptake was measured using [^{14}C]-glucose and calculated from the arteriovenous difference, the perfusate flow rate and the weight of the muscle perfused. As expected, glucose uptake was significantly different between NC and NCIns (NC 2.34 ± 0.54, NCIns 6.17 ± 0.44 μmol.g^{-1}.hr^{-1}, P = 0.001). No difference was found between HF and HFIns (HF 2.32 ± 0.85, HFIns 4.11 ± 0.56 μmol.g^{-1}.hr^{-1}), indicating insulin resistance.

4.4.3 3-O-MFPase activity

Maximal 3-*O*-MFPase activity in basal RG appeared to be reduced in the HF compared to NC and these results became significantly different when activity was expressed relative to muscle protein content (NC 6392 ± 397, HF 4041 ± 254 pmol.min^{-1}.mg^{-1} protein, $P = 0.001$). Results are shown in Figure 4.6. The submaximal concentration of insulin stimulation did not have an effect on 3-*O*-MFPase activity in the NCIns group (5800 ± 422 pmol.min^{-1}.mg^{-1} protein), however caused an elevation in maximal activity of the HFIns (HF 4041 ± 254, HFIns 5581 ± 420 pmol.min^{-1}.mg^{-1} protein, $P < 0.05$).

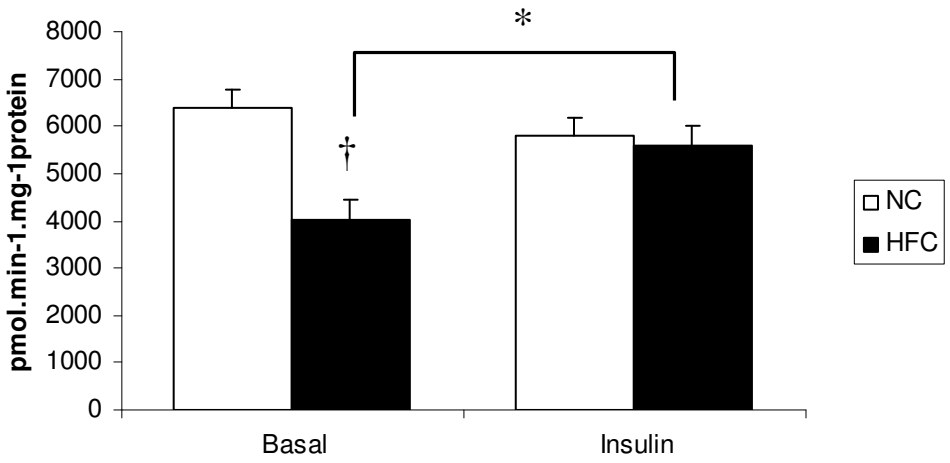

Figure 4.6 Na^+K^+-ATPase activity measured by 3-*O*-MFPase assay in basal and insulin stimulated RG from NC and HF rats. Data are means ± SE for 8 rats per group. Significance for NC > HF † P =0.001, HFIns > HF at * P <0.05,

4.4.4 Na^+K^+-ATPase content

Na^+K^+-ATPase content was 23% lower in red gastrocnemius of basal HF when compared to NC rats (HF 307 ± 18, NC 395 ± 18 pmol.g-1.wet wt $P < 0.01$, figure 4.7). Insulin stimulation with submaximal insulin concentrations had no effect on Na^+K^+-ATPase content in either HF or NC.

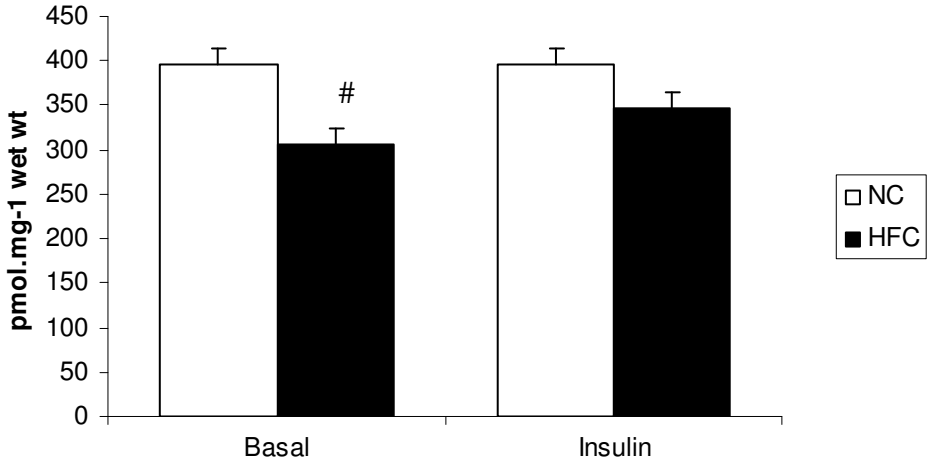

Figure 4.7 Na^+K^+-ATPase concentration measured by Vanadate facilitated [^{3}H]-ouabain binding assay in RG of LZ and OZ rats. Data are means ± SE for 8 rats per group. # Significance for NC v's HF at $p<0.01$.

4.5 Discussion

The underlying objective of this study was to analyse the effect of insulin resistance or type II diabetes on Na^+K^+-ATPase function in skeletal muscle. The findings of this study supported the two hypotheses that Na^+K^+-ATPase activity and concentration, respectively would be depressed with insulin resistance or type II diabetes in rat skeletal muscle.

4.5.1 Effect of Insulin Resistance on Na^+K^+-ATPase Function

One of the two most important findings from this study was that Na^+K^+-ATPase activity was depressed with insulin resistance and type II diabetes in rats when expressed relative to muscle protein content. This effect was evident in both the diabetic obese

Zucker rats and insulin resistant high fat fed Sprague Dawley rats. The other significant finding was the similar decrease in Na^+K^+-ATPase content in skeletal muscle of both animal models. These findings support the study involving identical twins discordant for type II diabetes (Djurhuus *et al.*, 2001) but oppose other previous findings in the literature for rat skeletal muscle (Banyasz & Kovacs, 1996; Ferrer-Martinez *et al.*, 1996).

4.5.1.1 Obese Zucker Rat

In the present study, obese Zucker rats displayed typical characteristics of obesity related type II diabetes, suffering from severe weight gain, hyperglycemia and dyslipidemia with elevated cholesterol, triglycerides and decreased HDL:LDL ratio. Obese Zucker rats also displayed a 29% drop in maximal 3-*O*-MFPase activity in skeletal muscle indicating depressed Na^+K^+-ATPase activity. In addition to this significant result, the content of functional Na^+K^+-ATPase measured in the sarcolemma was also decreased by 22% in obese Zucker rats. The reduced content offers an obvious explanation for the reduced Na^+K^+-ATPase activity found. Together, these findings suggest that Na^+K^+-ATPase function in skeletal muscle was compromised with type II diabetes. This opposes the "no change" result found in obese Zucker rats in a study conducted by Ferrer-Martinez and colleagues, however their study did show a non-significant decrease in Na^+K^+-ATPase activity using a small sample size (n=5) (Ferrer-Martinez *et al.*, 1996).

Structural alterations in the kinetics of the enzyme and/or the membrane lipids in which the protein is embedded may have contributed to the reduced activity but the decreased

Na^+K^+-ATPase content implies the membrane protein would have been compromised at either an earlier stage such as at gene transcription or translation, translocation to and/or insertion into the membrane or later on with a higher rate of enzyme degradation.

Decreased Na^+K^+-ATPase content with insulin resistance may be the result of prolonged elevated levels of circulating insulin and consequently insulin resistant diabetes itself. Therefore, it seemed reasonable to determine whether any association exists between muscle [^{3}H]-ouabain binding content and characteristic serum markers of type II diabetes. After plotting the [^{3}H]-ouabain binding content as a function of the serum markers, Na^+K^+-ATPase content was negatively correlated with glucose, triglycerides and total cholesterol, but positively correlated with the HDL:LDL ratio. These relationships suggest that the reduction in Na^+K^+-ATPase content could be associated with the development of insulin resistance and type II diabetes. Therefore, since Na^+K^+-ATPase is important for cell function in diverse tissues, this supports the literature implicating the Na^+K^+-ATPase in the development of diabetic complications such as neuropathy, nephropathy, retinopathy and myopathy. In addition, compromised Na^+K^+-ATPase function in skeletal muscle may offer a partial explanation for reduced exercise capacity and increased muscle fatigue associated with type II diabetes (Schneider *et al.*, 1984; Regensteiner *et al.*, 1995; Baldi *et al.*, 2003).

The fundamental cause for the alterations could not be narrowed down to the lack of insulin action as insulin stimulated muscle was not investigated in the obese Zucker

model. In addition, obese Zucker rats also display markedly elevated circulating leptin levels (Hardie *et al.*, 1996; Wang *et al.*, 1999) and decreased T_3 serum levels (Katzeff & Selgrad, 1993). These may offer other possible explanation for the decreased pump activity observed in these rats as leptin has been negatively associated with Na^+K^+-ATPase function in a variety of tissues (Cha & Jones, 1998; Sweeney & Klip, 2001; Beltowski *et al.*, 2004) and T_3 is known to be a chronic pump regulator and decreased levels are associated with decreased Na^+K^+-ATPase concentrations (Kjeldsen *et al.*, 1984c).

Compromised Na^+K^+-ATPase function in obesity related type II diabetes might lower cellular energy consumption, since Na^+K^+-ATPase is one of the biggest consumers of cellular ATP. It has been estimated that in resting humans, Na^+K^+-ATPase hydrolyses 23% of all cytoplasmic ATP (Lingrel & Kuntzweiler, 1994). Increased usage of ATP by the Na^+K^+-ATPase would influence resting metabolic rate which is a key factor in body weight maintenance. Therefore compromised Na^+K^+-ATPase function may lower metabolic rate thus increasing the likelihood of body fat accumulation. For that reason, we chose to examine any association that existed between rat muscle [^{3}H]-ouabain binding content and/or 3-*O*-MFPase activity and body weight, as an indicator of fat accumulation. Both Na^+K^+-ATPase activity and content were inversely correlated with body weight, indicating that Na^+K^+-ATPase function has a possible association with the development of obesity. On the other it may be that the obesity is causing reduced physical activity which may in turn be down regulating the Na^+K^+-ATPase function. It has been previously shown that reduced physical activity and muscle disuse will decrease

enzyme content (Kjeldsen *et al.*, 1986; Leivseth & Reikeras, 1994). Unfortunately the movement/physical activity of the rats was not measured and therefore the role of physical activity induced changes in pump function cannot be ruled out. Future studies might consider assessing the level of physical activity through the use of accelerometry or video movement analysis techniques.

4.5.1.2 High Fat Fed Rat

Insulin resistance was induced in Sprague Dawley rats with a high fat diet containing saturated fats. HF rats did not gain considerable weight but were still heavier than the lean chow fed controls. Epididymal fat pads were notably larger in HF rats indicating a substantial increase in body fat. HF rats did not have elevated serum insulin, glucose or FFA levels but were confirmed as insulin resistant, pre-diabetic phenotype, as insulin administration in HFIns rats did not significantly increase glucose uptake. Glucose uptake appeared to increase in the HFIns rats but not significantly. There was no difference in glucose uptake between diet groups in the basal states.

In agreement with the Zucker data, similar results were found with Na^+K^+-ATPase function in skeletal muscle from insulin resistant HF rats. HF rats had a 37% decrease in Na^+K^+-ATPase activity in basal skeletal muscle and a 23% reduction in Na^+K^+-ATPase content. Again, physical activity was not measured and therefore could not be ruled out as the cause of reduced Na^+K^+-ATPase function in the HF skeletal muscle. These results will be further discussed in Chapter 6.

4.5.2 Effect of Insulin Stimulation on Na^+K^+-ATPase in Insulin resistant skeletal muscle

Up regulation of Na^+K^+-ATPase activity upon insulin stimulation with large insulin concentrations (80-100 mM) is well known and documented (Weil *et al.*, 1991; Sampson *et al.*, 1994). However, no study has investigated the effect of insulin on Na^+K^+-ATPase function at a physiological concentration (500 μU/ml). Submaximal concentrations of insulin had no effect on Na^+K^+-ATPase activity in skeletal muscle of the NC rats. This result was not expected as insulin is a known acute activator of the pump. The *in vitro* assay appears to be sensitive to insulin stimulation as the HF rats showed a 38% increase in 3-*O*-MFPase activity. Logically one would expect to see the opposite result. The discrepancy between the two groups in Na^+K^+-ATPase response to insulin stimulation is confounding and potentially warrants further investigation. Future studies investigating the role of insulin stimulation of the pump need to consider the effect of insulin on phosphorylation status and Na+K +-ATPase enzyme kinetics.

The detrimental effect on Na^+K^+-ATPase activity seen with the HF rats could not be the result of deficient insulin action as seen with glucose uptake, and would appear to involve a different mechanism of inhibition. Na^+K^+-ATPase content was unaltered with insulin stimulation in both NC and HF rats, so the effect of insulin on maximal activity in HF group could not be due to an increase in enzyme translocation of to the sarcolemma. Instead, insulin's effect would be more of a direct alteration on the structure of the enzyme, such as phosphorylation of the ∝-subunit (Al-Khalili *et al.*, 2004) or enzyme kinetics by alleviating a potential inhibitor of the Na^+K^+-ATPase (McGill & Guidotti, 1991).

4.5.3 Conclusion

In summary, the results of this study demonstrate depression in maximal *in vitro* Na^+K^+-ATPase activity and content with insulin resistance in rat skeletal muscle. This was shown in both, the genetically predisposed and high fat diet induced animal models. The findings indicate compromised Na^+K^+-ATPase function causing a reduced reserve for Na^+K^+-ATPase activation in insulin resistant skeletal muscle. This may predispose diabetics to early muscle fatigue with associated impaired exercise tolerance. Whether the altered pump function is due to insulin resistance itself or due to obesity/reduced physical activity, the reduced exercise tolerance may further progress the development of type II diabetes and its associated complications as exercise is a well known antidiabetic treatment. The findings of the current study might also suggest that the altered Na^+K^+-ATPase function contributes to the development of obesity related insulin resistance and type II diabetes as the Na^+K^+-ATPase is one of the biggest ATP consumers in the body and reduced ATP consumption may increase fat accumulation.

Chapter 5

STUDY 2: ROSIGLITAZONE COMPROMISES NA^+K^+-ATPASE FUNCTION IN SKELETAL MUSCLE OF LEAN ZUCKER RATS.

5.1 Introduction

The treatment of obesity related insulin resistance involves a combination of diet, exercise and pharmacologic approaches. Thiazolidinediones (TZDs) are a group of oral antidiabetic compounds that function as insulin sensitisers (Saltiel & Olefsky, 1996). TZDs are agonists for peroxisome proliferator-activated receptor-γ, a nuclear hormone receptor, which when activated by natural ligands, fatty acids and their derivatives, regulates the transcription of genes involved in lipid and carbohydrate metabolism (Ricote *et al.*, 1998; Spiegelman, 1998; Wahli, 2002). TZD receptors are expressed in insulin target tissues; adipose tissue, liver and at low levels in skeletal muscle. Many studies have shown administration of TZDs improves hyperglycaemia and hyperinsulinemia in insulin resistant rodent models and Type II diabetic patients (Saltiel & Olefsky, 1996; Olefsky, 2000). Sensitised skeletal muscle might increase insulin-induced Na^+-K^+-ATPase function (Al-Khalili *et al.*, 2003), however, TZDs have also been shown to alter membrane phospholipids and fluidity (Kurebayashi *et al.*, 1997; Clore *et al.*, 2000; Wahl *et al.*, 2002). Since the Na^+K^+-ATPase is a membrane-embedded protein, the surrounding membrane phospholipids are important for the intrinsic function of the enzyme (Komers & Vrana, 1998).

Rosiglitazone, a TZD derivative, has been shown to restore insulin's ability to suppress hepatic gluconeogenesis and improve peripheral glucose uptake, without stimulating insulin production (Miyazaki *et al.*, 2001; Wagstaff & Goa, 2002). In addition, rosiglitazone has been shown to reduce circulating free fatty acids by promoting their uptake and storage in adipocytes (Mayerson *et al.*, 2002). However other TZDs, such as troglitazone and pioglitazone have been shown to repress the gene expression of stearoyl-CoA desaturase 1, a key enzyme involved in the biosynthesis of monounsaturated fatty acids, components of membrane phospholipids and maintenance of the fluidity of biological membranes (Kurebayashi *et al.*, 1997). Alterations in membrane fatty acyl chains and phospholipid compositions have been shown to influence membrane permeability, ion channels and transport, and cause changes in membrane-bound enzyme activities (Spector & Yorek, 1985).

Past experiments have demonstrated that the function and intrinsic activity of the membrane ion transporter, Na^+-K^+-ATPase is influenced by the lipid milieu in which it exists (Storch & Schachter, 1984; Else & Wu, 1999). However it is unclear whether the lipid-protein interactions are effective via direct contact and binding or indirectly by means of order and fluidity. Modifications that can affect protein function are alteration of the phospholipid head group, acyl chain length and unsaturation, and insertion of cholesterol (Cantor, 1999). The degree of unsaturation and length of acyl chains in adjacent phospholipids affects lateral movement and conformational change of an integral protein (Wu *et al.*, 2001). While the length of acyl chains simply increases bilayer thickness, the double bonds in unsaturated acyl chains inhibit freedom of rotation about

the bond and prevent phospholipids from compacting themselves, improving efficiency of ligand binding and activity of membrane-bound enzymes (Hollan, 1996). A study using hepatocyte membranes reported an increase in lipid fluidity which was accompanied by an increase in Na^+K^+-ATPase specific activity (Storch & Schachter, 1984). In a species membrane cross-over experiment, toad Na^+K^+-ATPase which was reconstituted in more unsaturated rat membrane showed a 30-40% increase in activity while rat Na^+K^+-ATPase positioned in toad membrane significantly decreased (~40%) enzyme activity in both kidney and brain (Else & Wu, 1999). Another double crossover study by Wu and colleagues used the same technique to show that membrane phospholipids play a significant role in determining the molecular activity of the Na^+K^+-ATPase (Wu *et al.*, 2004).

Membrane fluidity is increased by phospholipids composed of polyunsaturated fatty acids (PUFAs), in particular omega-3 PUFAs. Specifically, docosahexaenoic acid (DHA) contains 22 carbons and 6 double bonds and is the longest and most unsaturated fatty acid commonly present in biological membranes. DHA plays an important structural role in the cytoplasmic membrane of cells and has been shown to increase the unsaturation index, disordering and fluidity (Hashimoto *et al.*, 1999; Petrache *et al.*, 2001). Omega-6 PUFAs have been associated with inhibiting the absorbance of omega-3 PUFAs, in a competitive nature (Iritani & Fujikawa, 1982). The net result is an increase in omega-6/omega-3 ratio, which is associated with reduced membrane fluidity (Farkas *et al.*, 2002).

There is also evidence to suggest a high omega-6/omega-3 ratio is deleterious on insulin action in skeletal muscle (Storlien *et al.*, 1996). Dietary omega-3 and PUFAs have been shown to increase insulin binding to sarcolemma (Liu *et al.*, 1994), suggesting that changes in fatty acyl composition of phospholipids neighbouring the insulin receptor might be responsible for altered insulin sensitivity. The effect of membrane lipid composition on Na^+K^+-ATPase function may also explain irregular Na^+ ion transport observed in disease states with abnormal lipid metabolism such as hypertension, obesity and diabetes (Mazzanti *et al.*, 1990; Modan & Halkin, 1991; Zicha *et al.*, 1997).

Obesity-related insulin resistance and Type II diabetes are characterised by elevated plasma insulin and glucose levels (Goldstein, 2003), and frequently accompanied by altered regulation of the Na^+-K^+-ATPase enzyme (Jannot *et al.*, 2002). Individuals suffering obesity related insulin resistance or diabetes have compromised exercise capacity which could be partly due to inadequate Na^+K^+-ATPase function. Reduced Na^+K^+-ATPase function can cause hyperkalemia and contribute to early onset of fatigue, limiting the ability of these individuals to exercise as part of their treatment strategy.

In the previous study (Chapter 4), we showed a down regulation in Na^+-K^+-ATPase activity and concentration in skeletal muscle of two different rodent animal models of insulin resistance. The present study investigated Na^+-K^+-ATPase function and membrane phospholipids in skeletal muscle of more mature Zucker rats, aged 25 weeks. The effect of rosiglitazone on Na^+-K^+-ATPase function in skeletal muscle has not been

addressed, therefore the other purpose of the present study was to evaluate the effects of rosiglitazone on Na^+-K^+-ATPase function and membrane phospholipids in skeletal muscle of obese Zucker rats.

5.2 Methods

5.2.1 Animals and Drug treatment

This study used 25 week-old female obese Zucker rats and age-matched female lean Zucker rats. At 19 weeks of age, 7 obese Zucker rats (OZR) and 7 lean, littermates (LZR) received, via oral gavage, rosiglitazone (3 mg/kg/day suspended in 1% carboxymethylcellulose, CMC) for 6 weeks. This dose was chosen as it has previously been shown to protect against myocardial infarction in normal rats (Yue Tl *et al.*, 2001), is the threshold dose for lowering plasma triglycerides in dietary obese rats (Pickavance *et al.*, 1999) and improves insulin sensitivity without ill effects in obese Zucker rats (Sidell *et al.*, 2002). Another 7 obese Zucker (OZ) and 7 lean, littermate controls (LZ) received only vehicle (1% CMC) for 6 weeks. All animal handling procedures have been fully described earlier (Chapter 3).

5.2.2 Physiological & Biochemical Parameters

At the end of the 6-week treatment, body weight and blood pressure (tail-cuff plethysmography measured in conscious rats using Harvard rat tail blood pressure apparatus, Hadland Phototonics Pty Ltd, Australia) were recorded. An oral glucose tolerance test (OGTT) was performed where glucose 70% weight/volume was administered by oral gavage, 2.5 g/kg body weight. Non-fasting blood was drawn from

a cut at the tip of the tail at 0 and 10, 15, 30, 60, and 90 minutes after the glucose feed for blood glucose determinations (MediSense® glucometer with Precision Plus© blood glucose electrodes; MediSense Australia). 24 hours post last dose, rats were sacrificed by asphyxiation with CO_2 inhalation followed by decapitation. Mixed arteriovenous blood was collected in heparinised tubes for analysis of risk factors associated with diabetes, plasma insulin and lipids, and blood glucose.

5.2.3 Muscle analysis

All experimental procedures for muscle extraction and analysis have been fully described earlier (Chapter 3). Briefly, red gastrocnemius (RG) was extracted from the rat hind limb and analysed for 3-*O*-MFPase activity and [^{3}H]-ouabain binding site content. RG was further analysed for determination of the phospholipid fatty acid (FA) profile via gas chromatography.

5.2.4 Data Analysis

Descriptive statistics included means and standard error (SE). One-way analysis of variance (ANOVA) was used to determine significance between groups. Significance was accepted at $p<0.05$.

5.3 Results

5.3.1 General Characteristics

Clinical and metabolic characteristics for the four groups are shown in table 5.1. OZ were 77% heavier ($P < 0.001$) than LZ which was 17% ($P < 0.01$) further exacerbated with rosiglitazone treatment (OZR). Systolic blood pressure was significantly ($P < 0.01$)

higher in OZ compared with LZ, rosiglitazone caused a noticeable drop in systolic blood pressure in OZR, though this was not statistically significant. Despite the untreated OZ having normal glucose levels, they were considerably hyperinsulinemic (946 ± 292μM), consistent with an insulin resistant, pre-diabetic state. Rosiglitazone reduced plasma insulin by 88% ($P < 0.001$) in OZR. Total cholesterol ($P < 0.05$) and triglycerides ($P < 0.001$) were significantly higher in OZ compared to LZ. Rosiglitazone treatment had no effect on cholesterol levels but significantly ($P < 0.05$) improved triglyceride concentrations in OZR (see table 5.1). OZ displayed considerable glucose intolerance with a greater than 2 fold increase in the area under the curve (AUC) when compared to LZ ($P < 0.001$), while rosiglitazone completely restored normal glucose tolerance in OZR ($P < 0.001$).

Table 5.1 General and metabolic characteristics in lean and obese Zucker control and rosiglitazone treated rats.

	LZ	**OZ**	**LZR**	**OZR**
Body Mass (g)	212 ± 7	376 ± 7†	221 ± 5	440 ± 19$^{\#}$
Mean Arterial Blood Pressure (mmHg)	134 ± 5	174 ± 8$^{\#}$	141± 6	152 ± 8
Blood Glucose (mM)	6.7 ± 0.4	6.6 ± 0.4	6.0 ± 0.3	6.5 ± 0.2
Plasma Insulin (μM)	62 ± 18	946 ± 292†	45 ± 12	113 ± 30†
Total cholesterol (mM)	1.13 ± 0.06	1.58 ± 0.06*	1.46 ± 0.17	1.84 ± 0.11
Triglycerides (mM)	0.42 ± 0.07	2.52 ± 0.56†	0.48 ± 0.08	1.15 ± 0.19*
OGTT AUC (mM*min)	248 ± 22	529 ± 72†	225 ± 28	251 ± 29†

Values are means ± SE; n=7 with the exception of Blood pressure, n=5; Significance for LZ v's OZ; LZ v's LZR and OZ v's OZR at *p<0.05, # p<0.01, † p<0.001.

5.3.2 Na^+K^+-ATPase activity

To determine the effects of obesity-related insulin resistance and rosiglitazone on Na^+K^+-ATPase activity in skeletal muscle, maximal 3-*O*-MFPase activity in red gastrocnemius was measured. There was no difference between genotypes when 3-*O*-MFPase activity was expressed per gram of wet weight. However, after measuring protein levels in RG and expressing the results per mg of muscle protein, a significant 35% decrease was found with OZ (LZ 6814 ± 690, OZ 4428 ± 359 $pmol.min^{-1}.g^{-1}$ protein, $P < 0.01$) (Figure 5.1). When calculating the activity of an enzyme, the amount of muscle protein in the sample needs to be taken into account as added weight due to fluid or connective tissue, which does not contain Na^+K^+-ATPase, will underestimate the enzyme's activity level. Rosiglitazone treatment induced, again, a significant 35% decrease in 3-*O*-MFPase activity (LZ 6814 ± 690, LZR 4416 ± 404 $pmol.min^{-1}.g^{-1}$ protein, $P < 0.01$) (Figure 5.1). There was no change in OZR with treatment.

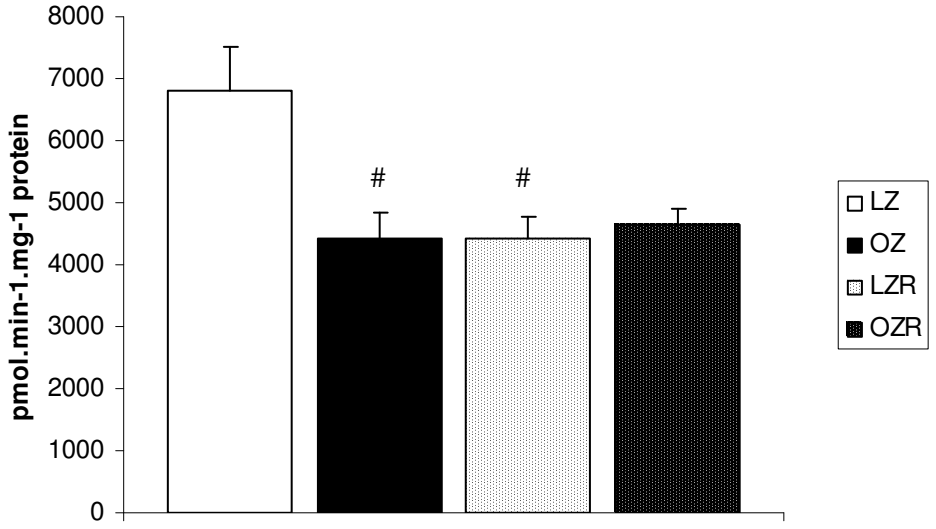

Figure 5.1 Na^+K^+-ATPase activity measured by 3-*O*-MFPase assays in RG. Data are means ± SE for 7 rats per group. Significance for LZ v's OZ and LZ v's LZR # $p<0.01$.

5.3.3 Na^+K^+-ATPase content

The concentration of Na^+K^+-ATPase was determined using the Vanadate facilitated [^{3}H]-ouabain binding method on whole muscle samples. Results are displayed in Figure 5.2. There was no detectable change in Na^+K^+-ATPase content between OZ and LZ rats, nor did rosiglitazone treatment have any affect on content.

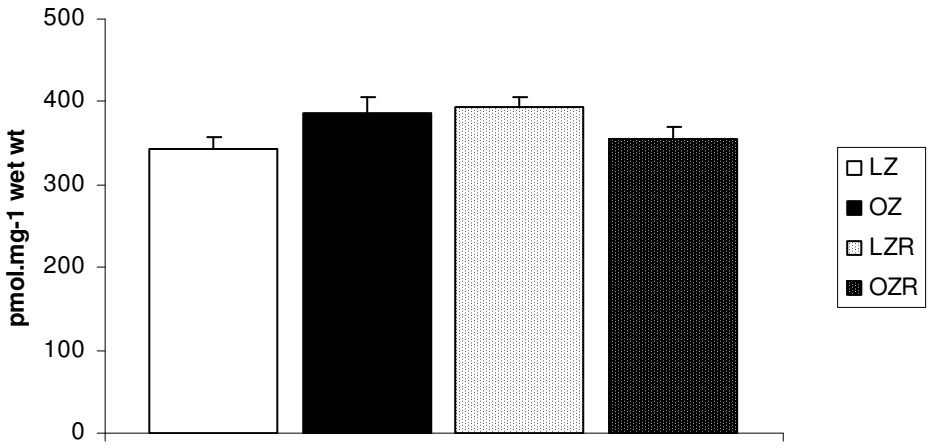

Figure 5.2 Na^+K^+-ATPase concentration measured by Vanadate facilitated [^{3}H]-ouabain binding assay in RG of control LZ and OZ rats and rosiglitazone treated LZR and OZR. Data are means ± SE for 7 rats per group.

5.3.4 Membrane Phospholipid analysis

The phospholipid fatty acid (FA) composition in RG membranes was determined by measuring the content of individual FA types and representing them as a percentage of the total FA content. Results are shown in Table 5.3. Saturated fatty acids, palmitic acid [16:0] and stearic acid [18:0] contents were not different across all groups. The

monounsaturated omega-9, oleic acid [18:1(n-9], was also unchanged. As a result, there were no differences found in percentage of saturated and mono-unsaturated FA (see Table 5.3). In contrast, variations were identified with different PUFA species, specifically between omega types. Of the omega-6 PUFAs, only linoleic acid [18:2(n-6)] was not significantly changed between animal groups, however there was a significant increase in the levels of dihomo-gamma-linolenic acid [20:3(n-6)] ($P < 0.001$) and arachidonic acid [20:4(n-6)] ($P < 0.01$) in OZ compared to LZ. Eicosapentaenoic acid (EPA) [20:5(n-3)], docosapentaenoic acid (DPA) [22:5 (n-3)] and docosahexaenoic acid (DHA) [22:6(n-3)] are long chained omega-3 PUFA. There was a significant ($P < 0.001$) decrease in DHA in the OZ compared to LZ, but only an indicated decrease in the content of EPA (non significant), while DPA was increased ($P < 0.01$). Together, the findings led to an increase in % omega-6 (n-6) and a decrease in % omega-3 (n-3), and therefore an overall 45% increase in the n-6/n-3 ratio ($P < 0.001$) in OZ compared with LZ.

Rosiglitazone only had a significant effect on membrane phospholipids in OZR, displaying lower arachidonic acid ($P < 0.001$) but elevated EPA ($P < 0.001$) (see Table 5.2). The OZR also had decreased % PUFA and % omega-6, resulting in a significant decrease in the unsaturation index ($P < 0.05$) compared to OZ.

Table 5.2 Phospholipid fatty acid composition of RG.

Fatty acid		LZ	OZ	LZR	OZR
16:0	Palmitic	13.2 ± 0.8	13.4 ± 0.8	14.9 ± 1.4	16.7 ± 1.3
18:0	Stearic	10.8 ± 0.5	10.9 ± 0.2	10.8 ± 0.3	11.1 ± 0.3
18:1(n-9)	Oleic	6.1 ± 0.3	6.7 ± 0.3	6.3 ± 0.1	7.3 ± 0.3
18:2(n-6)	Linoleic	22.3 ± 0.4	23.5 ± 0.5	21.7 ± 0.8	24.8 ± 1.2
20:3(n-6)	DHLA	0.70 ± 0.07	1.20 ± 0.04†	0.82 ± 0.04	1.20 ± 0.08
20:4(n-6)	Arachidonic	14.3 ± 0.2	17.7 ± 0.7#	14.7 ± 0.5	12.7 ± 0.9†
20:5(n-3)	EPA	0.78 ± 0.07	0.53 ± 0.03	1.00 ± 0.05	1.07 ± 0.14†
22:5(n-3)	DPA	1.53 ± 0.07	2.04 ± 0.13 #	1.43 ± 0.06	1.89 ± 0.11
22:6(n-3)	DHA	27.7 ± 0.7	20.8 ± 0.8†	25.1 ± 0.6	19.8 ± 1.1
% Saturated FA		23.9 ± 0.9	24.3 ± 0.9	25.7 ± 1.4	27.8 ± 1.4
% MUFA		8.3 ± 0.3	9.4 ± 0.4	9.0 ± 0.2	10.3 ± 0.4
% PUFA		67.7 ± 1.0	66.3 ± 1.0	65.3 ± 1.2	61.9 ± 1.2*
% n-6		37.8 ± 0.5	42.9 ± 0.9†	37.8 ± 0.8	39.2 ± 1.1*
% n-3		30.0 ± 0.7	23.4 ± 0.8†	27.5 ± 0.6	22.7 ± 1.0
% Unsaturated FA		76.1 ± 0.9	75.7 ± 0.9	74.3 ± 1.4	72.2 ± 1.4
Unsaturation index		292 ± 5	271 ± 5*	279 ± 6	251 ± 6*
n-6/n-3		1.26 ± 0.03	1.85 ± 0.08†	1.38 ± 0.03	1.75 ± 0.10

Values are means ± SE; n=7; Fatty acid composition is expressed as mole percentage of total fatty acids; DHLA, Dihomo gamma-linolenic acid; EPA, Eicosapentaenoic acid; DPA, Docosapentaenoic acid; DHA, Docosahexaenoic acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; Unsaturation index is the number of double bonds per 100 fatty acid chains; Significance for LZ v's OZ; LZ v's LZR and OZ v's OZR at *p<0.05, # p<0.01, † p<0.001.

The ratios of 20:4 to 20:3, 20:3 to 18:2, 18:1 to 18:0 and 18:0 to 16:0 are product-precursor ratios for the reactions catalysed by delta(sup 5)-desaturase (Δ^5desaturase), delta(sup 6)-desaturase (Δ^6desaturase), delta(sup 9)-desaturase (Δ^9desaturase) and elongase respectively. The estimated activity of Δ^5desaturase was significantly ($P < 0.01$) decreased in OZ compare to LZ, while Δ^6desaturase activity was significantly ($P < 0.01$) increased. Refer to Table 5.3.

Table 5.3 Estimated activity of desaturase and elongase enzymes in RG.

Enzyme		LZ	OZ	LZR	OZR
20:4/20:3	Δ^5desaturase	21.26 ± 1.49	14.75 ± 0.51#	21.26 ± 1.49	21.26 ± 1.49
20:3/18:2	Δ^6desaturase	0.032 ± 0.003	0.051 ± 0.002†	0.038 ± 0.003	0.049 ± 0.003
18:1/18:0	Δ^9desaturase	0.57 ± 0.02	0.61 ± 0.02	0.58 ± 0.02	0.66 ± 0.03
18:0/16:0	Elongase	0.84 ± 0.07	0.83 ± 0.05	0.77 ± 0.08	0.69 ± 0.06

Values are means ± SE; n=7; Significance for LZ v's OZ; at # p<0.01, † p<0.001.

5.4 Discussion

Obesity-related insulin resistance is a characteristic feature of type II diabetes and often the persistence of obesity interferes with conventional therapy. In these cases, a pharmacological approach is commonly used to reinstate normoglycemia. In this study, rosiglitazone substantially improved peripheral insulin sensitivity by decreasing insulin levels and improving glucose tolerance. However, as expected, rosiglitazone treatment also promoted weight gain and increased fat deposition. Na^+K^+-ATPase function was

found to be depressed with insulin resistance in the mature obese Zucker rats. Rosiglitazone's action on Na^+K^+-ATPase function in skeletal muscle of the lean Zucker rat was detrimental however in the obese Zucker rat, no drug effect was seen.

5.4.1 Effect of Insulin Resistance

In the present study, Na^+K^+-ATPase activity was, again, found to be depressed with insulin resistance in skeletal muscle of obese Zucker rats. However, in contrast to the previous study, Na^+K^+-ATPase content was found to be unaltered between the 25 week old lean and obese rat groups used for this study. This could possibly be explained by the use of much older rats as study 1 involved 15 week old rats. It may be that skeletal muscle attempts to rectify the Na^+K^+-ATPase disregulation seen in younger rats by upregulating the number of pumps in the membrane, just as excess insulin is produced when insulin resistance occurs. In addition, female obese Zucker rats are also known to be less susceptible to insulin resistant changes than their male equivalent and could also offer an explanation for this discrepancy. Finally it is also possible that the known age lowering effect on Na^+K^+-ATPase could mask any content differences between the groups (Kjeldsen *et al.*, 1984a). Nevertheless, these results indicate that the membrane protein is not compromised at a gene or protein level but at a functional level. On the other hand, many studies have shown different $\propto$-isoforms of the Na^+K^+-ATPase can display varying turnover rates in a variety of different tissues (Maixent & Berrebi-Bertrand, 1993; Segall *et al.*, 2001). Therefore, reduced Na^+K^+-ATPase activity without a change in content may be representing a change in Na^+K^+-ATPase isoenzymes, whereby the α_1 isoenzymes may have been reduced but not detected by the content

assay as this α isoform is ouabain resistant (Kjeldsen *et al.*, 1986). However, as reduced Na^+K^+-ATPase function can cause hyperkalemia and contribute to early onset of fatigue, the reduced exercise capacity seen with insulin resistant or diabetic patients may be, in part, explained by the compromised Na^+K^+-ATPase function as seen in obese Zucker rats.

The function of membrane enzymes, such as Na^+K^+-ATPase, is known to depend on membrane fluidity and lipidic components (Storch & Schachter, 1984). Specifically, DHA plays an important structural role in the plasma membrane and has been shown to increase fluidity (Hashimoto *et al.*, 1999; Petrache *et al.*, 2001). OZ had a reduced percentage of omega-3 PUFA, principally from decreased DHA content. In addition the percentage of omega-6 PUFAs was elevated. Omega-6 PUFA have been associated with inhibiting the absorbance of omega-3 PUFA, in a competitive nature (Iritani & Fujikawa, 1982). The net result was an increase in n-6/n-3 ratio, which is associated with reduced membrane fluidity (Farkas *et al.*, 2002). Na^+K^+-ATPase function in skeletal muscle from insulin resistant OZ rats may have been compromised due to the phospholipid profile, which indicated reduced fluidity in the plasma membrane. This is in agreement with other researchers who have used obese animal models and have found similar results with the n-6/n-3 ratio and Na^+K^+-ATPase activity in other insulin sensitive tissues (Izpisua *et al.*, 1989). PUFAs also allow a higher incorporation of cholesterol (rigidness) in the membranes to balance their fluidity, which would contribute to lower blood cholesterol levels (Colin *et al.*, 2003), which was found with the OZ compared with LZ.

In this study, the estimated activity determined for Δ^5desaturase was also found to be significantly decreased in the OZ when compared to the LZ. Δ^5desaturase is responsible for catalysing the addition of a double bond into a FA, and it's product-precursor ratio has been previously correlated with insulin sensitivity (Borkman *et al.*, 1993). Another enzyme responsible for inserting double bonds, Δ^6desaturase also displayed reduced activity in the OZ skeletal muscle. This is again in accordance with Borkman's study which found Δ^6desaturase to be negatively correlated with insulin sensitivity, although this was not statistically significant (Borkman *et al.*, 1993). Other studies of insulin deficiency have also shown that insulin has an effect on fatty-acid desaturase activity (Tilvis & Miettinen, 1985; el Boustani *et al.*, 1989). This could possibly suggest that reduced levels of unsaturated fatty acids in the membrane may be a result of reduced insulin action, caused by either insulin resistance or insulin deficiency.

It is also known that several protein kinases affect the function of Na^+K^+-ATPase. In particular, protein kinase C (PKC) has also been associated with the development of insulin resistance in skeletal muscle (Qu *et al.*, 1999). With insulin resistance, increased diacylglycerol-protein kinase C (DAG-PKC) signalling has been associated with reduced glucose transport and metabolism. However, several DAG-PKC isoforms were measured in muscle from these animals in an associated study and were shown to be not elevated (Lessard *et al.*, 2004).

5.4.2 Effect of Rosiglitazone

Rosiglitazone had a detrimental effect on Na^+K^+-ATPase function in skeletal muscle of lean Zucker rats. Na^+K^+-ATPase activity in LZR was reduced by the same degree that was seen with that of OZ. Maximal activity of OZR did not further decrease with rosiglitazone but remained similar to that of OZ. Rosiglitazone, therefore, had either no effect on Na^+K^+-ATPase in skeletal muscle of obese Zucker rats or, its effect was not seen because it was not additive to the decrease already observed with insulin resistance alone. Na^+K^+-ATPase content was unaltered with rosiglitazone treatment so the drop in maximal activity could not be due to lack of enzyme concentration but as a result of activation or functional inhibition, or isoenzyme changes.

Analysis of the phospholipids in LZR showed rosiglitazone had no significant effect. Rosiglitazone did significantly increase EPA content and percentage of omega-6 PUFAs, and decrease Arachnidonic acid content, the percentage of PUFA and unsaturation index in OZR when compared to the OZ. However, upon closer inspection of LZR lipid results, most of the FA's and composite parameters do appear to change in the same direction as the OZ despite the lack of statistical significance, for example DHLA, Arachnidonic acid and DHA content, percentages of FA groups, the unsaturation index and n-6/n-3 ratio. Nevertheless, we cannot conclude FA's or the degree of saturation in the sarcolemma was responsible for rosiglitazone's effect, and the inhibitory effect may involve a different mechanism to that of insulin resistance.

Rosiglitazone is thought to have its primary effects in adipose tissue and therefore any effect seen in muscle may be secondary. Leptin, an adipose-derived hormone, stimulates thermogenesis by its direct effects on leptin receptors in skeletal muscle (Friedman & Halaas, 1998; Dulloo *et al.*, 2002) and has been negatively correlated with Na^+K^+-ATPase function in kidney (Beltowski *et al.*, 2002; Beltowski & Wojcicka, 2002; Beltowski *et al.*, 2004), liver (Cha & Jones, 1998) and fibroblast cells (Sweeney & Klip, 2001). Obese Zucker rats display markedly elevated circulating leptin levels due to a mutation in the gene for the leptin receptor and the lack of auto-regulation (Hardie *et al.*, 1996; Wang *et al.*, 1999) which may offer a possible explanation for the decreased pump activity observed in these rats. Rosiglitazone has been shown to transcriptionally inhibit leptin expression in adipocytes (De Vos *et al.*, 1996) and decrease serum leptin levels (Boden *et al.*, 2003; Toruner *et al.*, 2004), however, in obese or diabetic Zucker (*fa/fa*) rats, leptin levels remain unchanged (Wang *et al.*, 1997; Cai *et al.*, 2000). This may explain why LZR responded to rosiglitazone and in OZR, where leptin signalling is dysfunctional, Na^+K^+-ATPase function remained unaffected.

Another possible explanation involves AMP-activated protein kinase (AMPK), a regulatory protein that monitors energy consumption and down-regulates ATP-consuming processes when activated, including enzyme activity. AMPK has been shown to be activated by rosiglitazone in cultured skeletal muscle cells (Fryer *et al.*, 2002) and by pioglitazone in rat liver and adipose tissue (Saha *et al.*, 2004). AMPK also has been shown to inhibit activity of a specific ATP-dependent Cl^- channel which also requires ATP hydrolysis by Na^+K^+-ATPase for chloride secretion (Hardie *et al.*, 2003).

Therefore, inhibition of Na^+K^+-ATPase by rosiglitazone may be secondary via AMPK activation. The AMPK activity in skeletal muscle of obese Zucker rats was shown to be normalised with chronic rosiglitazone treatment (Lessard *et al.*, 2006).

5.4.3 Conclusion

In conclusion, this study found Na^+K^+-ATPase activity, but not content, to be altered in insulin resistant obese Zucker rats and that this may be due to altered membrane phospholipid composition and desaturase activity as seen with this phenotype. Other possible explanations could involve PKC signalling or leptin concentrations. Despite insulin sensitivity increasing through rosiglitazone, Na^+K^+-ATPase activity was reduced only in the lean rats. This was not found to be due to decreased enzyme content or alterations in membrane lipids but may possibly be due to elevated leptin levels or AMPK activation, leading to inhibition of the membrane bound Na^+K^+-ATPase. Therefore, the treatment of insulin resistance with rosiglitazone may increase insulin sensitivity but may also affect Na^+K^+-ATPase, which may limit skeletal muscle function.

Chapter 6

STUDY 3: EFFECT OF ROSIGLITAZONE AND EXERCISE ON NA^+K^+-ATPASE FUNCTION IN INSULIN RESISTANT SKELETAL MUSCLE.

6.1 Introduction

Type II diabetics receive a range of treatment options to relieve the insulin resistance and or help reduce the associated risk factors for cardiovascular disease (Rennert & Charney, 2003). The treatment options include diet and exercise before the option of medication such as insulin sensitisers, such as Rosiglitazone, are administered (Rennert & Charney, 2003). The previous chapter showed that diabetic rats had lower Na^+K^+-ATPase activity and that rosiglitazone lowered Na^+K^+-ATPase activity in lean controls. It is postulated that exercise training would increase Na^+K^+-ATPase content and activity in diabetic rats. Further, exercise may counter any deleterious effects that rosiglitazone has on Na^+K^+-ATPase function. Therefore the third study of this dissertation examined the individual effects of rosiglitazone and exercise and their combined effect on the Na^+K^+-ATPase function in skeletal muscle of high-fat fed rats.

It has been well documented that regular exercise promotes physiological and health benefits (Karacabey, 2005). Studies have shown exercise training improves insulin sensitivity and increases insulin-stimulated skeletal muscle glucose transport in insulin resistant animal models (Wallberg-Henriksson & Holloszy, 1984; Ivy *et al.*, 1989; Cortez *et al.*, 1991) and Type II diabetics (Ivy *et al.*, 1989; Perseghin *et al.*, 1996). The increased insulin sensitivity with exercise training is mostly attributed to increased expression and

or activity of signalling proteins involved in the regulation of skeletal muscle glucose uptake (Zierath, 2002). These proteins include Glut 4 (Henriksen, 2002; Holloszy, 2005) and increased expression of PI-3 kinase activity (Houmard *et al.*, 1999) or AMPK protein (Sriwijitkamol *et al.*, 2006).

Chronic exercise training increases skeletal muscle Na^+K^+-ATPase concentration in both lean animal models (Kjeldsen *et al.*, 1986; Tsakiridis *et al.*, 1996) as well as healthy individuals (Green *et al.*, 1993; McKenna *et al.*, 1993; Madsen *et al.*, 1994; Fraser *et al.*, 2002). However, no one has investigated the potential benefits of exercise training on Na^+K^+-ATPase function in skeletal muscle of insulin resistant animals or Type II diabetics. These diabetic animals have impaired Na^+K^+-ATPase (see Chapter 4 and 5) so the beneficial effects of exercise training warrant investigation. The potential benefit of exercise training might be countered by any deleterious effects that co administered medication might have. Rosiglitazone increases insulin sensitivity and peripheral glucose uptake, without stimulating insulin production (Miyazaki *et al.*, 2001; Wagstaff & Goa, 2002). The intrinsic activity of the ion transporter, Na^+-K^+-ATPase is dependant upon the membrane lipid milieu. These TZDs such as rosiglitazone may also affect membrane fluidity and the function of membrane transport proteins (Kurebayashi *et al.*, 1997). This present study will investigate the effect of rosiglitazone and exercise on Na^+-K^+-ATPase function and membrane phospholipids in skeletal muscle of the high fat fed Sprague Dawley rat, an animal model of insulin resistance that develops type II diabetes.

6.2 Methods

6.2.1 Animals and Treatment groups

This study involved the use of 80 Sprague Dawley rats ordered at 4 weeks of age, 32 of which have already been described in Chapter 4. Of those the high fat (HF) group will represent the control group for the remaining 48 rats. 48 male rats were acclimatised for 2 weeks and allowed ad libitum access to water and standard rat chow (73% carbohydrate, 11% fat, 16% protein, D12328; Research Diets Inc., New Brunswick, NJ) before commencing a high fat diet containing hydrogenated coconut oil (58% of calories as fat and 26% as carbohydrate, D12330N; Research Diets). 4 weeks into the diet, 16 rats were randomly assigned to a rosiglitazone treatment group (HFR), an exercise treatment group (HFX) or a rosiglitazone plus exercise group (HFRX). At the end of their respective treatments, each group of 16 rats were divided into 2, one group receiving basal perfusate and the other group, insulin perfusate (500 μU/ml insulin concentration) for hind limb perfusion to measure insulin stimulated glucose uptake as previously described (Herr *et al.*, 2005).

Rats assigned to the rosiglitazone treatment groups had their high fat (D12330) diet replaced with high fat mixed with rosiglitazone maleate (SmithKline Beecham, Harlow, UK) diet (D04110901). The mixture was made into coloured pellets and dried by the manufacturer (Research Diets, Inc., New Jersey, US). The concentration of rosiglitazone in the food was 50 ppm. Drug-treated rats ingested an average daily dose of 2.08 ± 0.06 mg/kg body mass of rosiglitazone, a more therapeutic dose than the one

used in the previous study however the dose is still considered high when compared to doses used in humans.

Animals appointed to the exercise training groups underwent an endurance exercise training regime, carried out on a rodent treadmill. Rats were run 5 days per week for a period of 4 weeks. The work rates were gradually increased in the first week of training until the rats could run for a total time of 60 min at speed of 32 m/min on a 15% incline.

6.2.2 Physiological & Biochemical Parameters

All animal handling procedures have been fully described earlier (Chapter 3). At the end of their respective diets, animals were fasted for 8-12 h and exercised animals undertook their last acute training bout 36-48 h prior to undergoing hind limb perfusion. Rats were anaesthetised with pentobarbital (6.5 mg/100g body wt) and body weight was recorded. Left and right epididymal fat pad were quickly dissected out before preparing rats for hind limb perfusion, as previously described (Chapter 4). Blood was collected via cardiac puncture and rats were sacrificed via intracardiac injection of pentobarbital. After a stabilising washout period, 8 minutes of hind limb perfusion with a perfusate containing 8 mM of the non-metabolised glucose analogue 3-*O*-methylglucose (3-MG; 32 μCi 3-[^{3}H-*O*-MG/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, to measure glucose uptake. Red oxidative gastrocnemius was extracted from the perfused hind limb and classed as basal (HF, HFR, HFX or HFRX) or insulin

stimulated (HFIns, HFRIns, HFXIns or HFRXIns) tissue if perfusate contained 500μU/ml of insulin. Blood samples were immediately centrifuged and serum collected and frozen at -80ºC for later analysis of insulin, glucose and FFA concentrations.

6.2.3 Muscle analysis

All experimental procedures for muscle extraction and analysis have been fully described (Chapter 3). Briefly, basal and insulin stimulated RG was analysed for 3-*O*-MFPase activity and [^{3}H]-ouabain binding site content. Basal RG of all groups including the normal chow fed (NC, previously discussed in Chapter 4) group was further analysed for determination of the phospholipid fatty acid (FA) profile via gas chromatography.

6.3 Results

6.3.1 General Characteristics

Clinical and metabolic characteristics for the four high fat groups are shown in table 6.1. Rosiglitazone treatment did not have the expected detrimental effect on body or epididymal fat pad weight with no significant difference found after treatment. However exercise training caused a significant decrease in both body mass and epididymal fat pad weight. Body mass was reduced by 10% ($P < 0.01$) and 12% ($P < 0.001$) in the HFX and HFRX group respectively compared to the control HF group (HF 460 ± 10, HFX 413 ± 7 grams, HFRX 403 ± 8 grams, n=16) while epididymal fat pad weight were decreased by approximately 36% in HFX and HFRX (HF 10.6 ± 0.8, HFX 6.9 ± 0.6 grams, HFRX 6.7 ± 0.3 grams, n=16 $P < 0.001$). There was no

significant difference between HFX and HFRX but a significant decrease between HFR and HFRX in body mass and epididymal fat pad weights, indicating the reducing effect in the HFRX group was due to the exercise training and not rosiglitazone treatment.

There was no difference between glucose and insulin levels between groups. Glucose concentrations were elevated due to haemolysis (Kilpatrick *et al.*, 1995), caused when samples were collected via intracardiac puncture. Free fatty acids were significantly ($P < 0.05$) decreased in both exercised groups compared to the untreated HF group (HF 0.60 ± 0.10, HFX 0.33 ± 0.05 and HFRX 0.29 ± 0.4 mmol/L).

Table 6.1 General and metabolic characteristics in lean and obese Zucker control and rosiglitazone treated rats.

	HF	**HFR**	**HFX**	**HFRX**
Body Mass (g)	460 ± 10	458 ± 9§	413 ± 7$^{\#}$	403 ± 8†
Epidydimal Fat Pads	10.6 ± 0.8	9.7 ± 0.5¶	6.9 ± 0.6†	6.7 ± 0.3†
Blood Glucose (mM)	16.4 ± 1.0	15.7 ± 1.3	16.2 ± 1.0	11.8 ± 1.3
Plasma Insulin (ng/ml)	1.21 ± 0.24	1.25 ± 0.21	0.84 ± 0.16	0.88 ± 0.20
Free Fatty Acids (mM)	0.60 ± 0.10	0.50 ± 0.05	0.33 ± 0.05*	0.29 ± 0.4*

Values are means ± SE; n=16 with the exception of blood parameters, n=7-8;
Significance for HF v's HFX and HFRX is at * $p<0.05$, # $p<0.01$, † $p<0.001$ and for HFRX v's HFX and HFR is at, ¶ $p<0.01$, § $p<0.001$.

6.3.2 Glucose Uptake

Glucose uptake was measured using 3-*O*-methylglucose and calculated from the arteriovenous difference, the perfusate flow rate and the weight of the muscle perfused. As expected, glucose uptake in basal muscle did not differ between treatment groups. As previously stated in Chapter 4, insulin stimulation did not cause a significant increase in glucose uptake in RG in the high-fat fed control group (HF 2.32 ± 0.85 and HFIns 4.11 ± 0.56 $\mu mol.g^{-1}.hr^{-1}$), confirming established insulin resistance. However, all treatment groups showed significant increases in glucose uptake upon insulin stimulation. Rosiglitazone treatment caused a 3.6 fold increase (HFR 1.39 ± 0.31, HFRIns 5.05 ± 0.63 $\mu mol.g^{-1}.hr^{-1}$, $P < 0.01$), chronic exercise caused a 3.8 fold increase (HFX 2.11 ± 0.56, HFXIns 8.09 ± 1.22 $\mu mol.g^{-1}.hr^{-1}$, $P < 0.01$) and both treatments caused a 2.0 fold increase (HFRX 2.92 ± 0.65, HFRXIns 6.02 ± 0.77 $\mu mol.g^{-1}.hr^{-1}$, $P < 0.01$).

Table 6.2 3-*O*-methylglucose uptake in RG.

	Glucose Uptake (µmol.g-1.hr-1)			
Basal	**HF**	**HFR**	**HFX**	**HFRX**
	2.32 ± 0.85	1.39 ± 0.31	2.11 ± 0.56	2.97 ± 0.65
500 µU/ml	**HFIns**	**HFRIns**	**HFXIns**	**HFRXIns**
Insulin	4.11 ± 0.56	5.05 ± 0.63#	8.09 ± 1.22#	6.02 ± 0.77#

Values are means ± SE; n=8; Significance for HFR v's HFRIns; HFX v's HFXIns; and HFRX v's HFRXIns is at # $P < 0.01$.

6.3.3 Na^+K^+-ATPase activity

To determine the effects of rosiglitazone or exercise and their combined effect on Na^+K^+-ATPase activity in skeletal muscle, maximal 3-*O*-MFPase activity was measured in red gastrocnemius collected from treatment groups. There was a significant increase in 3-*O*-MFPase activity when insulin resistant rats were endurance exercise trained. Compared to the HF control group, exercise training caused a significant 58% ($P < 0.001$) and 46% ($P < 0.01$) rise in maximal 3-*O*-MFPase activity in HFX and HFRX groups respectively (HF 412 ± 27, HFX 652 ± 38 and HFRX 600 ± 24 $nmol.min^{-1}.g^{-1}$ wet wt, refer to Figure 6.1). The difference between groups was also seen when activity was made relative to muscle protein. Maximal 3-*O*-MFPase activity was significant 83% ($P < 0.01$) and 62% ($P < 0.05$) higher in HFX and HFRX groups respectively compared to HF controls (HF 4041 ± 254, HFX 7389 ± 687 and HFRX 6553 ± 558 $pmol.min^{-1}.mg^{-1}$ protein). Treating rats with rosiglitazone did not cause any significant change in 3-*O*-MFPase activity (HF 4041 ± 254 and HFR 5168 ± 785 $pmol.min^{-1}.g^{-1}$ protein). Insulin stimulation did not have the anticipated effect on Na^+K^+-ATPase with no significant difference in maximal activity compared to basal tissue.

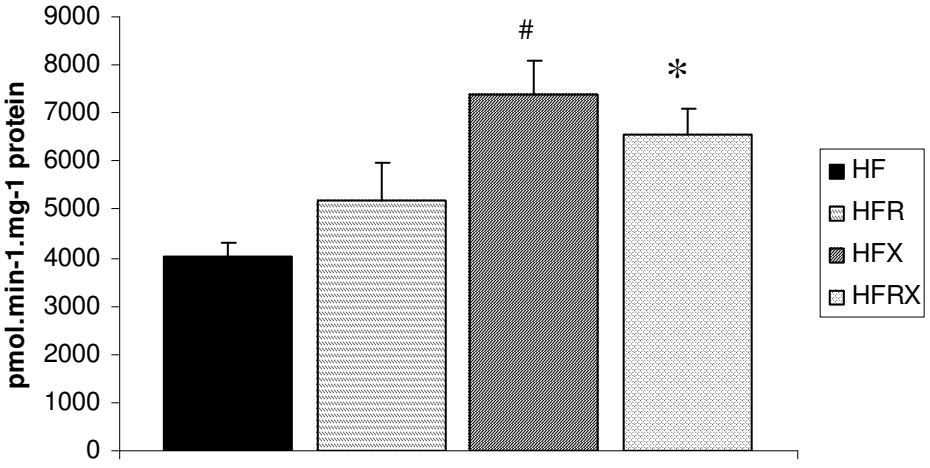

Figure 6.1 Na^+K^+-ATPase activity measured by 3-*O*-MFPase assays in basal RG. Data are means ± SE for 8 rats per group. Significance for HF v's HFX and HF v's HFRX at * p< 0.05 and # p<0.01.

6.3.4 Na^+K^+-ATPase content

The concentration of Na^+K^+-ATPase was determined using the Vanadate facilitated [^{3}H]-ouabain binding method on whole muscle samples. Exercise training had a significant effect on rats with HFX and HFRX having 53% ($P < 0.001$) and 79% ($P < 0.001$) respective increases in Na^+K^+-ATPase content when compared to HF control (HF 307 ± 18, HFX 471 ± 28 and HFRX 549 ± 23 pmol.g^{-1} wet wt). Rosiglitazone treatment did not seem to have an effect on content as there was no significant difference between HF and HFR but a significant difference between HFR and HFRX (HFR 328 ± 17 and HFRX 549 ± 23 pmol. g^{-1} wet wt, $P<0.01$). Results are displayed in Figure 6.2. There was no difference between basal and insulin stimulated muscle, indicating the insulin concentration used did not have the expected effect.

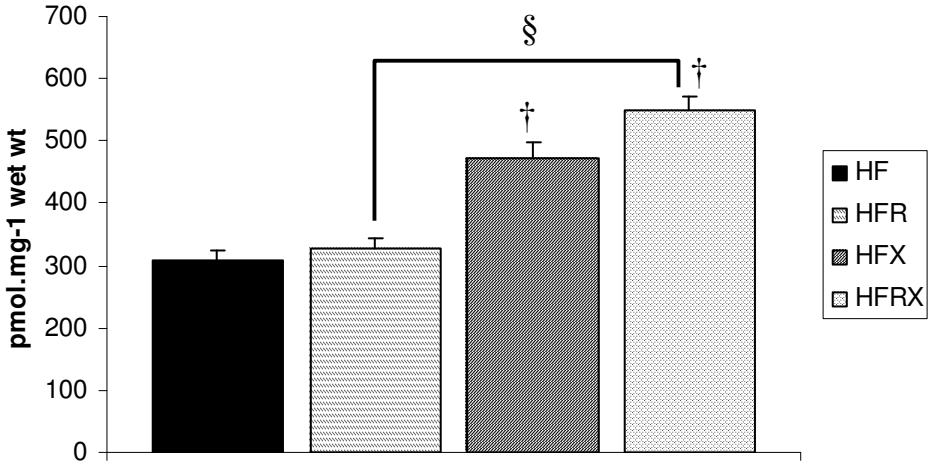

Figure 6.2. Na^+K^+-ATPase concentration measured by Vanadate facilitated [^{3}H]-ouabain binding assay in RG. Data are means ± SE for 8 rats per group. Significance for HF v's HFX and HF v's HFRX at † $p< 0.001$ and HFR v's HFRX is at § $p<0.01$

6.3.5 Membrane Phospholipid analysis

6.3.5.1 Effect of High-Fat Diet

The effect of the high-fat diet was investigated by comparing NC and HF groups. The content of saturated fatty acid palmitic acid [16:0] was significantly decreased ($P < 0.05$) while stearic acid [18:0] ($P < 0.001$) was increased in the HF group. All monounsaturated FA's were significantly decreased; palmitoleic [16:1(n-7)], vaccenic [18:1(n-7)] and oleic [18:1(n-9] acid, and of the omega 6 PUFAs, only dihomo-gamma-linolenic acid [20:3(n-6)] was significantly increased ($P < 0.001$) in HF compared to NC. No differences were found in the long chained omega 3 PUFA.

As a result, there were significant differences found in the percentages of saturated, mono-unsaturated, polyunsaturated and unsaturated FA but overall unsaturation index remained unchanged (refer to Table 6.3). No significant differences were found between omega groups.

Table 6.3 Phospholipid fatty acid composition of RG in NC and HF rats.

Fatty acid		NC	HF
16:0	Palmitic	19.4 ± 0.6	16.9 ± 1.0*
18:0	Stearic	17.2 ± 0.7	22.4 ± 0.7†
16:1(n-7)	Palmitoleic	1.22 ± 0.13	0.34 ± 0.04†
18:1(n-7)	Vaccenic	4.51 ± 0.33	1.93 ± 0.08†
18:1(n-9)	Oleic	5.1 ± 0.2	3.8 ± 0.1†
18:2(n-6)	Linoleic	24.0 ± 0.7	25.1 ± 0.5
20:3(n-6)	DGLA	0.61 ± 0.01	0.82 ± 0.04†
20:4(n-6)	Arachidonic	12.8 ± 0.3	13.3 ± 0.4
22:5(n-3)	DPA	2.53 ± 0.09	2.51 ± 0.07
22:6(n-3)	DHA	11.7 ± 0.5	12.0 ± 0.5
% Saturated FA		36.6 ± 0.3	39.3 ± 0.4†
% MUFA		10.8 ± 0.6	6.1 ± 0.2†
% PUFA		52.6 ± 0.5	54.6 ± 0.5#
% n-6		38.4 ± 0.5	40.1 ± 0.7
% n-3		14.2 ± 0.5	14.5 ± 0.5
% Unsaturated FA		63.4 ± 0.3	60.7 ± 0.4†
Unsaturation index		199 ± 2	201 ± 2
n-6/n-3		2.73 ± 0.12	2.80 ± 0.12

Values are means ± SE; n=8; Fatty acid composition is expressed as mole percentage of total fatty acids; DGLA, Dihomo gamma-linolenic acid; DPA, Docosapentaenoic acid; DHA, Docosahexaenoic acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; Unsaturation index is the number of double bonds per 100 fatty acid chains; Significance for NC v's HF at *p<0.05, # p<0.01, † p<0.001.

The ratios of 20:4 to 20:3, 20:3 to 18:2, 18:1 to 18:0 and 18:0 to 16:0 are product-precursor ratios for the reactions catalysed by delta(sup 5)-desaturase (Δ^5desaturase), delta(sup 6)-desaturase (Δ^6desaturase), delta(sup 9)-desaturase (Δ^9desaturase) and elongase respectively. The estimated activities of Δ^5desaturase and Δ^9desaturase were significantly ($P < 0.001$) decreased, while activities of Δ^6desaturase and elongase were significantly ($P < 0.01$) increased in HF compared to NC group. Refer to Table 6.4.

Table 6.4 Estimated activity of desaturase and elongase enzymes in RG.

Enzyme		NC	HF
20:4/20:3	Δ^5desaturase	21.00 ± 0.62	16.37 ± 0.54†
20:3/18:2	Δ^6desaturase	0.026 ± 0.002	0.033 ± 0.002#
18:1/18:0	Δ^9desaturase	0.30 ± 0.02	0.17 ± 0.01†
18:0/16:0	Elongase	0.90 ± 0.07	1.38 ± 0.14#

Values are means ± SE; n=8. Significance for NC v's HF at # p<0.01 and † p<0.001.

6.3.5.2 Effect of Exercise

Chronic exercise training had a mixed effect on the membrane phospholipid profile, results are shown in Table 6.5. No change was seen in saturated and monounsaturated fatty acids. Of the omega 6 PUFAs, the essential linoleic acid [18:2(n-6)] was significantly ($P < 0.001$) increased while arachidonic acid [20:4(n-6)] was significantly ($P < 0.001$) decreased in HFX compared to HF. Overall there was a significant ($P < 0.05$) decrease and ($P < 0.05$) increase in the % of saturated and unsaturated fatty acids respectively. However, when unsaturation was expressed as the number of double

bonds per 100 fatty acid chains, unsaturation index was significantly ($P < 0.05$) decreased. Together, the findings led to an increase in % omega-6 (n-6) and a decrease in % omega-3 (n-3), and therefore an overall 19% increase in the n-6/n-3 ratio ($P < 0.05$) in HFX compared with HF.

6.3.5.3 Effect of Rosiglitazone

Rosiglitazone treatment significantly ($P<0.01$) increased the monounsaturated omega 9, oleic acid [18:1(n-9] leading to a significant ($P<0.05$) increase in the % of monounsaturated fats (see Table 6.5). The % of omega 3 fatty acids was significantly ($P<0.05$) decreased however the n-6/n-3 ratio was unchanged.

6.3.5.4 Effect of Combined treatments

In the HFRX group, treatments had an added effect on the phospholipid composition with changes displayed by the HFR and HFX groups repeated in the HFRX group when compared to HFC (refer to Table 6.5). Oleic and linoleic acid were significantly ($P<0.01$) increased and arachidonic acid was significantly ($P < 0.001$) decreased. A significant ($P<0.001$) decrease in docosapentaenoic acid (DPA) was also seen. Overall, there was a significant ($P < 0.05$) decrease in % of saturated FAs and significant ($P < 0.001$) increase in % of monounsaturated FAs. Like the HFX group, the % of unsaturated FA was significantly ($P < 0.05$) increased but then significantly ($P < 0.05$) decreased when expressed as the unsaturation index. In addition, the % of omega 3 fatty acids was significantly ($P<0.05$) decreased and an overall 19% increase in the n-6/n-3 ratio ($P < 0.05$) was seen in HFRX compared with HF.

Table 6.5 Phospholipid fatty acid composition of RG.

Fatty acid		HF	HFR	HFX	HFRX
16:0	Palmitic	16.9 ± 1.0	16.5 ± 0.6	15.2 ± 0.5	15.4 ± 0.5
18:0	Stearic	22.4 ± 0.7	22.2 ± 0.4	22.7 ± 0.4	22.6 ± 0.3
16:1(n-7)	Palmitoleic	0.34 ± 0.04	0.43 ± 0.06	0.34 ± 0.03	0.40 ± 0.03
18:1(n-7)	Vaccenic	1.93 ± 0.08	1.84 ± 0.03	1.91 ± 0.05	1.97 ± 0.07
18:1(n-9)	Oleic	3.8 ± 0.1	4.6 ± 0.2#	4.3 ± 0.1‡	4.9 ± 0.2†
18:2(n-6)	Linoleic	25.1 ± 0.5	27.2 ± 0.6	29.6 ± 0.6†	29.0 ± 0.7†
20:3(n-6)	DGLA	0.82 ± 0.04	0.94 ± 0.04	0.84 ± 0.03	0.86 ± 0.07
20:4(n-6)	Arachidonic	13.3 ± 0.4	12.6 ± 0.3‡	11.3 ± 0.2†	11.3 ± 0.3†
22:5(n-3)	DPA	2.51 ± 0.07	2.30 ± 0.08	2.28 ± 0.08	2.03 ± 0.08†
22:6(n-3)	DHA	12.0 ± 0.5	10.6 ± 0.3	10.6 ± 0.3	10.7 ± 0.4
% Saturation		39.3 ± 0.4	38.6 ± 0.3	37.9 ± 0.2*	37.9 ± 0.3*
% MUFA		6.1 ± 0.2	6.9 ± 0.2*	6.5 ± 0.1‡	7.3 ± 0.2†
% PUFA		54.6 ± 0.5	54.5 ± 0.4	55.5 ± 0.3	54.8 ± 0.3
% n-6		40.1 ± 0.7	41.7 ± 0.5	42.6 ± 0.5*	42.0 ± 0.5
% n-3		14.5 ± 0.5	12.9 ± 0.2*	12.9 ± 0.3*	12.8 ± 0.4*
% Unsaturation		60.7 ± 0.4	61.4 ± 0.3	62.1 ± 0.2*	62.1 ± 0.3*
Unsaturation index		201 ± 2	193 ± 2	192 ± 2*	192 ± 2*
n-6/n-3		2.80 ± 0.12	3.25 ± 0.09	3.33 ± 0.12*	3.33 ± 0.16*

Values are means ± SE; n=8; Fatty acid composition is expressed as mole percentage of total fatty acids; DGLA, Dihomo gamma-linolenic acid; DPA, Docosapentaenoic acid; DHA, Docosahexaenoic acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; Unsaturation index is the number of double bonds per 100 fatty acid chains; Significance for HF v's HFR; HFX and HFRX at *p<0.05, # p<0.01, † p<0.001 and HFRX v's HFR and HFX is at ‡ p<0.05.

6.3.5.5 Estimated enzyme activities

The estimated activity of Δ^5desaturase was significantly ($P < 0.05$) decreased in all treatment groups compared to HF control group while Δ^9desaturase activity was significantly ($P < 0.01$) increased in rosiglitazone treated groups. Δ^6desaturase and elongase activities were unchanged. Refer to Table 5.3.

Table 6.6 Estimated activity of desaturase and elongase enzymes in RG.

Enzyme		HF	HFR	HFX	HFRX
20:4/20:3	Δ^5desaturase	16.37 ± 0.54	13.63 ± 0.59*	13.64 ± 0.43*	13.55 ± 0.84*
20:3/18:2	Δ^6desaturase	0.033 ± 0.002	0.035 ± 0.002	0.028 ± 0.001	0.030 ± 0.003
18:1/18:0	Δ^9desaturase	0.17 ± 0.01	0.21 ± 0.01#	0.19 ± 0.00	0.22 ± 0.01#
18:0/16:0	Elongase	1.38 ± 0.14	1.36 ± 0.07	1.51 ± 0.07	1.48 ± 0.06

Values are means ± SE; n=8; Significance for HF v's HFR; HF v's HFX and HF v's HFRX at *p<0.05.

6.4 Discussion

The most important finding from this study was the positive effect exercise training had on the down regulation of Na^+K^+-ATPase found with insulin resistance. However the antidiabetic drug, rosiglitazone did not have the same desired effect in this study.

6.4.1 Effect of Exercise Training

The benefits of exercise training to treat insulin resistance are well known (Ivy *et al.*, 1989; Perseghin *et al.*, 1996). As expected this study showed, exercise significantly

reduced body mass and epididymal fat pad weight, and increased insulin-stimulated skeletal muscle glucose uptake in the high fat fed animal model of insulin resistance. There was no effect on blood glucose and insulin concentrations as the HF rats were still in the early stages of developing insulin resistance and hence parameters were already normal. Only FFA levels significantly improved with exercise. However, the effect of exercise training on Na^+K^+-ATPase function in skeletal muscle has never been studied. In Chapter 4 and 5, insulin resistance was repeatedly shown to down regulate Na^+K^+-ATPase activity. Exercise training is known to increase Na^+K^+-ATPase content in healthy rats and humans (Kjeldsen *et al.*, 1986; McKenna *et al.*, 1993; Madsen *et al.*, 1994). This study found moderate exercise training for a period of 4 weeks caused a significant increase in Na^+K^+-ATPase content in the sarcolemma, plus a corresponding significant increase in Na^+K^+-ATPase maximal activity. It may be safe to assume the rise in content was ultimately the reason for an increase in maximal Na^+K^+-ATPase activity.

Surprisingly, insulin stimulation of exercise trained muscle did not have the anticipated effect on Na^+K^+-ATPase activity or content. This may have been a result of the submaximal insulin concentration used, as the muscle did show a significant increase in insulin stimulated glucose uptake into red gastrocnemius. Further, the amount of insulin exposure (dose 500uU/ml and duration 8 min) may have been sufficient to stimulated glucose uptake but not affect Na^+K^+-ATPase function.

Chronic exercise training had a mixed effect on the membrane phospholipid profile. No change was seen in saturated and monounsaturated fatty acids however with respect to the omega 6 PUFAs, only the content of linoleic acid was increased while arachidonic acid was decreased compared to the high fat diet group. The overall effect resulted in a decrease in the percentage of saturated fatty acids and an increase in the percentage of unsaturated fatty acids. These changes appear to be beneficial however, when unsaturation was expressed as the number of double bonds per 100 fatty acid chains, there was a decrease in the unsaturation index. Exercise training, also, had the opposite effect on omega PUFA percentages than what was expected. An increase in the percentage of omega-6 PUFA and a decrease in the percentage of omega-3 PUFA, leading to an overall increase in the n-6/n-3 ratio in HFX compared with HF. This effect in the omega ratio is known to reflect reduced membrane fluidity (Farkas *et al.*, 2002).

6.4.2 Effect of Rosiglitazone

Rosiglitazone is currently used as an antidiabetic drug to treat hyperglycemia, hyperlipidemia without causing further secretion of insulin (Miyazaki *et al.*, 2001; Wagstaff & Goa, 2002). This study showed rosiglitazone improved insulin stimulated glucose uptake in skeletal muscle of insulin resistant rats. As reported in our previous study (Chapter 5), rosiglitazone did not significantly alter Na^+K^+-ATPase activity or content in insulin resistant muscle. Na^+K^+-ATPase maximal activity did seem to display a trend to suggest increased activity. The mean activity in skeletal muscle from rosiglitazone treated rats was 28% greater than the high fat fed control rats. However,

this difference was not found to be statistically significant, probably due to the large variation within the treatment group.

Similarly to the exercised muscle, insulin stimulation of rosiglitazone treated muscle did not have any effect on Na^+K^+-ATPase activity or content.

Rosiglitazone treatment had little effect on membrane phospholipid composition of the HF rats which was consistent with those found with the obese Zuckers in study 2 (Chapter 5). The content of only one fatty acid, oleic acid was altered when rosiglitazone treatment was taken at this dosage. This fatty acid was most likely responsible for the increase in the percentage of monounsaturated fatty acids. The overall percentage of omega 3 PUFA was significantly decreased but this was not reflected in the n-6/n-3 ratio.

6.4.3 Combined effect of Rosiglitazone & Exercise Training

Since the effect of rosiglitazone treatment alone was found to be non-significant but exercise training alone caused significant improvements in Na^+K^+-ATPase activity and content, the effect seen in the group of rats receiving both treatments was most likely due to the exercise training. In some instances, the combined treatment group was found to be significantly different from the rosiglitazone treated group, confirming the effect to be as a result of the exercise training regime. Like the exercise trained rats, rats undergoing both treatments showed a significant increase in Na^+K^+-ATPase content in the sarcolemma and a matching increase in Na^+K^+-ATPase maximal activity.

Phospholipid analysis of the sarcolemma in these rats showed most of the changes seen with the individual treatments. Hence the combined treatment group showed an increase in oleic acid and the percentage of monounsaturated fatty acids when compared to the normal control rats. These two measures were also significantly decreased when compared to the exercise group, supporting the notion these changes were due to the rosiglitazone treatment. Mimicking the alterations seen in the exercise group, the combined treatment group also displayed increased linoleic acid, percentage of unsaturated fatty acids and percentage of omega-6 PUFA, decreased arachidonic acid, percentage of saturated fatty acids, unsaturation index and percentage of omega-3 PUFA, and the net increase in n-6/n-3 ratio compared with HF.

6.4.4 Membrane Phospholipid Analysis

The high-fat diet consumed by the rats contained hydrogenated coconut oil, of which 93.3% was saturated fatty acids, 2.4% monounsaturated fatty acids, and 4.3% polyunsaturated fatty acids. Although, it contains seven different saturated fatty acids in total, it contains primarily 44.3% lauric acid, 16.7% myristic acid and 8.8% palmitic acid. The only monounsaturated and polyunsaturated fatty acid present is oleic acid and linoleic acid respectively. Therefore, a significant increase in percentage of saturated FA was expected. From the content analysis, the fall in palmitic acid may have caused the rise in stearic acid, as it is the direct elongase product of palmitic acid. The other products of palmitic acid, palmitoleic and vaccenic acid were significantly lower in the HF group, however the contents of these FA's are so low, they would not have much

impact. Two elongase reactions with linoleic acid produce linolenic acid, which is turned into Arachidonic acid by Δ^5desaturase were present in the diet, they also were present in low amounts and would not significantly affect overall results. The overall affect of the diet on the rats was a significant decrease in unsaturated FA.

6.4.5 Conclusion

Based upon previous results from this thesis, we demonstrated that maximal Na^+K^+-ATPase function in skeletal muscle is compromised with insulin resistance. The present study demonstrated chronic exercise training significantly increased Na^+K^+-ATPase content and activity, restoring maximal function in insulin resistant skeletal muscle, and this increase may have beneficial effects on the exercise capacity and reducing the fatiguing effects of exercise for diabetic patients as well as helping to reduce the associated risk factors for cardiovascular disease. Although no effect was demonstrated on Na^+K^+-ATPase function with rosiglitazone, a larger study may be required to confirm this observation. This study indicates that exercise has beneficial effects on Na^+K^+-ATPase function as well as glucose disposal in this diabetic population. Therefore it strengthens the recommendation that exercise is the preferable treatment option for diabetic patients.

Chapter 7

GENERAL SUMMARY

7.1 Introduction

Na^+K^+-ATPase function is vital to the preservation of membrane excitability and impaired function has been implicated with insulin resistance and diabetes, and associated complications. In this thesis, the first study investigated Na^+K^+-ATPase activity and content in skeletal muscle of two insulin resistant animal models, genetically predisposed obese Zucker rats and high fat diet induced Sprague Dawley rats. The subsequent studies investigated the effects of two therapeutic treatments for type II diabetes, rosiglitazone and exercise training, on Na^+K^+-ATPase function and membrane phospholipids in insulin resistant skeletal muscle.

7.2 Major Findings

7.2.1 Decreased Na^+K^+-ATPase activity and content with insulin resistance in rat skeletal muscle

One of the most important findings from this thesis was that Na^+K^+-ATPase function was compromised with insulin resistance in rat skeletal muscle. Maximal *in vitro* Na^+K^+-ATPase activity, expressed relative to muscle protein content, was decreased by 29% in 15 week old obese Zucker rats and 37% in 14 week old high fat fed Sprague Dawley rats. A similar decrease was found in sarcolemmal Na^+K^+-ATPase content of both animal models. The content of functional Na^+K^+-ATPase was reduced by 21.5% in

obese Zucker rats and 23% in high fat fed Sprague Dawley rats. Structural alterations in the kinetics of the enzyme and/or the membrane lipids in which the protein is embedded may have contributed to the reduced activity but the reduced content implies Na^+K^+-ATPase is compromised at gene transcription or translation, translocation to or insertion into the membrane, or has a higher rate of enzyme degradation.

Despite high fat fed Sprague Dawley rats showing deficient insulin action as seen with glucose uptake, insulin stimulation with submaximal concentrations of insulin increased Na^+K^+-ATPase activity by 38%. Hence, the compromised Na^+K^+-ATPase function seen with insulin resistant Sprague Dawley rats could not have been caused by the lack of insulin action and would appear to involve a different mechanism of inhibition. Possibly reduced physical activity in obese or diabetic rats contributes to a downregulation in Na^+K^+-ATPase function. To investigate this further, the level of physical activity of rats in future studies would need to be measured with the use of accelerometry or video movement analysis techniques.

Together, these findings indicate Na^+K^+-ATPase content is compromised in insulin resistant skeletal muscle, resulting in a reduced reserve capacity for Na^+K^+-ATPase activation. To some degree, this may help explain the predisposition to early onset of fatigue and reduced exercise capacity seen with type II diabetics, however, this outcome is not the result of reduced insulin action on the Na^+K^+-ATPase. Unfortunately the movement/physical activity of the rats was not measured and therefore the role of physical activity induced changes in Na^+K^+-ATPase function cannot be ruled out.

Na^+K^+-ATPase function in skeletal muscle from insulin resistant obese zucker rats may have been compromised due to reduced fluidity in the sarcolemma, as indicated by membrane phospholipid profile analysis, reported in Chapter 5. The estimated activity for Δ^5desaturase, which catalyses the addition of a double bond into a FA, was also found to be decreased in the obese Zucker rat. Studies of insulin deficiency have shown insulin to have an effect on fatty-acid desaturase activity, suggesting that reduced levels of unsaturated fatty acids in the membrane may be a result of reduced insulin action, caused by either insulin resistance or insulin deficiency.

7.2.2 Relationships of Na^+K^+-ATPase activity and content with blood parameters and body mass of rats

As discussed in Chapter 4, Na^+K^+-ATPase content in Zucker rats had strong inverse correlations with type II diabetic serum markers; glucose ($r = -0.75$, $P < 0.001$), triglycerides ($r = -0.8$, $P < 0.001$) and total cholesterol ($r = -0.9$, $P < 0.001$). These relationships and the positive correlation between Na^+K^+-ATPase content and HDL:LDL ratio ($r = 0.7$, $P < 0.001$) suggest that the reduction in Na^+K^+-ATPase content could be associated with the development of type II diabetes. Na^+K^+-ATPase activity also had moderate correlations with serum triglycerides and cholesterol, but no relationship was found with glucose or HDL:LDL ratios. This thesis also examined any association between rat muscle [^{3}H]-ouabain binding content or 3-*O*-MFPase activity and body weight, as an indicator of fat accumulation. Both, Na^+K^+-ATPase activity ($r = -0.8$, $P < 0.001$) and content ($r = -0.7$, $P < 0.01$) had a strong negative correlation with body weight, indicating that Na^+K^+-ATPase function has a possible association with the development of obesity in the obese Zucker rats.

7.2.3 Rosiglitazone depresses maximal Na^+K^+-ATPase activity in lean rat skeletal muscle

Chronic rosiglitazone treatment had a detrimental affect on the Na^+K^+-ATPase function in skeletal muscle of lean Zucker rats. Maximal *in vitro* 3-*O*-MFPase activity was decreased by 35%, to the same activity level as seen in obese Zucker rats. Maximal activity of obese Zucker rats did not further decrease with rosiglitazone treatment. Rosiglitazone, therefore, had either no effect on Na^+K^+-ATPase in skeletal muscle of obese Zucker rats or, its effect could not seen because it was not additive to the decreased activity already observed with insulin resistance. Na^+K^+-ATPase content was unaltered with rosiglitazone, hence decreased activity could not be due to reduced sarcolemmal content but as a result of activation or functional inhibition. Analysis of the membrane phospholipids showed alterations in membrane lipids and the degree of saturation was also not responsible for rosiglitazone's inhibitory effect. It would appear that the detrimental effects of rosiglitazone, involves a different mechanism to that of insulin resistance. Chronic rosiglitazone treatment may increase insulin sensitivity but it could also affect Na^+K^+-ATPase activity which may limit skeletal muscle function. Future studies using a therapeutic dose would need to be carried out to determine if the same effect would be seen. Rosiglitazone treatment at more therapeutic doses, although still high, did not significantly alter Na^+K^+-ATPase activity or content in insulin resistant skeletal muscle.

7.2.4 Exercise training restores Na^+K^+-ATPase function in skeletal muscle of insulin resistant rats

In Chapter 4 and 5, insulin resistance was repeatedly shown to be associated with down regulation of Na^+K^+-ATPase activity. In the final study, the most important finding was the beneficial effect exercise training had on reduced Na^+K^+-ATPase function

associated with insulin resistance. Moderate exercise caused a 53% increase in Na^+K^+-ATPase content in the sarcolemma and an 83% increase in Na^+K^+-ATPase maximal activity in insulin resistant rats. This is the first report of restored Na^+K^+-ATPase function with exercise training in insulin resistant skeletal muscle. Comparable with the exercise trained rats, rats undergoing both rosiglitazone treatment and exercise training showed a 79% increase in Na^+K^+-ATPase content in the sarcolemma and a subsequent 62% increase in Na^+K^+-ATPase maximal activity. The effect in this group was almost certainly due to the exercise training as rosiglitazone treatment alone was no effect.

7.3 Recommendations for further research

Studies conducted in this thesis have raised important questions regarding the effects of insulin resistance and obesity, rosiglitazone and exercise training on skeletal muscle Na^+K^+-ATPase function and membrane phospholipids. Further work is required to determine the mechanism(s) responsible for compromised Na^+K^+-ATPase function in skeletal muscle with insulin resistance and type II diabetes.

Insulin resistance or deficiency may be responsible for decrease desaturase enzyme activity resulting in reduced unsaturation in membrane phospholipids, which is known to affect Na^+K^+-ATPase function. However this hypothesis warrants further investigation. Analysis focusing on the expression of Na^+K^+-ATPase isoforms may be of great benefit. In addition the measurement of rat physical activity or movement would be of great benefit to identify its potential role in affecting the Na+K+-ATPase.

Research in this area may provide further insight into the mechanisms responsible for the effects of the disease.

Another study is required to investigate the mechanism causing the effect of rosiglitazone on Na^+K^+-ATPase in skeletal muscle. The inhibition of Na^+K^+-ATPase by chronic rosiglitazone treatment, may be secondary to other mechanisms such as AMPK activation.

Taking these finding from animal models and investigating the effects in humans would be of great benefit. The growing epidemic of obesity and diabetes poses may challenges. Further research that elucidates the effects of diabetes on skeletal muscle function and the interaction with treatment option such as exercise, diet and medication is paramount.

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