

The Effects of Dietary Supplements on Skeletal Muscle Function in Type 2 Diabetic Rats

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ABSTRACT

Type 2 Diabetes is considered to be a heterogeneous disease, and whilst its pathogenesis is currently unknown, it is often characterized by high plasma free fatty acids (FFAs), hyperglycaemia, and altered insulin secretion from pancreatic β -cells. Whilst multiple organ systems are often affected by the disease, the effects on skeletal muscle in particular are pertinent, as this is the main organ responsible for glucose disposal, and also has a major role in basal and altered metabolism. The overall aim of this thesis is to examine the extent of any changes to skeletal muscle morphology and (dys)function in Type 2 Diabetes and how these can be improved by the use of dietary supplements. Specifically, the potential of Creatine Monohydrate, Whey Protein Isolate, and Aspirin on the contractile apparatus and specific intramyocellular organelles will be tested in order to reverse some of the maladaptations found in skeletal muscle of Type 2 Diabetic patients.

It has been well established that skeletal muscles undergo atrophy in Type 2 Diabetes, with the direct mechanism and complete effects to muscle function yet to be fully elucidated. Calcium dysregulation causing the activation of the calpain system is one potential pathway leading to skeletal muscle protein breakdown and hence muscle atrophy, leaving muscle cells more susceptible to damage. In addition, the increases in FFA content can lead to excessive production of reactive oxygen species (ROS) and reduced antioxidant defences resulting in damage to proteins, lipids and nucleic acids. Therefore study 1 aims to determine if the skeletal muscle atrophy associated with obesity and insulin-resistance is a result of enhanced membrane fragility mediated by excess ROS and/or increased fibre damage due to excess calpain activity. Overall, it was determined that the atrophy observed in Type 2 Diabetes is not caused by sarcolemmal damage, elevated lipid peroxidation and degradative calpain activity.

Given the large amount of controversy in the literature pertaining to skeletal muscle function in insulin resistance and Type 2 Diabetes, and the heterogeneity of the disease, the next series of studies looked at contractile function in fast- and slow-twitch fibres. Some of the intracellular processes potentially influencing contractile function were also looked at in terms of sarcoplasmic reticulum (SR) function, mitochondrial ATP production rates (MAPR), as well as how inflammatory processes may play a role in muscle dysregulation, and if supplementation with creatine, whey and aspirin could reverse any deleterious effects.

In order to study the efficacy of the supplementation regime pertaining to these specific parameters in skeletal muscle, the Zucker Diabetic Fatty rat was utilised as an animal model of Type 2 Diabetes due to its close resemblance to the human form of the disease. Specifically they have been bred to develop hyperglycaemia, hyperinsulinaemia, insulin resistance, glucose intolerance and hyperlipidaemia.

In determining contractile function, muscle peak twitch and tetanic forces were calculated per muscle cross-sectional area. Overall, no differences between any of the animal groups were detected, despite slight increases in relative force production in both supplemented groups of EDL and Soleus muscles. The Time-to-Peak (TTP) interval and half relaxation time ($\frac{1}{2}$ RT) was also determined between groups, however no significant differences were found in neither EDL nor Soleus. A right shift in the force-frequency curve was observed in Obese compared to Lean animals of EDL, but not Soleus, and once again, supplementation had little effect. Fatigue was shown to be the same in EDL muscle of Obese and Lean animals with the supplements showing some improvement in the Obese animals, albeit insignificant. In Soleus, Lean animals were significantly more fatigue resistant than Obese animals ($p < 0.01$). Supplementation of the Obese animals (ObSupp) showed significant improvements ($p < 0.01$) such that fatigue resistance was not significantly different from the Lean animals. Recovery

from fatigue in EDL, was about 10% greater in the ObSupp group compared to the Lean group ($p < 0.05$), and almost 20% higher than the supplemented Lean (LSupp) group. In Soleus muscle, Obese animals showed a 30% reduction in force production throughout the recovery period compared to Lean animals ($p < 0.05$). The supplementation significantly improved recovery in ObSupp animals such that they were no different to Lean littermates.

In relation to SR function, Obese rats showed no significant alterations in Ca^{2+} uptake rates, slightly lower leak rates and significantly lower release rates compared to Lean littermates in red- and white Gastrocnemius muscles. Creatine, whey protein and aspirin supplementation appear to have no beneficial effects on skeletal muscle SR function. MAPR was found to be impaired in red Gastrocnemius muscle fibres of Obese rats, which was attenuated with creatine, whey and aspirin supplementation. This improvement occurred independently of mitochondrial protein content, intramyocellular lipid content and whole body insulin resistance. Finally, the pro-inflammatory cytokines IL-6 and TNF- α were analysed in skeletal muscle samples, and were shown to be significantly elevated in Obese rats compared to Lean littermates ($p < 0.05$), and further that this elevation was specific to fast-twitch muscle fibres. Whilst supplementation with aspirin, creatine and whey protein reduced IL-6 and TNF- α in EDL, and plasma IL-6, this did not result in improvements to insulin resistance or muscle atrophy of diabetic animals.

Overall this thesis has shown that the main alterations that occur in skeletal muscle as a result of obesity and Type 2 Diabetes, are that they are significantly smaller, more fatigueable, particularly the slow twitch muscle fibres, with recovery also being impaired. This may contribute to the exercise intolerance observed in Type 2 Diabetics. The underlying cause of the fatigue and recovery processes may be a result of the impaired ATP production capacity and thus reduced energy available to the contractile apparatus. Supplementation was

beneficial in improving MAPR and as such returned fatigue and recovery processes back to control conditions. This gives further evidence that mitochondria may play a significant role in disease progression, given that inflammation and SR function had little influence on the outcomes observed above, and that this can be reversed by appropriate dietary interventions. The mechanisms behind the altered mitochondrial function however require further investigation as well as the specific modes of action of each supplement used herein and its applicability to human models.

COMMUNICATIONS

Publications:

1. Pompeani, N., Rybalka, E., Latchman, H., Murphy, R.M., Croft, K., and Hayes, A. (2014). Skeletal muscle atrophy in sedentary Zucker Obese rats is not caused by calpain-mediated muscle damage or lipid peroxidation induced by oxidative stress. *J Neg Res Biomed.*, 13 (19), 1-11.
2. Williams, A.D., Capitanio, N¹., and Hayes, A. (2010). *Skeletal Muscle in Heart Failure and Type 2 Diabetes*. Nova Science Publishers, New York.
3. Williams, A.D., Capitanio, N., and Hayes, A. (2010). Skeletal Muscle in Heart Failure and Type 2 Diabetes: Maladaptations and Potential Treatment. In: *Muscle Strength: Types, Efficiency and Drug Effects*. Eds: Marvin S. Donato and Jahiem E. Marini, pp 1-56, Nova Publishing.

Presentations:

Pompeani, N., Rybalka, E., Latchman, H., Murphy, R.M., Croft, K., and Hayes, A. Skeletal muscle atrophy in sedentary Zucker Obese rats is not caused by calpain-mediated muscle damage or lipid peroxidation induced by oxidative stress. Poster Presentation at Australian Physiological Society (AuPS) 2010, Adelaide, SA, Australia.

¹ It is noted that at the time of publication, N Pompeani (maiden name) was N Capitanio (married name) and has since reverted to the maiden name for further publication purposes.

Pompeani, N., Rybalka, E., Latchman, H., Murphy, R.M., Croft, K., and Hayes, A. Skeletal muscle atrophy in sedentary Zucker Obese rats is not caused by calpain-mediated muscle damage or lipid peroxidation induced by oxidative stress. Oral Presentation to the University of Texas at El Paso (UTEP) 2010, video conference.

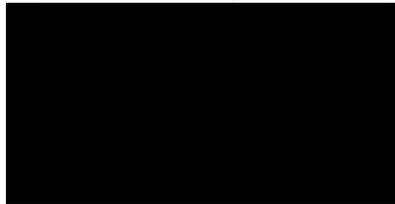
Pompeani, N., Rybalka, E., Latchman, H., Murphy, R.M., Croft, K., and Hayes, A. Skeletal muscle atrophy in sedentary Zucker Obese rats is not caused by calpain-mediated muscle damage or lipid peroxidation induced by oxidative stress. Oral presentation at Victoria University Postgraduate Research Conference 2010, Melbourne, VIC, Australia.

Pompeani, N., Rybalka, E., and Hayes, A. The effects of Creatine, Whey and Aspirin supplementation of mitochondrial and sarcoplasmic reticulum function in skeletal muscle of ZDF rats. Oral presentation at Victoria University Postgraduate Research Conference 2011, Melbourne, VIC, Australia.

DECLARATION

“I, Nancy Pompeani, declare that the PhD thesis entitled The Effect of Dietary Supplements on Mitochondrial and Sarcoplasmic Reticulum Function is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Signature:



Date: 11 / 02 / 2016

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LIST OF ABBREVIATIONS

4EBP-1	4E-binding 1
α	Alpha
Akt/PKB	Protein Kinase B
ADP	Adenosine Diphosphate
AIS	Analytical Imaging Station
ATP	Adenosine Triphosphate
BCAA	Branched Chain Amino Acids
β	Beta
Ca ²⁺	Calcium
CK	Creatine Kinase
CoA	Coenzyme A
Cr	Creatine
CS	Citrate Synthase
CSQ	Calsequestrin
DHP	Dihydropyridine
EAA	Essential Amino Acids
EBD	Evan's Blue Dye
EBD+	Evan's Blue Dye positive
EDL	Extensor Digitorum Longus
ELISA	Enzyme Linked ImmunoSorbent Assay
EMCL	Extramyocellular Lipids
ETC	Electron Transport Chain
FA	Fatty Acid
FAT	Fatty Acid Translocase
FFA	Free Fatty Acids
G-6-P	Glucose-6-Phosphate

GADD45 α	Growth Arrest and DNA Damage-45 alpha
Glut-4	Glucose Transporter
GSH	Glutathione
H ⁺	Hydrogen ion
H&E	Haemotoxylin and Eosin
HRP	Horseradish Peroxidase
HRT	½ Relaxation Time
IL	Interleukin
IGF-1	Insulin Growth Factor-1
Ikk β	Inhibitor of nuclear factor kappa-B kinase subunit beta
IMCL	Intramyocellular Lipids
IRS-1	Insulin Receptor Substrate 1
IRS-2	Insulin Receptor Substrate 2
K _d	Dissociation Constant of Indo-1
LBM	Lean Body Mass
LSupp	Supplemented Lean ZDF Rats
MAPR	Mitochondrial ATP Production Rate
MgATP	Magnesium Adenosine Triphosphate
MHC	Myosin Heavy Chain
MnSOD	Manganese Superoxide Dismutase
MR	Magnetic Resonance
MS	Mitochondrial Suspension
mTOR	mammalian target of rapamycin
NADH	Nicotinamide Adenine Dinucleotide Hydride
NAD ⁺	Nicotinamide Adenine Dinucleotide
NF- $\kappa\beta$	Nuclear Factor Kappa Beta
NO	Nitric Oxide

iNOS	Inducible Nitric Oxide Synthase
ObSupp	Supplemented Obese ZDF Rats
OCT	Optimal Cutting Temperature
PCr	Phosphocreatine
PFK	Phosphofructokinase
PI 3-kinase	Phosphatidylinositol 3-kinase
³¹ P-MRS	31-phosphorus MR spectroscopy
QUICKI	Quantitative Insulin Sensitivity Check Index
RG	Red Gastrocnemius
Rheb	Ras homolog enriched in brain
Rmin	Minimum Fluorescence Ratio
Rmax	Maximum Fluorescence Ratio
ROS	Reactive Oxygen Species
RyR	Ryanodine Receptor
SEM	Standard Error of Mean
SERCA	Sarco(Endo)plasmic Reticulum Calcium ATPase
S6K1	S6 kinase 1
SOD	Superoxide Dismutase
SR	Sarcoplasmic Reticulum
STZ	Streptozotocin
TA	Tibialis Anterior
T2D	Type 2 Diabetes
TNF α	Tumor Necrosis Factor
TSC1/2	tuberous sclerosis complex 1/2
TTP	Time-To-Peak
UCP	Uncoupling Proteins
V _{max}	Maximum Velocity

WG	White Gastrocnemius
ZDF	Zucker Diabetic Fatty

CHAPTER 1

Literature Review²

² Significant portions of this review have been published as indicated in references 2 and 3 on page iv.

1.1 Introduction: Type 2 Diabetes Mellitus

Although not exclusively the cause, the growing obesity epidemic (Cameron et al., 2003; Shaw et al., 2006; Caballero, 2007) combined with decreases in physical activity, has also seen the rise of diabetes mellitus to epidemic proportions. It has been estimated that the number of people with diabetes worldwide will have doubled from the year 2000 to 2030, such that it is expected that 366 million people will be affected (Wild et al., 2004; Maruthur, 2013). Epidemiological data have shown that the increased prevalence of diabetes is particularly evident in young and middle-aged people (Alberti et al., 2004). Therefore the associated complications of the disease, such as nephropathy, retinopathy, cardiomyopathy, and vascular complications will rise, inevitably imposing a higher financial burden on health care costs due to the morbidity and mortality that is associated with the disease (American Diabetes Association, 2003).

Over 90% of all diabetes cases are type 2, formerly known as Non-Insulin Dependent Diabetes Mellitus (NIDDM)³. Type 2 Diabetes Mellitus (T2D) is considered to be a heterogeneous disease, and while its pathogenesis is currently unknown, it is often characterized by high plasma free fatty acids (FFAs), hyperglycaemia, altered insulin secretion from pancreatic β -cells (either high, normal or low depending on the stage of the disease) (Wallberg-Henriksson et al., 1998), and demonstrable peripheral insulin resistance (Scheuermann-Freestone et al., 2003; Boden, 2011). In fact, insulin resistance has been shown to develop one to two decades before the onset of T2D (Lillioja et al., 1993; Caprio, 2012). Insulin is in part, responsible for maintaining glucose homeostasis, by acting predominantly on skeletal muscle and adipose tissue to promote glucose uptake and inhibit the production of glucose in the liver. Therefore, any disruptions to either the production of

³ For consistency purposes, the term Type 2 Diabetes (T2D) will be used throughout this thesis however it should be noted that the term NIDDM was used for publication of this chapter only.

insulin or the response of insulin-sensitive cells (such as skeletal muscle) to insulin, will result in hyperglycaemia and ultimately disease states such as T2D (Cheng et al., 2002). The effects on skeletal muscle in particular are pertinent, as this is the main organ responsible for glucose disposal (De Fronzo et al., 1982), and also has a major role in establishing basal and altered metabolism (Phielix and Mensink, 2008). In particular, impaired fat (Unger, 1995) and protein (Nair et al., 1983; Gougeon et al., 2000) metabolism are key features of T2D, with any resultant trend towards obesity further exacerbating insulin resistance.

1.2 Insulin Resistance

Skeletal muscle is a primary site of insulin-stimulated glucose utilisation (Krook et al., 2000). In fact, glucose transport is the rate-limiting step in glucose utilisation (Kubo and Foley, 1986). The transport of glucose occurs via facilitated diffusion, using a carrier protein for transport across the cell membrane. These carrier proteins are expressed in a tissue-specific manner (Bell et al., 1993), whereby GluT-4 is the major isoform present in both human and rat skeletal muscle (Hayashi et al., 1997).

During resting conditions, GluT-4 transporters are located in the cytoplasm and are translocated to the plasma membrane upon stimulation via muscle contractions and/or insulin binding receptors on the skeletal muscle cell membrane (Ploug et al., 1984; Wallberg-Henriksson and Holloszy, 1985; Kennedy et al., 1999; Thorell et al., 1999). It has been suggested that there are different signalling pathways involved in exercise stimulated GluT-4 translocation compared to insulin-stimulated GluT-4 translocation (Hayashi et al., 1997). Interestingly, patients suffering from T2D have been shown to exhibit normal exercise-stimulated GluT-4 translocation, compared to a decrease in insulin-stimulated translocation (Kennedy et al., 1999). One potential cause of the decrease in glucose uptake via insulin

stimulation has been shown to be due to elevations in plasma FFAs (Boden and Chen, 1995). However it has been argued as to whether triglyceride accumulation is simply a marker or plays a causative role in insulin resistance (Lewis et al., 2002). Understanding the mechanism of the insulin resistant response, and thus the underlying pathogenesis of T2D, will lead to therapies directed at increasing skeletal muscle glucose uptake and metabolism, providing an avenue for increased energy metabolism. By doing so, the exercise intolerance (Burns et al., 2007; Green et al., 2007) found in diabetic patients may be circumvented, thus improving muscle function.

Under normal conditions, insulin binds to insulin receptors on target organ cells such as skeletal muscle (Petersen and Shulman, 2002; Menting et al., 2013). In obesity and T2D, FFAs have been shown to induce insulin resistance yet the precise mechanism is still unclear. Figure 1.0 summarises some of the ways FA may be involved in contributing to insulin resistance. One mechanism thought to be involved with insulin resistance is impaired insulin signal transduction in skeletal muscle due to elevated FA levels (Yu et al., 2002). The defects have been found to occur at the level of the insulin receptor substrates (IRS-1 and IRS-2) and at the level of Phosphatidylinositol (PI) 3-kinase, a signalling transducer that mediates the metabolic effects of insulin such as glucose transport (Okada et al., 1994; Dresner et al., 1999). The expression of both have been shown to be reduced in T2D subjects (Krook et al., 2000).

Another theory regarding the mechanism of FA-induced insulin resistance is known as the glucose FA cycle, postulated by Randle et al. (1963). This theory states that FFAs compete with glucose for substrate oxidation. Thus an increase in fat oxidation causes insulin resistance with obesity because of an increase in the intramitochondrial acetyl CoA/CoA and

NADH/NAD⁺ ratios. This inactivates the action of pyruvate dehydrogenase, which then increases the intracellular citrate concentrations. High citrate concentrations lead to the inhibition of Phosphofructokinase (PFK), a rate-controlling enzyme in glycolysis, thus decreasing glycolysis and increasing Glucose-6-Phosphate (G-6-P). The increase in G-6-P would inhibit hexokinase activity, and result in increased glucose concentrations and reduced glucose uptake (Perseghin et al., 2003). However this type of activity had not been tested in human experiments.

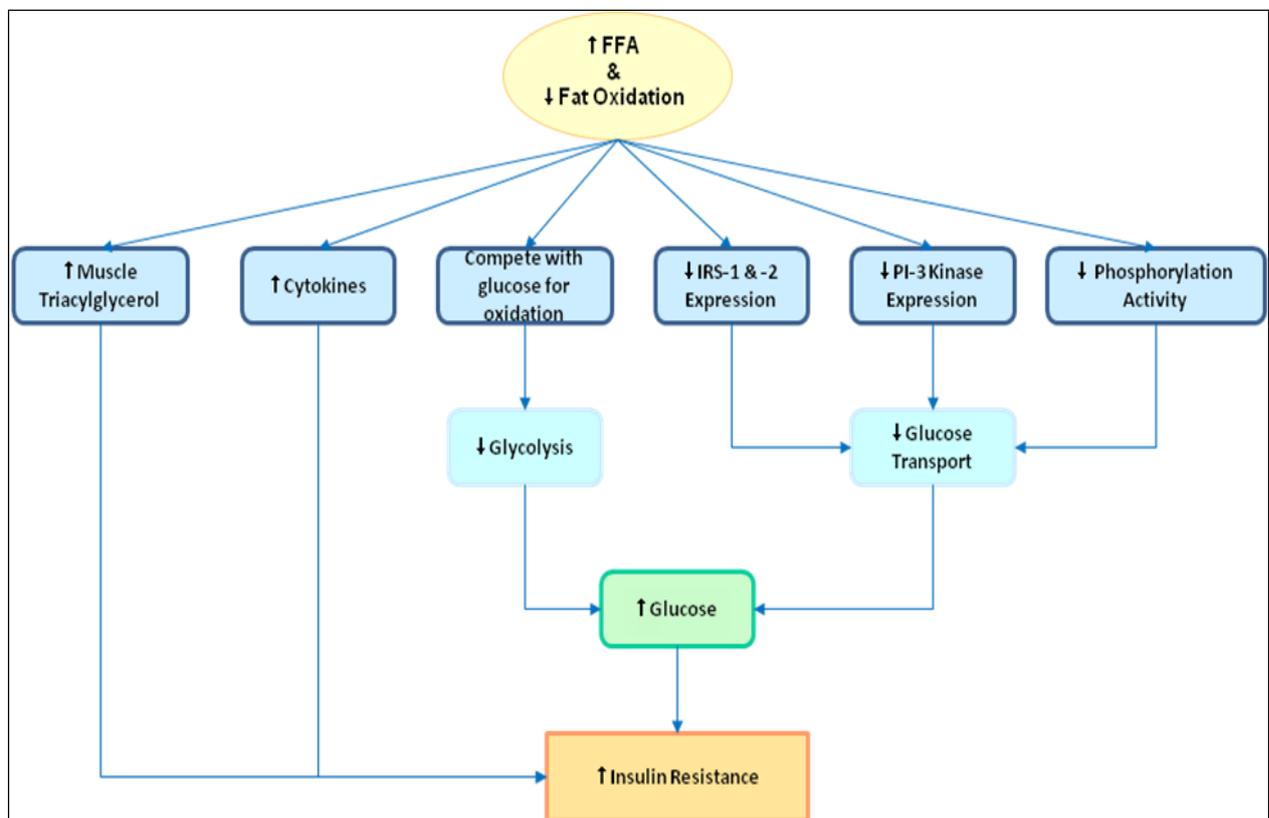


Figure 1.0 Potential mechanisms resulting in insulin resistance due to high FFA.

Roden et al. (1996) studied the glucose FA cycle in humans using ¹³C and ³¹P MRS. It was shown that increases in FA concentration initially induced insulin resistance by inhibiting glucose transport or phosphorylation activity, thus reducing the intramuscular G-6-P concentration and causing a decrease in glycogen synthesis and glucose oxidation. This is similar to what has been seen in humans with obesity and T2D (Rothman et al., 1992; Roden

et al., 1996; Petersen et al., 1998). The reduced glucose transport activity found in these studies also gives merit to the theory mentioned earlier regarding reduced glucose transport caused by defects in the insulin signalling pathway.

While the lack of insulin sensitivity and subsequent hyperglycaemia has been the primary concern of researchers (Petersen et al., 1998; Perseghin et al., 2003), there is a growing body of evidence that places a vital role for metabolism, and thus the mitochondria and reactive oxygen species, and their subsequent effects on skeletal muscle, that even if not the primary cause, are the major reason for the continuation and extent of the disease. Certainly, while cytokines secreted from adipose tissue have been shown to affect insulin sensitivity (Lazar, 2005), there is a reduced fat oxidative capacity in diabetic sufferers (Kelley and Simoneau, 1994). Increased plasma FFA, in combination with the reduced oxidative capacity, produces an increase in skeletal muscle triacylglycerol, which has a strong correlation with insulin resistance (Krssak et al., 1999; Perseghin et al., 1999). Muscular fat accumulation, in the form of fatty acyl coenzyme A (fatty acyl CoA) and diacylglycerols (Shulman, 2000; Itani et al., 2002; Yu et al., 2002) can directly inhibit insulin-stimulated glucose uptake (Dresner et al., 1999). Indeed, elevating FFA in the plasma at the same time as hyperinsulinaemia, while maintaining stable plasma glucose, is enough to cause insulin resistance (Boden et al., 1991; Boden and Chen, 1995).

Ultimately, insulin resistance has a substantial effect on skeletal muscle structure and function, leading to the exercise intolerance associated with the disease, therefore it is important to understand the mechanisms behind the changes occurring so appropriate interventions can be put in place to improve the morbidity and mortality associated with T2D.

1.3 Skeletal Muscle Function and Type 2 Diabetes

Skeletal muscle constitutes approximately 30% of total body mass and is a highly plastic tissue that adapts rapidly to changing functional demands. Muscles are the contraction specialists of the body. They attach to the skeleton and during contraction apply a force to the long bones causing these bones to act as levers and initiate movement. For this to occur, the cytoplasm of skeletal muscle fibres is packed with contractile and metabolic apparatus, with the contractile apparatus accounting for approximately 75% of the volume of the fibre (see review by Luff and Atwood, 1971).

Not surprisingly, insulin resistance has a substantial effect on the metabolism and growth of skeletal muscle. Skeletal muscle atrophy and alterations in contractile, morphological and biochemical properties are characteristic features that are associated with diabetes. Figure 1.1 shows the interaction of insulin with its membrane-bound receptor, and the subsequent molecular regulation of muscle growth and metabolism. Insulin resistance has been shown to decrease muscle mass and exacerbate muscle weakness (Andersen et al., 2004; Sayer et al., 2005; Barzilay et al., 2009), and then the lack of subsequent physical activity, or sarcopenia associated with ageing (Stenholm et al., 2008; Zamboni et al., 2008), further disrupts metabolic regulation, particularly glucose uptake and removal.

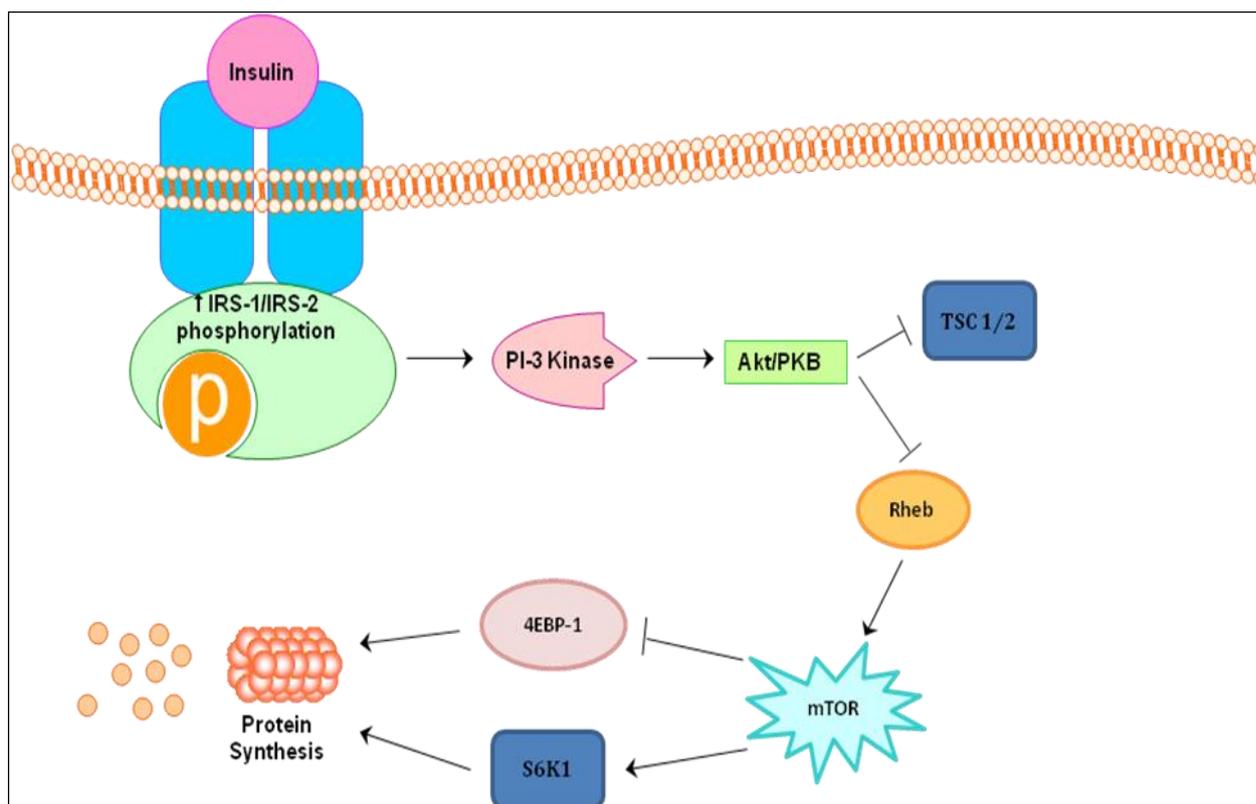


Figure 1.1 Schematic diagram showing the interaction of insulin with its membrane-bound receptor, and the subsequent molecular regulation of protein synthesis. Pointed arrowheads indicate stimulatory effects; Flat arrowheads indicate inhibitory effects; IRS-1/IRS-2, insulin receptor substrate 1/2; PI-3-kinase, phosphatidylinositol 3-kinase; Akt/PKB; protein kinase B; TSC1/2, tuberous sclerosis complex 1/2; Rheb, Ras homolog enriched in brain; mTOR, mammalian target of rapamycin; S6K1, S6 kinase 1; 4EBP-1, 4E-binding 1

1.3.1 Contractile Properties

Several studies have suggested that diabetes affects muscle fibres differently. The bulk of the studies suggest type I fibres are preferentially affected (Hickey et al., 1995; Gaster et al., 2001), with decreased proportions of type I fibres, and an elevated percentage of type IIX fibres in the vastus lateralis muscle of human subjects with T2D (Marin et al., 1994; Nyholm et al., 1997). Furthermore, a study by Tanner et al. (2002) showed an increase in type IIX and lower percentage of type I fibres in the rectus abdominus muscle of obese women. Although these results have not been universally observed (Holten et al., 2004; van Loon et al., 2004), changes towards a reduced type I fibre content could explain some of the decreased oxidative enzyme activities observed in diabetic mitochondria (see section 1.3.2). Although, these changes should also cause alterations to the contractile properties of diabetic muscles, studies

of this nature in humans have not been conducted and thus much of our information comes from animal models of diabetes, in particular models of Type I Diabetes.

To date, the effects of diabetes on skeletal muscle contractile force have been controversial. In chemically-induced diabetic rats, no difference in twitch and tetanic tensions were found in the soleus muscle (Grossie, 1982; Cameron et al., 1990; Cotter et al., 1993; McGuire and MacDermott, 1999). However, as will be discussed in section 1.3.7 it has been shown that it is the fast-twitch muscles, rather than the slow-twitch soleus, which appear to be more affected by muscle atrophy associated with diabetes (Cotter et al., 1993) and thus changes in contractile function could be more likely to be observed in fast twitch muscles. It is also likely that the duration of diabetes can influence the results, as Cotter et al. (1989) and McGuire and Macdermott (1998) have shown there to be a decrease in the twitch and tetanic tensions in the soleus and diaphragm muscle, respectively, 3 months after rats were made diabetic with a streptozotocin (STZ) injection.

While any effects of diabetes on contractile force in the soleus are equivocal, it is conceded that there is slowing of the $\frac{1}{2}$ relaxation times ($\frac{1}{2}$ RT) and increases in the Time-to-Peak tensions (TTP) (Cotter et al., 1989; Cameron et al., 1990; Cotter et al., 1993; McGuire and MacDermott, 1999). The changes of $\frac{1}{2}$ RT and TTP tensions are shown to be evident within the soleus muscle, but not within the EDL muscle. This is somewhat surprising given the already slow-twitch nature of the soleus muscles. Findings of no change in $\frac{1}{2}$ RT and TTP in the EDL seem to be consistent with many of the studies mentioned above. This is perhaps surprising, given that muscle mass changes appear to be more prevalent in the fast-twitch fibres (Cotter et al., 1993) which are predominantly found in muscles such as the EDL (Ariano et al., 1973).

On the other hand, EDL in diabetic rats did show decreases of up to 21% in tetanic tension (Paulus and Grossie, 1983; Cotter et al., 1989; Cameron et al., 1990; Cotter et al., 1993; McGuire and MacDermott, 1999). This decrease in force output has been attributed to the higher rate of atrophy seen in EDL compared to soleus (Cotter et al., 1989; Cotter et al., 1993), suggesting that the contractile proteins in the EDL are not only broken down more regularly than the soleus, but in the process, may also lose their force-generating ability (Paulus and Grossie, 1983).

To date, most studies on contractile function have looked at STZ-induced diabetic rat, which, as mentioned earlier are more representative of the Type I Diabetic condition. The only studies testing contractile function in the commonly used animal model of T2D, the Zucker Diabetic Fatty (ZDF) rat (Shafir, 1992; Sima and Shafir, 2001), involve cardiac muscle, in which decreases in function were found and attributed to activation of iNOS and NO as a result of hyperglycaemia (Song et al., 2008).

Therefore given that there are many differential effects on skeletal muscle morphology and function throughout the progression of the disease, further research would be beneficial in ascertaining any relationship between plasma insulin and glucose concentrations, insulin resistance and the succession of skeletal muscle maladaptations in T2D.

1.3.2 Mitochondria and Reduced Oxidative Capacity

Whilst researchers have focused attention on the mechanisms of insulin resistance in target tissue of T2D patients, others have focused on the dysfunction of the mitochondria (for review see Lamson and Plaza, 2002). Given that optimal mitochondrial function is required

for normal glucose metabolism, any impairment would reduce this metabolic capacity and hinder muscle contractile function.

Mitochondria generate approximately 90% of the energy required for normal cell function. This energy is generated in the form of adenosine triphosphate (ATP) and about 80% of the energy is produced by oxidative phosphorylation (Lamson and Plaza, 2002). This is achieved through a series of five multi-subunit enzymes or complexes which catalyse the redox reactions to produce ATP and reactive oxygen species (ROS). The enzyme complexes are found on the inner mitochondrial membrane (Refer to Figure 1.2).

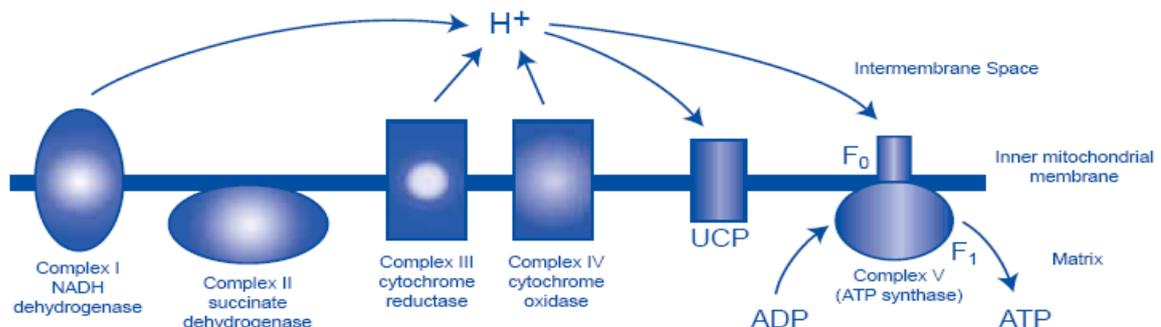


Figure 1.2 Mitochondrial Respiratory Chain (Lamson and Plaza, 2002: pp 2).

Mitochondria have their own DNA (mtDNA) which code for the proton pumping proteins/subunits. The mtDNA is transiently attached to the inner mitochondrial membrane and is in close proximity to harmful ROS by-products of oxidative phosphorylation (Lamson and Plaza, 2002). The ROS products have a short half-life and react rapidly with DNA, protein and lipids, causing oxidative damage of these cell components (James and Murphy,

2002; Schrauwen and Hesselink, 2004), thus reducing the mitochondrial ATP production rate (MAPR) and hence the energy required for muscle contraction. Fortunately, under normal conditions, mitochondria have a range of defences against oxidative damage as well as mechanisms involved to repair or degrade oxidatively damaged lipids, proteins and DNA (Green et al., 2004). However, the inability of cells to detoxify ROS products may result in mutations to mtDNA thus impairing mitochondrial transcription, resulting in impaired oxidative capacity, impaired Ca^{2+} homeostasis and ultimately cell death and conditions like Diabetes (Leinonen et al., 1997; Hinokio et al., 1999; Suzuki et al., 1999; James and Murphy, 2002). Dysregulated ROS can also occur due to high FA content, and this can lead to mitochondrial uncoupling (Echtay et al., 2002a; 2002b) resulting in impaired metabolism. Uncoupling proteins (UCP) drain protons from the intermembrane space into the mitochondrial matrix, bypassing ATP synthase (Lamson and Plaza, 2002). Studies have shown Obese mice and Obese ZDF rats to have increased levels of UCP, and this has been implicated in the pathogenesis of T2D (Zhang et al., 2001). However this may also be viewed as a potential protective mechanism in an attempt to alleviate any excess ROS (Brand and Esteves, 2005).

It has been found that there is reduced mitochondrial oxidative function in T2D (Kelley et al., 2002; Lowell and Shulman, 2005). This in turn would lead to some of the conditions found in the disease. Indeed, a strong correlation between decreases in mitochondrial oxidative activity, ATP production and insulin resistance have been observed (Petersen et al., 2003). Moreover, higher levels of triglycerides were also detected in both muscle and liver, suggesting an impairment in mitochondrial fatty acid oxidation, and the subsequent increase in fatty acyl CoA and diacylglycerols can directly affect insulin signalling.

In T2D, the reduced oxidative enzyme capacity has been shown to be accompanied with changes such as decreased citrate synthase (Simoneau and Kelley, 1997; Kelley et al., 2002; Ortenblad et al., 2005), succinate dehydrogenase (Simoneau and Kelley, 1997; Kelley and Mandarino, 2000; He et al., 2001), and mitochondrial complexes I (Kelley et al., 2002) and II (Ritov et al., 2005). These changes were possibly related to morphological abnormalities and a decreased mitochondrial size in Obese and T2D participants (Kelley et al., 2002), and are independent of fibre type differences (He et al., 2001). In addition, the increase in FA content associated with obesity and T2D can lead to several other mitochondrial defects. These include, 1) increased production of ROS such as superoxide and hydrogen peroxide, and reduced antioxidant defences available such as Manganese Superoxide Dismutase (MnSOD) and Glutathione (GSH) (Johansen et al., 2005); 2) increase in inflammatory mediators leading to insulin resistance (Dandona et al., 2004) and apoptosis/necrosis (Shoelson et al., 2007); and 3) reduced membrane fluidity which inhibits complex V (ATP synthase) of the Electron Transport Chain (Lamson and Plaza, 2002). These defects result in a cascade of events leading to reduced ATP synthesis, thereby inducing Calcium (Ca^{2+}) overload due to poor regulation by Ca^{2+} -ATPase driven pumps, reduction in protein synthesis and overall muscle damage and dysfunction. High intracellular [Ca^{2+}] has also been found to diminish cellular sensitivity to insulin and might participate in the pathogenesis of insulin resistance in T2D (Draznin et al., 1989). If Ca^{2+} is not sequestered efficiently into the Sarcoplasmic Reticulum (SR), as has been shown in T2D (Belke et al., 2004), it may result in the accumulation of intracellular [Ca^{2+}], which has long been known to trigger a cycle of cell damage (Armstrong, 1990), resulting in muscle degradation and proteolysis (Berchtold et al., 2000) and impaired contractile function. The role of Ca^{2+} regulation by the SR will be discussed in more detail in section 1.3.3.

1.3.2.1 Measuring Mitochondrial ATP Production Rate

The functionality of mitochondria have, in the past, been determined by measuring mitochondrial ATP production rate (MAPR) in skeletal muscle homogenates using spectrophotometric analysis (Ruitenbeek et al., 1981). However, interpretation of the results using this method has been difficult due to complicating side reactions (Wibom and Hultman, 1990). Subsequently, it has been found that a bioluminescence technique can be used to accurately measure MAPR utilising small amounts of muscle (Wibom et al., 1990). Thus, the technique can be applied to muscles from small rodents, such as rats and mice, as well as human muscle biopsy samples.

The bioluminescence technique involves light emission produced by enzyme-catalysed reactions (Lundin and Thore, 1975). Lundin and Thore (1975) state that the method is highly sensitive to low ATP levels making studies in muscle biopsies (or small animal muscles) to investigate mitochondrial function in biological systems possible. The method incorporates the use of the firefly luciferase reaction to emit light (refer to Figure 1.3). While other measurements of mitochondrial function exist, concentrating on oxygen consumption (van den Broek et al., 2010; Wessels et al., 2014), MAPR is a direct measurement of the ultimate output of ATP by the mitochondria, such that it is a powerful and relevant experimental tool that has been successfully used in other disease states, such as chronic heart failure (Williams et al., 2004; Williams et al., 2007).

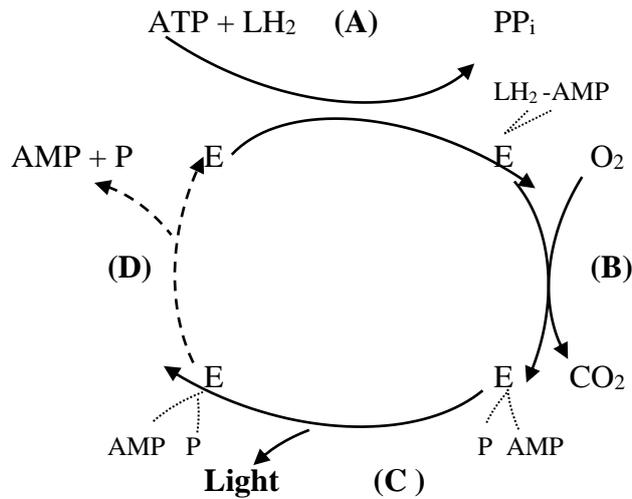


Figure 1.3 Schematic mechanism of the firefly luciferase reaction (Lundin and Thore, 1975: pp 47).

- A. In an initial activating reaction, similar to the activation of amino acids in protein biosynthesis, luciferin (LH₂) is adenylated by ATP to form luciferyl adenylate (LH₂-AMP) bound to the luciferase enzyme (E), and free pyrophosphate (PP_i).
- B. In the next step, the luciferyl adenylate enzyme complex (LH₂-AMP-E) is oxidised by molecular oxygen resulting in CO₂, AMP, and electronically excited oxyluciferin (P). AMP and oxyluciferin remain bound to the enzyme.
- C. On returning to the ground state the excited complex emits light, the colour of the light depends on the firefly species (i.e. the structure of the luciferase) and the pH: it varies between greenish yellow (560nm) and red (620nm).
- D. In a final step luciferin and AMP are released from the luciferase enzyme (E) which is then free to enter another reaction cycle.

1.3.3 SR Calcium Regulation

Calcium (Ca²⁺) is an important ion required for the normal function of muscle cells. It helps regulate muscle contraction and relaxation as well as modulate other cellular functions (Shamoo, 1985). One of the main membrane transport systems involved in the regulation of intracellular Ca²⁺ is known as the Sarcoplasmic Reticulum (SR). The function of the SR is to release Ca²⁺ into the myoplasm, so Ca²⁺ can bind with troponin C on the contractile apparatus and allow muscle contraction to take place. When Ca²⁺ dissociates from troponin C and is pumped back into the SR muscle relaxation can occur (Berchtold et al., 2000).

The process of Ca^{2+} release from the SR occurs via the Ryanodine Receptor (RyR), also known as the Ca^{2+} release channel. The activation process of Ca^{2+} release in skeletal muscle is primarily induced by depolarisation of the T-tubule (Fabiato, 1985; Hurwitz et al., 1991; Lamb, 2000). Depolarisation-induced Ca^{2+} release is a process where upon the T-tubule is depolarised, causing the voltage sensors on the Dihydropyridine (DHP) receptor to move up thereby opening the RyR of the SR, and initiating contraction (Berchtold et al., 2000).

Calcium resequestration into the SR is a metabolic process requiring the use of ATP and the Ca^{2+} -ATPase pump (Berchtold et al., 2000). SR Ca^{2+} -ATPase (SERCA) pumps undergo conformational changes during the hydrolysis of ATP, which result in the transport of Ca^{2+} into the SR lumen. The steps include: 1) the binding of Ca^{2+} , ATP and the enzyme; 2) phosphorylation of the enzyme complex, resulting in conformational movements of the enzyme domains and a weaker bond between the enzyme and Ca^{2+} ; 3) Ca^{2+} is then contained in the polar cavity of the transmembrane segment; 4) further conformational changes allows release of Ca^{2+} into the SR lumen; and 5) dephosphorylation of the enzyme, completing the Ca^{2+} transport cycle (refer to Figure 1.4).

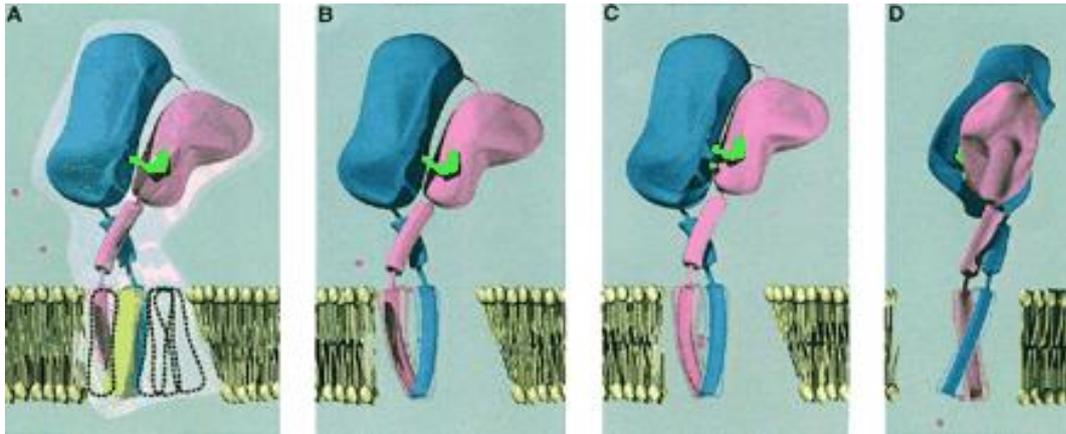


Figure 1.4 Mechanism of Ca²⁺ transport by SERCA.

- A) the structural model reveals some of the interacting domains. M4, S4, and the phosphorylation domain are blue; the nucleotide binding/hinge domain, S5, and M5 are pink; M6 is yellow; ATP is green; Ca²⁺ is red.
- B) the transmembrane domain is simplified by removal of all helices but M4 and M5 so that the essential contribution of M6 to the Ca²⁺ binding sites must be imagined. In the E1 conformation, high affinity Ca²⁺ binding sites located near the center of helices M4, M5, and M6 are accessible to cytoplasmic Ca²⁺ but not to luminal Ca²⁺.
- C) phosphorylation from ATP, following the occupation of both sites by Ca²⁺, leads to linked movements of both cytoplasmic and transmembrane domains, resulting in occlusion through closure of the entry gate. Ca²⁺ is now contained in a polar cavity formed near the center of the transmembrane domain by relatively small amino acids and blocked from exit by the juxtaposition of relatively large residues at both ends of the transmembrane helices.
- D) further conformational changes open the gate allowing exit of the two Ca²⁺ ions to the lumen. In this conformation Ca²⁺ affinity is very low. This conformation also activates dephosphorylation and returns the pump to the high Ca²⁺ affinity form, completing the cycle. (MacLennan et al., 1997: p28818)

1.3.4 SR Ca²⁺ Sequestration and the Phosphocreatine System

If Ca²⁺ is not sequestered efficiently into the SR, not only does this delay muscle relaxation, it results in the accumulation of intracellular [Ca²⁺], which has been found to trigger a cycle of cell damage, resulting in muscle degradation and proteolysis (Berchtold et al., 2000). Therefore it is imperative to ensure optimal SR Ca²⁺ uptake. A potential method for improving Ca²⁺ reuptake in the SR is by use of the phosphocreatine (PCr) system. This is because the efficiency of the Ca²⁺ pump, and hence muscle function, depends on an adequate energy supply and the removal of ATPase reaction products such as Adenosine Diphosphate (ADP) and Hydrogen ion (H⁺) (Minajeva, 1996; de Groof et al., 2002). The PCr system, with the aid of the creatine kinase (CK) enzyme, helps maintain cytosolic ATP levels in skeletal muscle by the following reaction:



In fact, it has been found that CK is specifically and tightly bound to the SR membranes, and that a significant portion of the Ca^{2+} uptake is supported by this system in the presence of PCr and ADP (Rossi et al., 1990).

Furthermore, it has been reported that Ca^{2+} uptake is more efficient when fuelled by PCr plus ADP, as opposed to ATP alone (Minajeva, 1996), indicating the importance of the proximity of CK to the Ca^{2+} pump. Depletion of PCr stores have been found to impair SR Ca^{2+} uptake during conditions of skeletal muscle fatigue (Duke and Steele, 1999). In particular, lower levels of PCr have been found in patients with T2D (Scheuermann-Freestone et al., 2003), which contribute to poor muscle function. Hence the potential role of increasing PCr as a means of improving muscle function in patients suffering from various myopathies and exercise intolerance, such as T2D, is enormous.

1.3.5 Measuring Ca^{2+} Transport in the SR

In order to determine the efficiency of Ca^{2+} regulation it is important to analyse the functionality of the SR (Warmington et al., 1996). It has been found that upon brief mechanical homogenisation, membrane systems of most muscle tissues are able to spontaneously reform into functionally intact vesicles, primarily of SR type (Kargacin et al., 1988; Simonides and Van Hardeveld, 1990). This therefore permits the analysis of Ca^{2+} transport in the SR.

Such assays require the utilisation of Ca^{2+} -sensitive fluorescent probes such as Fura-2 or Indo-1 in order to obtain quantitative data. Warmington et al. (1996) describes a method for measuring Ca^{2+} uptake and release processes in fresh skeletal muscle homogenates, using Fura-2 (Refer to figure 1.5). It was found that as Ca^{2+} was pumped into the SR via MgATP

hydrolysis, the fluorescence ratio declined, because Fura-2 is impermeable to the membrane and remained in the bathing fluid. Additionally, the sequestered Ca^{2+} reversibly binds to oxalate within the SR (mimicking the role of calsequestrin *in vivo*), and can be released through the RyR or Calcium Release Channel. The same can also be said for Indo-1.

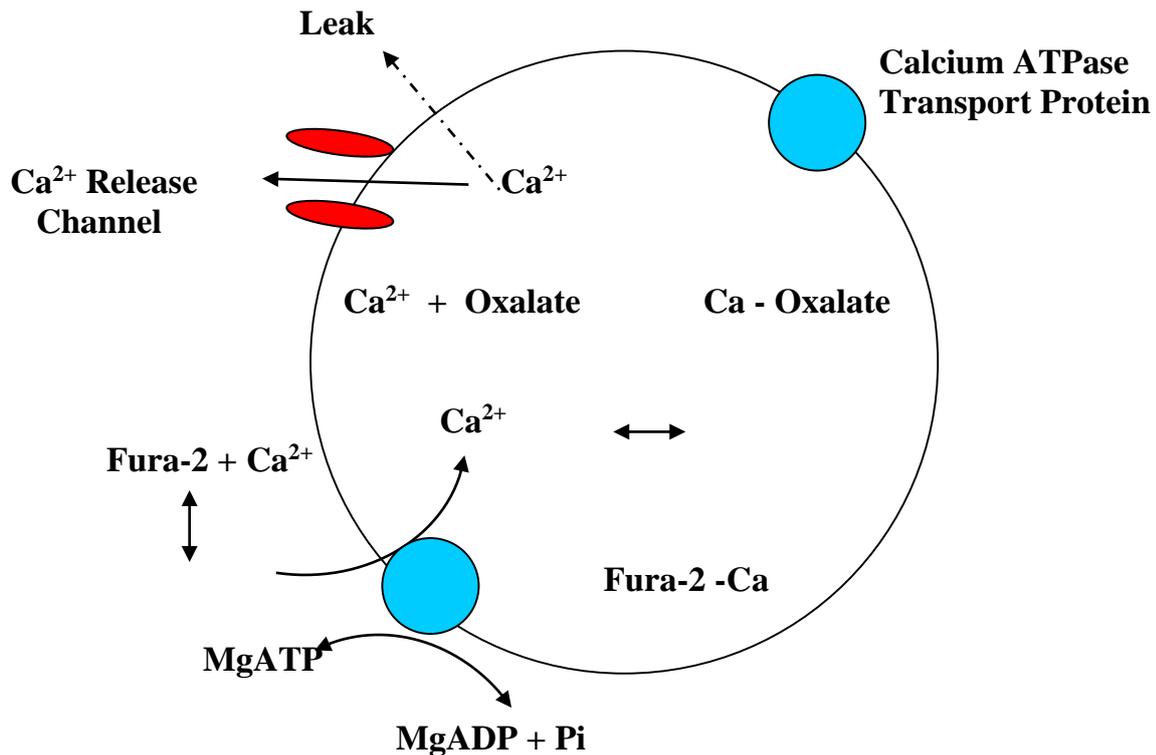


Figure 1.5 Schematic Model of the SR Vesicles in the Assay Mixture.

The Ca^{2+} -ATPase transports Ca^{2+} into the vesicles via hydrolysis of MgATP. Oxalate within the vesicles sequesters transported Ca^{2+} which can be released through the intrinsic Ca^{2+} channels. Fura-2 outside the vesicles alters its fluorescence according to the level of Ca^{2+} remaining after uptake or release (Warmington et al., 1996: p80).

Approximately 40% of the ATP utilised by skeletal muscle during physical activity is required for ion transport necessary for the alternating cycles of activation and relaxation (Homsher and Kean, 1978) and consequently any impairment in calcium handling may be secondary to impaired oxidative function. While this may be due to issues that are intrinsic to the SR, any decrements may also be due to alterations in the ability to generate sufficient ATP to meet the demands for optimal Ca^{2+} handling. Substantial evidence now exists that

intracellular Ca^{2+} handling is altered in the myocardium in heart failure (Yano et al., 2005). Nevertheless the alterations in Ca^{2+} handling in the myocardium, which is striated muscle like skeletal muscle, suggest that alterations in Ca^{2+} handling may contribute to skeletal muscle dysfunction in diabetes.

Changes in contractile activity are eminent in T2D, and may be a result of impaired SR Ca^{2+} regulation. In fact, slowing of the half relaxation time ($\frac{1}{2}\text{RT}$) can indicate slower resequestration of Ca^{2+} by the SR, thereby leaving high intracellular Ca^{2+} levels leading to a series of events causing damage of skeletal muscles (Gissel and Clausen, 2001). Several studies have found a correlation between T2D and defective Ca^{2+} cycling (Yu et al., 1994; Pierce and Russell, 1997; Choi et al., 2002; Zheng et al., 2004), with disruptions to the SR and t-tubules in both skeletal and cardiac muscle (Afzal et al., 1988; Cotter et al., 1993). Impaired Ca^{2+} handling has also been reported to be a result of reduced expression of SERCA pumps and RyRs (Bidasee et al., 2001; Netticaden et al., 2001; Zhong et al., 2001; Choi et al., 2002). Trost et al. (2002) conducted a study to determine if SERCA pump overexpression could ameliorate the diabetes-induced contractile failure in STZ-induced diabetic rat hearts. The results indicated that contractile function was enhanced by SERCA overexpression. However they could not determine if this was due to improved Ca^{2+} sequestration of the SR, or simply a result of the increased expression of SERCA pumps.

An earlier study by Eibschutz et al. (1984) looked at Ca^{2+} transport function in the SR of skeletal muscle. They also used the Streptozotocin animal model of diabetes and found that there was in fact reduced Ca^{2+} ATPase activity, suggesting this is the reason for the impaired muscle function in Diabetic animals. However, the reasons for these results remain elusive with contradictory evidence showing there to be enhanced SERCA pump activity resulting in

hyperfunction of skeletal muscle in diabetes (Ganguly et al., 1986; Ganguly et al., 1987; Cotter et al., 1993). However these rats were not made chronically Diabetic compared to the study by Eibschutz et al. (1984) and this may account for the differences in muscle function. Indeed, the relative stage of the disease is important. In a study using coronary artery ligation to induce heart failure in rats (Shah et al., 2004), maximum velocity (V_{\max}) of skeletal muscle SR Ca^{2+} uptake and the maximal activity of the Ca^{2+} stimulated ATPase enzyme were significantly increased at 8 weeks (Shah et al., 2004) but the values had fallen below those of the control animals by 16 weeks post-surgery. This is consistent with reduced Ca^{2+} ATPase activity only in the chronically diabetic muscles, and likely indicates a compensatory response early in disease progression, with reductions occurring only in the later stages of the diseases.

Another reason that may account for the differences found was the type of skeletal muscle employed. Ganguly et al. (1986) predominantly used fast-twitch skeletal muscle fibres however Eibschutz et al. (1984) did not provide this type of information, making comparisons difficult. In addition, Eibschutz et al. (1984) did not examine the initial stages of the disease, nor was information provided on the methods used to measure the Ca^{2+} transients, again making comparisons difficult.

Taken together, research to date indicates that there may be a compensatory mechanism in the early stages of the diseases and hence the hyperfunction of the SR, whilst during the chronic stages of the disease a reduction in function is eminent. However, no studies to date have looked at the time course of the progression of changes, thus this would warrant further investigation. Nevertheless, it is apparent that whether there are increases or decreases in SR function, there are changes in diabetic skeletal muscle which may contribute to the changes

observed in contractile activity and impaired exercise tolerance that has been reported previously.

1.3.6 Reactive Oxygen Species and Inflammation

Oxidative balance is the balance between levels of oxidants such as free radicals or Reactive Oxygen Species (ROS), and antioxidants or the enzymes such as superoxide dismutase (SOD), Glutathione Peroxidase, and Catalase, which counteract their negative effects. Hence oxidative stress can be defined as an imbalance between ROS production and antioxidant defences resulting from either increased ROS production and/or impaired defence mechanisms. There is substantial evidence to indicate that chronic elevations in oxidative stress may be a mechanism of T2D disease progression (Brownlee, 2001; Ceriello and Motz, 2004).

Impaired mitochondrial function in T2D has been described in earlier sections, and this impairment often leads to oxidative stress and the production of free radicals. While only recently getting more attention, it has long been observed that increased free radical production occurs in diabetic sufferers, and thus oxidative stress has been implicated in the pathogenesis and progression of the disease (Baynes, 1991; Baynes and Thorpe, 1999; Rosen et al., 2001; Vincent et al., 2002). Further to this, impaired antioxidant capacity, and thus ability to counteract free radical production, has also been observed (Halliwell and Gutteridge, 1990; McLennan et al., 1991; Saxena et al., 1993). There is a clear association between hyperglycaemia and oxidative stress (Rosen et al., 2001), with hyperglycaemia resulting in the generation of free radicals via a superoxide-dependent pathway by promoting lipid peroxidation (Kawamura et al., 1994; Tsai et al., 1994).

Thus there is substantial evidence that ROS production is elevated in T2D. However there is also increasing evidence that the elevated levels of oxidative stress are not solely due to increased ROS production, but are also due to impaired antioxidant defences in the skeletal muscle. Studies have reported activities of free radical scavenger enzymes such as superoxide dismutase, catalase and glutathione peroxidase to be lower in T2D compared to healthy controls (Maritim et al., 2003; Ramirez-Sanchez et al., 2013; Fabre et al., 2014). Any decreases in the levels of antioxidant defences only serve to leave the cell more susceptible to the increased levels of ROS produced in response to everyday physical activity and disease progression. Even at normal levels of ROS production without physical activity, the reduction in antioxidant defences may still render cells susceptible to the effects of the imbalance of ROS.

Oxidative stress has also been shown to activate the process of inflammation by activating NF- κ B, which in turn leads to pro-inflammatory changes by increasing TNF- α and Il-6. This has been shown to contribute to insulin resistance and reducing β -cell function in the pancreas, hence causing T2D (Mohamed et al., 1999; Dandona et al., 2004).

Inflammation is likely to play an important role in the pathogenesis and progression of T2D (Dandona et al., 2004). Many of the features of T2D are consistent with the biological effects of inflammatory markers, such as the cytokine tumor necrosis factor alpha (TNF- α), that have been noted to be elevated in T2D (Hohmeier et al., 2003; Patsouris et al., 2014) and levels of which correlate with disease severity (Anker et al., 1997). Cytokines are protein molecules with relatively small molecular weights which are secreted by cells in response to a variety of different inducing stimuli. Specifically it has been contended that when cytokines are expressed in high concentrations, there may be release of these molecules into the circulation

where they may exert endocrine effects (Torre-Amione et al., 1996). Thus this provides a possible mechanism by which a disease with central origins may result in the range of peripheral maladaptations that have been observed in T2D. However there is also evidence that local expression of a range of pro-inflammatory cytokines such as TNF- α , interleukin-1 beta (IL-6), and nuclear-factor-kappa-B (NF- $\kappa\beta$) may be elevated in skeletal muscle in T2D (Yu et al., 2002; Dandona et al., 2004).

Regardless of the mechanisms responsible for the inflammation in T2D, elevated tissue levels of inflammatory cytokines including TNF- α , IL-6, JNK and NF- $\kappa\beta$ initiate a cascade of events leading to further cytokine expression (Baeuerle and Baltimore, 1996) and eventually to impaired muscle structure and function via several potential pathways. These include decreased muscle oxidative capacity (Hambrecht et al., 1999), impaired muscle contractility due to reduced phospholamban (a SR regulatory protein) expression (McTiernan et al., 1997) as well as protein degradation, atrophy and apoptosis (Niebauer et al., 1998; Dalla Libera et al., 2001; Langen et al., 2002; Cai et al., 2004; Hunter and Kandarian, 2004; Mourkioti et al., 2006; O'Connor et al., 2008; Lenk et al., 2009). Recently, diabetic alterations to the contents and activation of NF- $\kappa\beta$ were found to be reduced in slow-twitch muscle fibres but elevated in fast-twitch fibres (Frier et al., 2008), potentially explaining the fibre type differences in atrophy found in T2D (Armstrong et al., 1975).

1.3.7 Skeletal Muscle Atrophy and Weakness

Maintaining lean muscle mass is imperative for longevity. While primary myopathies such as the muscular dystrophies are well known for their ability to increase morbidity and mortality (Burnham et al., 2005), a number of other diseases, such as diabetes and CHF, subsequently cause a loss of lean muscle mass which becomes a major contributor to the disease process,

and hence contributes to morbidity and mortality. Certainly, T2D patients have been shown to suffer from muscle weakness (Andersen et al., 2004; Sayer et al., 2005). However, the diabetic complication of loss of lean body mass (LBM) (Price et al., 1996) has not been widely studied, perhaps as the loss of mobility and strength prevents people with diabetes from being active in daily life, which could then result in further muscle mass loss due to the inactivity, thus becoming a cycle of muscle inactivity and atrophy, leading to further decreases in LBM and inactivity. Thus, muscle atrophy has often been seen as secondary to the inactivity usually associated with obesity and glucose intolerance. However, there appears to be a link between insulin levels and skeletal muscle protein turnover.

Denne et al. (1991) has shown in healthy adult men, euglycaemic hyperinsulinaemic conditions were able to suppress skeletal muscle proteolysis. Denne et al. (1995) also investigated protein turnover in euglycaemic hyperinsulinaemic T2D patients, finding there to be a reduction in skeletal muscle proteolysis. This suggests a possible compensatory mechanism in T2D, particularly in the earlier stages of the disease when hyper secretion of insulin is inherent. It may also suggest that hyperglycaemia may play a significant role in protein degradation. Recently, studies have shown a link between insulin resistance, hyperglycaemia, protein degradation and overall muscle atrophy in the earlier stages of the disease. In particular, Wang et al. (2006) looked at *db/db* mice (another model of T2D) with increased blood glucose and insulin levels. Compared with controls, insulin resistant mice showed significantly smaller muscle weights and cross-sectional areas, which were a result of activation of the major proteolytic systems. These abnormalities were improved by administering rosiglitazone, a drug that improves indices of insulin resistance, and therefore plasma glucose concentrations.

Studies of chemically-induced diabetes have shown that insulin deprivation with hyperglycaemia, as seen in the later stages of T2D, decreases protein synthesis and increases protein degradation (Pain and Garlick, 1974; Garlick et al., 1981), resulting in overall muscle atrophy. These changes have been shown to be fibre-type specific, predominantly affecting fast glycolytic fibres, with slow oxidative fibres being least affected (Armstrong et al., 1975; Cotter et al., 1989; Cotter et al., 1993; Price et al., 1996; Farrell et al., 1999). Whilst the animal models used in these experiments are more representative of Type 1 Diabetes, and potentially the advanced stages of T2D, similar findings have been shown in the Obese Zucker rat, a more representative model of T2D. Wheatley et al. (2004) showed that calf muscle mass comprising of soleus, plantaris, gastrocnemius, extensor digitorum longus (EDL) and tibialis anterior (TA) of the Obese Zucker rat were significantly lower than that of the lean non-diabetic controls. However, fibre type differences were not determined in this study due to the measurement of the calf muscle mass as a whole. Thus, it is clear that the characteristic pattern of muscle atrophy is eminent in T2D and further work in establishing fibre type alterations could provide the framework for therapeutic targets.

1.4 Potential Therapies In Type 2 Diabetes

Given the considerable alterations that have been observed in skeletal muscle in T2D, and the likely role of the maladaptations in the profound exercise intolerance and poor outcomes associated with this disease, discussion of potential treatment modalities is warranted. A range of pharmaceutical treatments are routinely prescribed to treat T2D patients. Given that these medications are prescribed for the treatment of disease, it is difficult to determine the exact effects on skeletal muscle as the diseases themselves have effects on skeletal muscle as we have described above. However, one medication (Aspirin) in particular may have the

potential to prevent or reverse some of the skeletal muscle maladaptations that have been observed in this condition.

1.4.1 Aspirin

Aspirin is widely prescribed as an anti-platelet medication in T2D. It may have a direct role in reducing skeletal muscle maladaptations by preventing thromboses in the microcirculation (Schorr, 1997) thereby assisting in the maintenance of blood supply to skeletal muscle. However aspirin also has antioxidant properties which may affect skeletal muscle function in this disease.

The production of ROS by the mitochondria, in particular superoxide, has been shown to activate the process of inflammation by activating NF- κ B, which in turn leads to pro-inflammatory changes by increasing TNF- α and Il-6. This has been shown to contribute to insulin resistance and reducing beta-cell function in the pancreas hence causing T2D (Dandona et al., 2004). The antioxidant defences available to the cell include superoxide dismutase, glutathione peroxidase and catalase (Ye et al., 2004). Therefore these authors tested the effects of catalase overexpression on cardiomyocyte function using the Agouti mouse model of T2D. They found that this was useful in reducing oxidative stress in Diabetic mouse hearts. Although it did not prevent other ROS damage from occurring, such as from superoxide, because catalase only targets hydrogen peroxide (Ye et al., 2004), it does highlight that antioxidant therapy may be a useful treatment option. Indeed, aspirin, which is already commonly used as a medication in T2D, has been shown to reduce superoxide production and oxidative stress (El Midaoui et al., 2002; Bobik, 2004), as well as improving insulin resistance (Barreiro et al., 2004). Thus aspirin may be useful in alleviating the cascade of events caused by superoxide anions that can cause and/or worsen T2D pathology.

However, given there are more than one ROS impeding muscle function, further research needs to be undertaken on this and other possible antioxidants to help improve muscular function and exercise tolerance in T2D, and provide further data about the underlying pathogenesis.

Whilst exercise training may be beneficial in ameliorating some of the associated skeletal muscle changes and conditions in T2D, it is important to consider combining these exercise regimes with supplements which are known to help prevent or even reverse some of these maladaptations, to ensure that the benefits of exercise alone are not overcome by the dysfunctional aspects of each disease, but rather have an additive and therapeutic effect, and may decrease reliance on pharmacological interventions.

1.4.2 Creatine Monohydrate

Creatine monohydrate is a supplement that has been consistently demonstrated to promote gains in lean body mass and strength in resistance trained individuals (Brose et al., 2003; Rawson and Volek, 2003). The supplementation period across the various studies in this review ranged from 7 to 91 days, with a dosage ranging from 20 to 25 g of creatine per day for the entire supplementation period, including a loading dose for 3–7 days, followed by a maintenance dose for the remainder of the supplementation period. Nevertheless each regime ensured that muscle creatine stores were increased. Furthermore, the gains in lean body mass and strength were shown to improve through increases in muscle fiber size and contractile protein content (Cribb et al., 2007). As such creatine monohydrate may be a supplement that can assist in reversing the loss of skeletal muscle in T2D. Indeed, creatine supplementation has proven to be an effective means in which to treat muscle impairments in

Duchenne Muscular Dystrophy (Tarnopolsky et al., 2004; Kley et al., 2007) whereby increases handgrip strength and muscle fat-free mass were significantly improved.

In patients with T2D, depletion of PCr stores has been found to impair SR Ca^{2+} uptake (Scheuermann-Freestone et al., 2003). As mentioned previously, elevated cytoplasmic Ca^{2+} initiates the damage-repair cycle within muscle, with repeated bouts of this rendering the muscle susceptible to further necrosis. Thus, elevating PCr is one potential therapeutic means of improving muscle function in patients suffering from various myopathies and exercise intolerance, such as T2D. Given that Ca^{2+} regulation is impaired in T2D, and a prime target involves SERCA activity, a potential way to improve SERCA function is by increasing the fuel available to it, particularly that which is used by the PCr system. It has been found that total muscle creatine increases as a result of creatine supplementation (Harris et al., 1992). Greenhaf et al. (1993) have suggested that this is due to an increase in PCr resynthesis and results in an overall improvement in muscle performance by improving SR Ca^{2+} regulation. In addition, a study by Casey and Greenhaff (2000) found that ingestion of creatine monohydrate at a rate of 20 g/d for five to six days was shown to increase the total creatine concentration of human skeletal muscle. Further the observed improvements in performance are due to parallel improvements in ATP resynthesis during exercise as a consequence of increased phosphocreatine availability. Therefore, the use of creatine supplementation in treating poor muscle performance in diabetic patients may be of use in terms of providing additional fuel for SR Ca^{2+} regulation, given that these patients show impaired Ca^{2+} regulation (Allo et al., 1991; Pierce and Russell, 1997).

Another benefit of creatine supplementation is that it may reduce insulin resistance by improving glucose uptake (Ferrante et al., 2000), although these effects were observed in

transgenic Huntington mice, not diabetic rats. A study on healthy humans conducted by Eijnde et al. (2001) found that oral creatine supplementation prevents the decline in muscle glucose transporter (GluT-4) protein content that occurs in immobilisation, and increases GluT-4 protein content during rehabilitation training. By increasing GluT-4 protein expression, glucose uptake is enhanced, thereby reducing the severity of insulin resistance. Therefore Eijnde et al. (2001) suggested that creatine supplementation could be a potential treatment for T2D. However, the effects shown in this study may not be solely attributable to creatine supplementation, and may not necessarily indicate an improvement in insulin resistance given that exercise alone has been shown to enhance GluT-4 translocation via a different mechanism to the insulin-stimulated GluT-4 translocation (Wallberg-Henriksson and Holloszy, 1985; Hayashi et al., 1997). More recently, Ju et al. (2005) looked at the effects of creatine supplementation on GluT-4 gene expression in normal Wistar rats. They found a significant positive correlation between the two variables i.e. that creatine supplementation increased GluT-4 expression without the influence of exercise. This indicates the potential for creatine to also enhance glucose uptake into muscle cells for metabolism to provide fuel to maintain mitochondrial function and hence enhance muscle contraction. Furthermore, the use of creatine supplementation has not been looked at in animal models of T2D, and so it would be useful to determine if creatine can ameliorate the abnormalities in skeletal muscle function arising from impaired glucose transport.

1.4.3 Whey Protein

Protein supplementation is commonplace to enhance the effects of resistance training, with maintenance of lean muscle mass being essential for healthy longevity (Hayes and Cribb, 2008). Whey proteins are the most desirable, due to their high biological value and fast absorption kinetics (Dangin et al., 2003). Due to the prevalence of essential amino acids,

particularly leucine, a key regulator of the protein synthesis pathway (Norton and Layman, 2006), whey proteins are able to activate mTOR, thus increasing protein synthesis (see Figure 1.1) and thereby potentially assisting in the reversal of muscle atrophy that has been observed in T2D. While the exact mechanisms are not yet understood, it does appear that stimulating the mTOR signalling pathway with amino acids occurs independently of Akt (Liu et al., 2002). Thus, even in insulin-resistant tissues, protein supplementation is likely to still exert its effects. Indeed, whey protein has been found to increase lean muscle mass, fibre area, protein synthesis, glucose uptake, ATP production, and glutathione levels, whilst reducing body weight gain, muscle fat content, oxidative stress caused by ROS, and insulin resistance (Bounous and Gold, 1991; Lands et al., 1999; Belobrajdic et al., 2004).

Whey protein is also a rich source of cysteine that provides antioxidant protective benefits and may therefore reduce the vicious cycle of inflammation and subsequent free radical production that was described earlier. A single study has investigated the effect of whey protein on antioxidant status in an animal model of iron overload cardiomyopathy (Bartfay et al., 2003). The authors reported that the mice that received the whey protein had significantly decreased concentrations of malondialdehyde and increased glutathione concentration and glutathione peroxidase activity in the cardiac tissue following four weeks of supplementation indicating improved antioxidant status.

Thus, while it is clear that the effects of whey proteins could provide several beneficial results for diabetes, more work is required before general recommendations regarding supplementation with whey protein should be made. However, given that it is clear that exercise is a useful therapy for T2D, it is more than likely that the augmentation similar to those shown in young, fit individuals of the effects of supplementation such as creatine and

whey protein, particularly in the hours surrounding the exercise bout (Cribb and Hayes, 2006b), would provide even greater benefits. Increased lean mass will increase the capacity for further exercise training at higher intensities, decreasing adiposity and reducing circulating free fatty acids, thus decreasing the pro-inflammatory effects of these free fatty acid intermediates, improving mitochondrial function and increasing insulin sensitivity, improving glucose disposal even further, creating a positive feedback mechanism which could reverse the deleterious effects and severity of these diseases. Further decreases in inflammation and improved mitochondrial function may also be achieved by anti-oxidant therapy. For the purposes of this thesis however, the benefits of whey protein must be looked at prior to incorporating the additive benefits of exercise and supplementation to determine any underlying mechanisms.

1.5 Use of Animal Models in Type 2 Diabetes

When looking at potential therapeutic agents and their effects in T2D, there are many complex genetic and environmental interactions to consider. Therefore in order to control for some of these, animal models that best represent the human condition are considered ideal in such research.

There are several categories of different animal models to choose from in T2D including genetically derived, diet-induced, chemically-induced, and surgical- and transgenic-knockout models. Each category has its advantages and disadvantages and has been previously reviewed (Srinivasan and Ramarao, 2007).

Many studies to date have made use of the chemically-induced model – the Streptozotocin-induced rat to study various features of Diabetes Mellitus. In fact, it is the most commonly

used animal model of Diabetes (Yu et al., 1997). After injection with streptozotocin, which acts to selectively destroy the pancreatic β cells (for review see Yu et al., 1997), rats show characteristics of diabetes such as lowered body weight, elevated plasma glucose and depressed insulin levels (Wallberg-Henriksson and Holloszy, 1985; Ganguly et al., 1986; Zhong et al., 2001). However, these characteristics mimic those of Type 1 Diabetes and not of Type 2. This is because, in T2D, insulin levels are usually increased as the pancreatic β cells are still able to produce and secrete insulin (although not in some patients). The other reason that this model does not sufficiently mimic T2D is because Type I Diabetic rats are not insulin resistant, and it has been found that insulin treatment ameliorates the effects of the streptozotocin injection (Ganguly et al., 1987; Taira et al., 1991).

In order to study the effects of T2D on skeletal muscle function, it is therefore imperative to incorporate the appropriate animal model for the disease. The Zucker Obese rat has been an extensively used animal model of human obesity, and they also express many features of T2D (Peterson, 2001). These features include hyperinsulinaemia, insulin resistance, glucose intolerance, and hyperlipidaemia. However, it does not include hyperglycaemia (for review see Corsetti et al., 2000). They are useful for many insulin sensitising and anti-obesity agents (Himms-Hagen and Danforth, 1996; Ramarao and Kaul, 1999; Nuss and Wagman, 2000).

The Zucker Diabetic Fatty (ZDF) rat is a substrain of the Zucker Obese rat specifically bred for hyperglycaemia, thus more closely resembling the human form of the disease. In this model, the *fa* mutation results in impaired leptin signalling (Pickavance et al., 1998), and thus impaired appetite suppression and thermogenic effects (Terrettaz and Jeanrenaud, 1983). It is specifically the male ZDF rats that are more prone to develop hyperglycaemia 7-10 weeks after birth (Peterson et al., 1990; Kuhlmann et al., 2003) and is commonly used to investigate

the mechanisms associated with insulin resistance as well as testing insulin sensitisers. Another characteristic seen in human T2D is that patients have significant reductions in skeletal muscle GluT-4 transporters, particularly in slow muscle fibres (Gaster et al., 2001). It is these ZDF rats who also show similar reductions in GluT-4 transporters in skeletal muscle (Srinivasan and Ramarao, 2007). As such, using the ZDF rat allows for comparisons to be made with other studies, which have not, to date, tested the effects of aspirin and, creatine and whey supplementation on skeletal muscle function of T2D.

1.6 Significance

Skeletal muscle is one of the main organs responsible for glucose disposal and hence its role in contractile performance and metabolism can be pivotal in the pathogenesis of disease states such as T2D. Some of the overall effects of obesity and T2D on muscle structure and function are summarised in Figure 1.6. However, given disease progression varies in both time and severity, not to mention the additional effects of the complications of T2D, there is still controversy in the literature pertaining to how muscle function is affected and the mechanisms involved. Therefore the significance of this project was to give further insights into skeletal muscle pathophysiology and their mechanisms in an animal model of T2D, which not only will help direct us towards the causes of muscle impairments, but would provide a target for potential therapies to help improve muscle function and exercise tolerance in this condition.

Provided that the null hypothesis is rejected, results from this thesis will help discern the potential uses of Creatine, Whey and Aspirin in ameliorating muscle dysfunction and hence be applied to human populations suffering from the debilitating effects of T2D. This will enhance exercise tolerance, and encourage a more active lifestyle which in turn will improve

the deleterious effects of insulin resistance and the negative connotations of a hyperglycaemic environment. Indeed, many healthy individuals currently benefit from utilising Creatine and Whey protein in developing muscle strength, size and improved performance, as well as reducing fat mass, therefore the use of these supplements can be advantageous to other populations, including those suffering from T2D. Additionally, given recent reviews suggesting exercise as a treatment in several disease states including T2D (Pedersen and Saltin, 2015), utilising these supplements in combination with appropriate exercise prescription can result in additional benefits to this population, and decrease mortality associated with the disease.

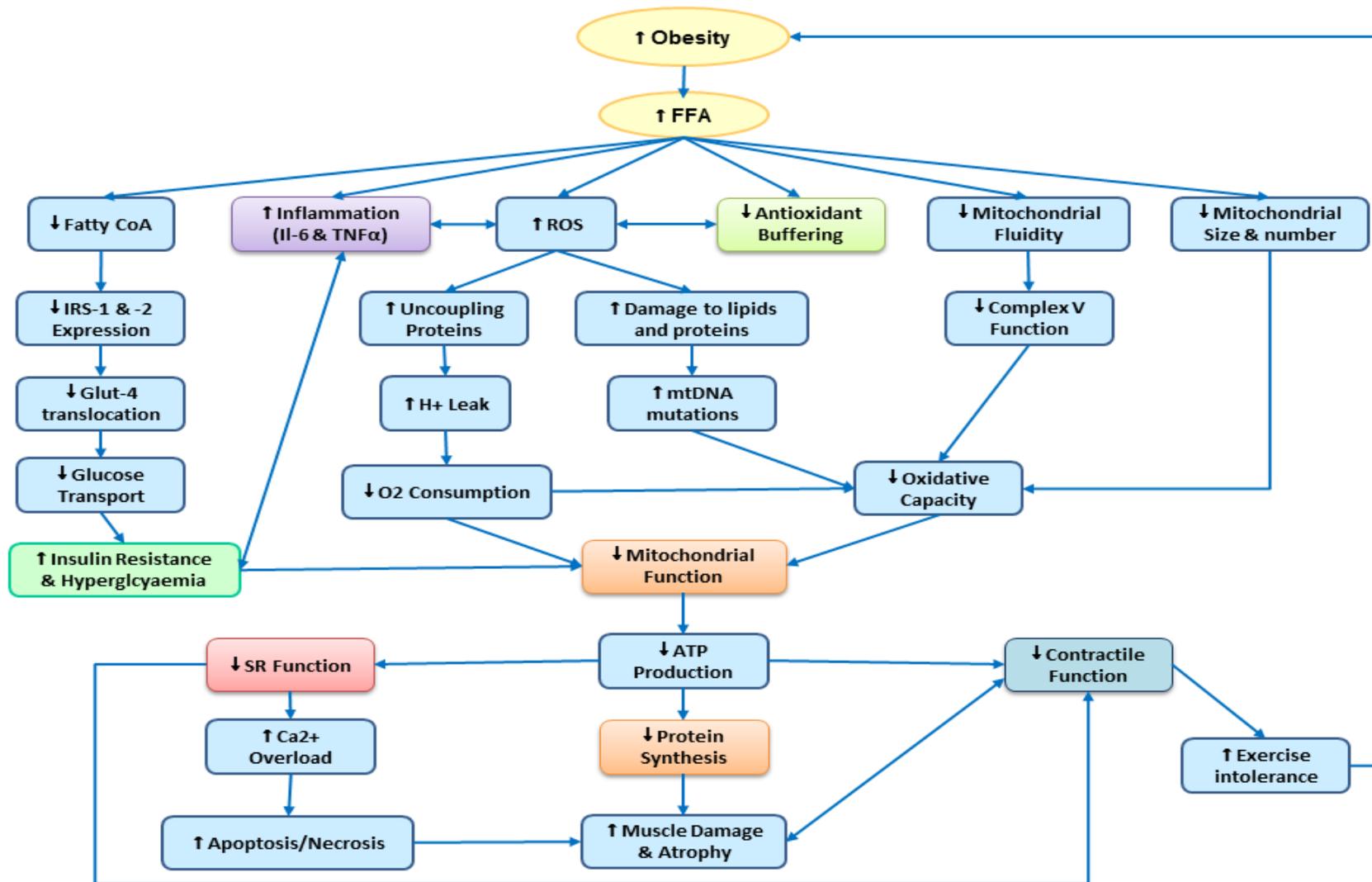


Figure 1.6 Summary of Pathways Resulting in Skeletal Muscle Impairments. Target areas of each supplement to reverse the effects those shown above.

■ Primary target of all three supplements.
 ■ Primary target of Whey and/or Aspirin.
 ■ Primary target of Whey.
 ■ Primary target of Creatine.
 ■ Primary target of Aspirin.

1.7 General Aims

Therefore the overall aims of this thesis are to examine the extent of skeletal muscle morphology alterations and/or (dys)function in T2D and how these can be improved by the use of dietary supplements. Specifically, muscle atrophy and/or damage will be examined and how this may impact upon contractile function. Some of the mechanisms likely to be involved such as SR and mitochondrial dysfunction, and inflammation will be examined, as well as how insulin resistance may be impacting on skeletal muscle. Finally, it is aimed to determine how Creatine, Whey Protein Isolate, and Aspirin can be used to potentially reverse any of the maladaptations found in skeletal muscle of the ZDF rat model of T2D.

1.8 General Hypotheses

It is hypothesised that there will be more skeletal muscle atrophy and damage found in sedentary Obese ZDF rats compared to Lean ZDF littermates as a result of an increase in oxidative stress and excessive calpain activation. This in turn will impact on skeletal muscle contractile function in T2D, where the main mechanism contributing to this impairment will be a reduction in SR and mitochondrial function, causing Ca^{2+} dysregulation and impaired energy production for optimal cell function, respectively. These detriments will further be exacerbated by increases in ROS production such as superoxide thereby activating inflammatory processes, thus contributing to insulin resistance. It is also hypothesised that treatment with creatine, whey and aspirin will reverse any impairments found and enhance overall muscle morphology and function whilst improving insulin resistance.

CHAPTER 2

Methods

2.1 Animals

For the muscle atrophy and EBD experiment, animals were sourced from the School of Medicine Animal House (Flinders University Adelaide, Australia). A total of 28 rats were used in this study: 14-week-old male Obese Zucker rats and age-matched, non-diabetic male lean Zucker littermates served as the control group. Obese Zucker rats have been shown to have developed insulin-resistance by this age (Durham and Truett, 2006). All experiments were approved by the Animal Ethics Experimentation Committee, Victoria University (AEETH 09/07).

For all other experiments, animals were sourced from Monash Animal Services (Monash University Melbourne, Australia). Obese Zucker Diabetic Fatty (ZDF) rats and Lean Zucker littermates at 8 weeks of age were divided into four groups, two supplemented (ObSupp and LSupp) and two unsupplemented (Obese and Lean). A total of 10 Lean, 12 Obese, 11 Lean supplemented, and 13 Obese supplemented ZDF rats comprised the groups. This age was chosen because ZDF rats have been shown to develop T2D between 6-8 weeks of age (Sima and Shafrir, 2001). The supplemented group were fed a special chow and water diet as detailed in section 2.3 for 6 weeks. The unsupplemented group were fed a normal chow (Barastoc, Ridley Corporation, Melbourne) (comprising Protein 20.00%, Total Fat 4.80%, Crude Fibre 4.80%, Acid Detergent Fibre 7.60%, Neutral Detergent Fibre 16.40%, Total Carbohydrate 59.40%) and water diet. Experiments herein were approved by the Animal Ethics Experimentation Committee, Victoria University (AEETH 09/07 and 10/10).

All animals of the same phenotype were housed in pairs and allowed access to food and water *ad libitum*. They were housed in a temperature controlled (22°C) room with a 12:12hr light-

dark cycle. Experiments conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2 Animal Anaesthesia and Tissue Acquisition

Rats were approximately 14 weeks of age at the time of analysis for all projects. 24 hours prior to sampling, animals were weighed then received an intra-peritoneal injection containing 1% EBD (refer to chapter 3.2.4). Food and water was removed for an overnight fast for the purposes of determining insulin resistance (refer to section 2.10). On the day of experimentation, animals were weighed again then anaesthetised with Sodium Pentobarbital (60mg/kg body weight) (pentobarbitone sodium, 60mg/ml, Therapon, Burwood, Vic, Australia). Hind limb and eye blink reflexes were checked regularly to ensure appropriate level of anaesthesia for tissue acquisition. Top-up doses were applied as required. Once all required tissue was extracted (refer to ensuing sections for specific details of tissue acquisition for each study), up to 4mls of blood was collected via cardiac puncture using 25G x 3/4" gauge needles (Terumo, Australia), dispensed in plain Eppendorf tubes and immediately centrifuged for 2 minutes at 12000RPM (13709 g-force) (Heraeus megafuge 40R centrifuge, Thermofisher Scientific, Australia). Cardiac tissue was removed and snap frozen in liquid nitrogen. Plasma was collected from centrifuged blood samples and likewise snap frozen in liquid nitrogen for later analysis of cytokines (Il-6 and TNF- α), insulin, and glucose.

2.3 Supplementation

2.3.1 Creatine and Whey Protein Supplementation Rates

Rats in the supplemented groups were fed a standard chow diet reconstituted to contain a combination of powdered creatine monohydrate and whey protein isolate (AST Sports

Science Inc., CO, USA) for 6 weeks. The supplements were combined as it has been found that the combination of creatine and whey showed greater improvements in muscle function compared to ingesting creatine or whey alone (Burke et al., 2001). In addition, as this was a novel study to determine the effects of supplementation on muscle function in ZDF rats, and thus our supplementation strategy/regime maximised the likelihood of obtaining an effect, a combined supplement was used. The concentration of creatine and whey was designed to deliver 0.09g creatine/kg/day and 1.5g whey/kg/day, respectively. The dose of creatine was equivalent to the maintenance dose employed in human studies as it has been found that over a period of 28 days, total muscle creatine content reaches similar values to those given a higher loading dose over 6 days (Hultman et al., 1996). Similarly, the dose of whey chosen was also based on doses regularly used in human studies (Laight et al., 1999; Burke et al., 2001; Middleton et al., 2004; Cribb and Hayes, 2006a). The doses of creatine and whey were scaled for the higher metabolic rates of rats as per calculations below:

70kg man expends ~2000cal/day = 28.57 cal/kg/day

Adult rat expends ~130cal/kg/day

Therefore, (130cal/kg/day) / (28.57cal/kg/day) = 4.5

i.e. a rat expends 4.5 times more energy than man. So:

Whey dose in man of 1.5g/kg/day x 4.5 = 6.75g/kg/day equivalent for rat

Creatine dose in man of 0.1g/kg/day x 4.5 = 0.45g/kg/day equivalent for rat.

These doses were further adjusted according to the average food consumption and average weight of lean and obese animals, to ensure they weren't under- or over-dosed.

The exact supplement dosage was calculated by measuring total chow consumption, however because the animals were housed in pairs thereby making true dosage calculations less accurate, the weights of each rat were also recorded every 3 days and compared.

2.3.2 Aspirin Supplementation Rate

Acetylsalicylic acid (Aspirin) (Catalogue #A2093 – 100G, Sigma, Australia) was administered to the supplemented groups in drinking water, for which access was *ad libitum*. The target was 100mg/kg/day for 6 weeks. This dosage was chosen as the reductions in insulin resistance, inflammation (Yuan et al., 2001) and oxidative stress (El Midaoui et al., 2002; Wu et al., 2002) were greatest compared to other doses. In addition, an increasing effect was shown with a longer supplementation period (Yuan et al., 2001; Wu et al., 2002). Whilst Yuan et al. (2001) conducted their treatment regime over 3-4 weeks, and Wu et al. (2002) over 53 days, both resulted in similar significant improvements, as such the period of 6 weeks herein was chosen as a mid-point and would be in line with the supplementation regime required for creatine and whey protein as discussed in the previous section.

2.4 Contractile Function

2.4.1 Muscle Acquisition and Preparation

Once rats were sufficiently anaesthetised as described in section 2.2, Extensor Digitorum Longus (EDL) comprising of mostly fast twitch type II fibres, was excised and tested followed by soleus comprising of mostly slow twitch type I fibres. Soleus remained intact in the animal throughout testing of EDL in order to maintain blood supply and preserve the muscle.

The proximal tendon of the EDL was located first and surrounding tissue was cleared away. Surgical silk size 3.0 (SP118, Critical Assist, VIC, Australia) was tied securely at the point of insertion of the EDL muscle. The same procedure was performed at the distal point of insertion. The muscle was carefully dissected free from other tissues, starting from the proximal end, and placed into a horizontal custom-built plexiglass bath containing Krebs Henseleit Ringer solution (NaCl 118mM; KCL 4.75mM; Na₂HPO₄ 1mM; MgSO₄·7H₂O 1.18mM; NaHCO₃ 24.8mM; CaCl₂ 2.5mM and D-Glucose 11.0mM), pH 7.4. This pH was maintained by having the buffer solution aerated with carbogen (95% O₂ and 5% CO₂) (BOC gases, Melbourne, Australia). All contractile experiments were conducted at 25°C as isolated muscles do not remain viable for long periods of time if tested at 37°C. These experimental conditions facilitate optimal oxygen diffusion throughout the muscle, and maintain stability of functional measurements *in vitro* (Ryall et al., 2002).

To mimic the direction of skeletal muscle force development *in vivo*, the proximal end of the EDL muscle was tied to a micromanipulator, whereas the distal end was attached to the isometric force transducer (Research Grade 60-2999, Harvard Apparatus, South Natick, MA), which had been previously calibrated with weights of known mass. The muscle was contracted by field-stimulating platinum plate electrodes attached to a stimulator (Grass S11 stimulator, Quincy, MA) coupled to an amplifier (CE-1000, Crown Instruments, Elkhart, IN, USA) to ensure supramaximal stimulation, and thus maximal muscle fibre recruitment. Deflection of the transducer with a muscle contraction produces a measurable electric signal, proportional to the force produced. Electrical signals were converted to digital signal by Powerlab4510 (ADInstruments, Castle Hill, NSW, Australia) running Chart V5.0.2 for windows.

To prepare the muscle for the contractile protocol employed in this study, optimal muscle length (L_0), voltage and frequency was established in order to make comparisons with other

muscles. L_o was generated by a series of twitch contractions (0.2msec pulse duration) at slightly increased muscle lengths to establish where maximal twitch force (P_t) is produced. Once L_o was found, it was measured using a vernier caliper at the point at which the muscle is connected to its tendons.

Following L_o , a tetanic contraction at 100Hz (350msec train duration for EDL; 500msec for soleus) was then elicited to ensure the knots were secure and the muscle remained intact. Three minutes of recovery was allowed to prevent fatigue of the muscle. A single twitch was next elicited to ensure optimal force had been maintained. If not, optimal length was re-established until optimal force could be maintained.

To determine the optimal stimulation frequency that produced maximal tetanic force (P_o), muscle was stimulated tetanically at increasing frequencies ranging from 10Hz to 150Hz until a complete fused tetanus was performed, with a three minute recovery period (Goodman et al., 2009) between stimulations to ensure no muscle fatigue. This ensured the maximal effects of Calcium (Ca^{2+}) on tetanic force production. Optimal length, frequency were then used for the remainder of the test protocol.

2.4.2 Contractile Protocol

The contractile protocol began with measuring a series of three P_t from which the TTP and $1/2RT$ was also determined (see figure 2.0). TTP indicates the time taken for Ca^{2+} to be released from the sarcoplasmic reticulum, bind to troponin-C to allow actin and myosin filaments to engage in cross-bridge cycling. $1/2RT$ is the time it takes the muscle to reach half of its maximum force thus indicating how efficiently Ca^{2+} is resequenced into the sarcoplasmic reticulum by the Ca^{2+} -ATPase pump.

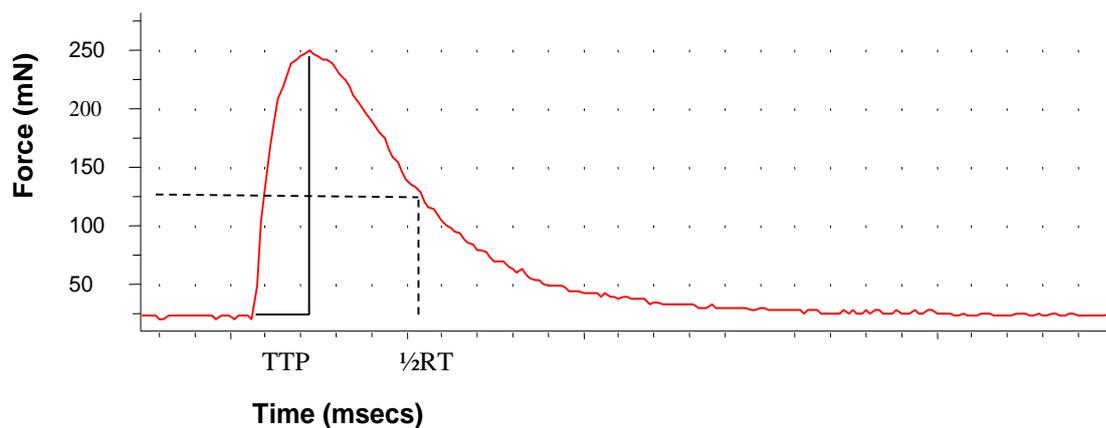


Figure 2.0 Example of Pt in skeletal muscle of ZDF rat indicating the measurements of TTP and $\frac{1}{2}RT$.

Following the twitch protocol, a fatigue and recovery protocol was next tested. The fatigue protocol utilised in this project involved 30Hz stimulations of 300ms duration every 2 seconds for 2.5 minutes. 30Hz was chosen as this mimics the physiological range (Grimby et al., 1981). Previous studies often allow fatigue protocols to run for 5 minutes (Cotter et al., 1993; van Lunteren, 1996; Warmington et al., 2000; McGuire et al., 2001), however upon initial testing, 5 minutes proved too damaging to ZDF rat skeletal muscle such that the recovery process would not occur. As such 2.5 minutes was sufficient to elicit fatigue from which recovery was still possible.

Recovery was measured at intervals of 30s, 1, 2, 5, 10, 15, 30, 45 and 60 minutes. Some studies have only looked at recovery after 5 minutes (Warmington et al., 2000; O'Neill et al., 2010). However given that muscle recovery can take anywhere from seconds to hours depending on the protocol employed (Jones, 1996; Bruton et al., 1998; Allen et al., 2008), more recent studies (Goodman et al., 2009) have looked at additional time points to ensure a more thorough investigation of the recovery process. As such, given no prior studies have measured fatigue and recovery in the ZDF rat, a more thorough investigation of recovery was

warranted and hence the same additional time points used in Goodman et al., (2009) were measured herein.

Once the contractile protocol was complete, muscles were removed from the bath and blotted dry on filter paper, the knots and tendons were removed, and muscle weighed on an analytical balance (OHAUS, Galaxy™ 160D, Ohaus Corporation, NJ, USA). The muscle was cut and half was snap frozen in liquid nitrogen, with the remainder embedded in Optimal Cutting Temperature (OCT) embedding medium (OCT compound, Cat. #4583, Scigen Scientific Gardena, CA 90248, USA) and snap frozen in isopentane pre-cooled in liquid nitrogen. All muscle samples were then stored at -80°C.

All forces were expressed relative to the muscle cross-sectional area allowing for comparisons between muscles of different area and length. Cross-sectional area (X-A) was calculated by the following formula:

$$X-A = \frac{\text{Muscle Mass}}{L_o \times \text{Fibre Length:Muscle Length ratio} \times \text{Density}}$$

The fibre length: muscle length ratio was previously established to be 0.44 in EDL and 0.71 in soleus) (Brooks and Faulkner, 1988), and density to be 1.06 g/cm³ (Close, 1972).

2.5 Sarcoplasmic Reticulum Function

2.5.1 Muscle Acquisition and Preparation

Once the contractile protocol outlined in section 2.4 was complete, the right Gastrocnemius muscle was removed from the anaesthetised rat and placed on a glass plate that was cooled on ice. The red (RG, slow-twitch) and white (WG, fast-twitch) portions of the muscle were

separated, dissected free from visible fat and connective tissue, and part of it was used for the SR Ca^{2+} uptake assay, with the remainder used for the MAPR assay (see section 2.6.1 for specific details on muscle preparation for Mitochondrial function).

For the SR uptake assay, each portion of RG and WG was weighed separately and $8\mu\text{L}$ homogenising solution per mg wet muscle was added. The homogenising solution consisted of homogenate buffer (40mM KCl, 40mM sodium HEPES and 250mM sucrose; pH 7.1); N,N,N',N'-tetrakis(2-pyridyl-methyl)ethylenedi-amine (TPEN) ($5\mu\text{M}$); sodium azide (10mM); and oxalate (5mM). After homogenisation with a motorised stick tissue homogeniser (Omni homogeniser, Japan) on ice using 3×10 second half power bursts and a 1×10 second full power burst, oxalate becomes sealed inside the vesicles and functions to mimic the intracellular calcium binding protein calsequestrin. It has also been found that upon brief mechanical homogenisation, membrane systems of most muscle tissues are able to spontaneously reform into functionally intact vesicles, primarily of SR type (Kargacin et al., 1988; Simonides and Van Hardeveld, 1990). This therefore permits the analysis of Ca^{2+} transport into and out of the SR.

The homogenate was stored on ice until the assay was performed (no more than 30 minutes). Upon completion of the assay for both muscles, samples were snap frozen in liquid nitrogen and stored for later use in protein determinations (refer section 2.7.3).

2.5.2 Use of Indo-1 in the SR Ca^{2+} Uptake Assay

The Ca^{2+} SR uptake assay in this experiment was similar to that detailed in Rybalka (2001) as modified from Warmington *et al.* (1996), however the fluorescent probe Indo-1 was used instead of Fura-2 and it was recorded on a Photon Technology International (PTI)

spectrophotometer and associated software (Felix32, PTI Version 1.10). The assay was performed in a cuvette housed in a sealed chamber with a 10mm injection port in the lid through which homogenate samples were introduced.

Indo-1 was excited at 338nm and the emitted light from the muscle homogenate was collected at 405 and 485nm. This provided ratiometric data points (100 per second), which were used for subsequent analysis. For all samples, the cuvette chamber was heated with circulated, temperature-regulated water at 37°C, and cuvette contents were constantly mixed via a magnetic stirrer.

Indo-1, a Ca^{2+} -sensitive fluorophore was used for the assay because of its ability to provide quantitative information about the Ca^{2+} concentration (Williams, 1995). This indicator has a specific emission spectrum for a given concentration of ionised Ca^{2+} , which results in the production of a distinct isosbestic point in the spectra (refer to figure 2.1). The isosbestic point is the point where fluorescence is independent of the Ca^{2+} ion concentration (Williams, 1995) and was determined in this study at ~457nm, which is very close to the 450nm isosbestic point stipulated by Molecular Probes (2001). The important aspect of dual excitation/emission dyes is that at either side of the isosbestic point, dye fluorescence increases and decreases with increasing calcium, respectively. This allows for the collection of ratiometric data to calculate ionic Ca^{2+} concentrations irrespective of dye concentration and distribution.

In addition, Indo-1 has a dissociation constant (K_d) that lies within the range of the physiological concentrations of calcium in skeletal muscle. Fluorescent dyes are able to

detect changes in ionic concentrations in the range $0.1 \times K_d - 10 \times K_d$ which means that Indo-1 is capable of measuring Ca^{2+} concentrations usually observed in skeletal muscle function.

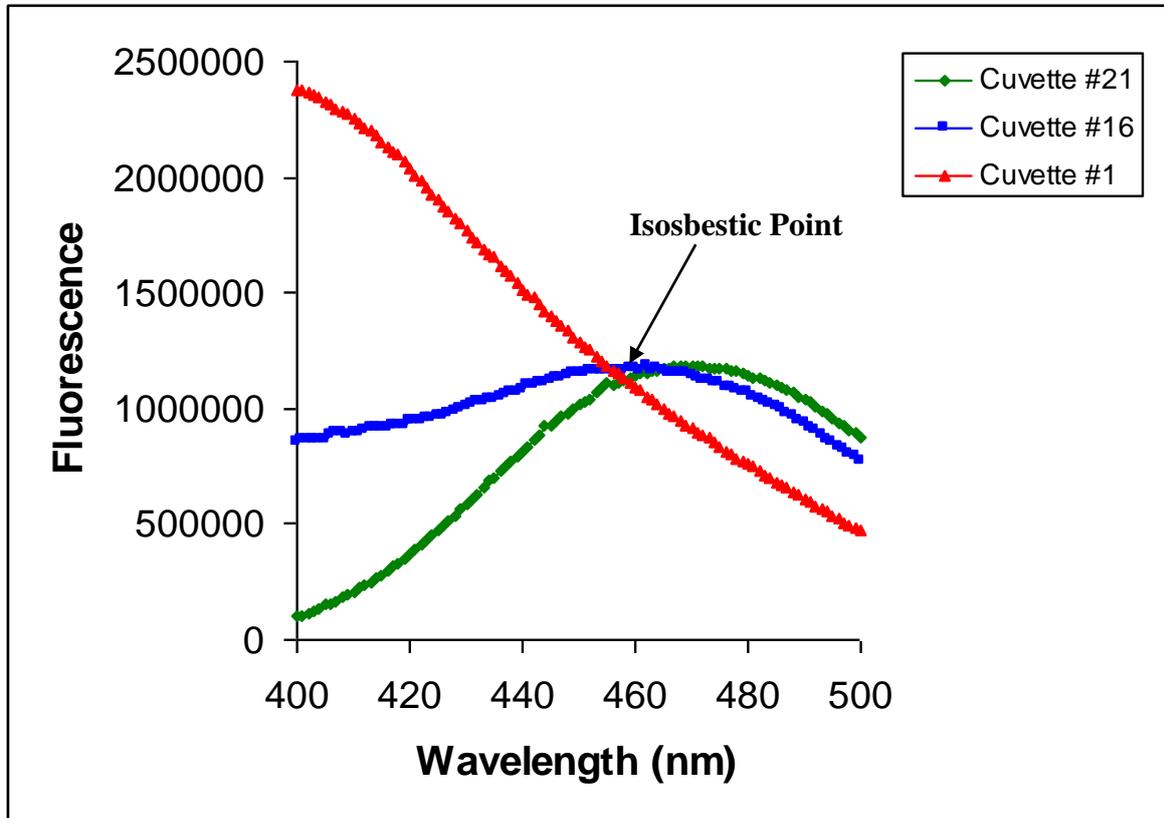


Figure 2.1 The Ca^{2+} Dependent Fluorescence Emission Spectra of Indo-1. The isosbestic point was found to be $\sim 457\text{nm}$.

2.5.3 Calibration and K_d of Indo-1

Given that the response of Indo-1 must be accurately known over a range of Ca^{2+} concentrations at which emission can be measured, the K_d (dissociation constant for Ca^{2+} binding to Indo-1) of the fluorophore was determined by the calibration technique described in Warmington *et al* (1996). Calibration of the dye is required because the biochemical environment may affect the properties of fluorophores, especially the ionic strength (Williams and Fay, 1990) and the protein binding characteristics (Baker *et al.*, 1994).

Freshly prepared buffers (A & B) both containing 40mM KCl, 40mM NaHEPES, 10mM ethyleneglycolbis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) and 7.5 μ M Indo-1 were used in the calibration process. Buffer A also contained 10mM CaCl₂ whereas Buffer B did not. A series of standards comprising known, variable Ca²⁺ concentrations were prepared via the combination of Buffers A and B in specific proportions. Free [Ca²⁺] were calculated (refer to Table 4.0). using the following equation:

$$\text{Free [Ca}^{2+}] = C_d \times \frac{[\text{Ca}^{2+}\text{-EGTA}]}{[\text{EGTA}]} \times \frac{\text{Volume Buffer B}}{\text{Volume Buffer A}},$$

where C_d is the dissociation constant of [Ca²⁺-EGTA] = 200nM

$$\rightarrow \text{Free [Ca}^{2+}] = 200\text{nM} \times \frac{\text{Volume Buffer B}}{\text{Volume Buffer A}}$$

The Indo-1 fluorescence of each standard was measured at both 405 and 485nm and the relative fluorescence of each standard was calculated using the following equation:

$$\text{Rel. Fluor.} = \log_{10} \left[\frac{(\text{Fluorescence} - \text{Fluorescence}_{\min})}{(\text{Fluorescence}_{\max} - \text{Fluorescence})} \right]$$

The relative fluorescence was plotted as a function of solution pCa(-log free [Ca²⁺]), and by use of linear slope equations of the relative fluorescence at 405 and 485nm wavelengths (obtained from the pCa graph in Figure 2.3), a K_d of 356.45nm for Indo-1 was determined (Refer to Appendix A for calculations). The resultant K_d was used in all calculations of SR Ca²⁺ uptake rates.

Table 2.0 Calibration Cuvette Constituents and Associated Free Ca²⁺ Concentrations

Calibration Cuvette #	Volume Buffer A (mL)	Volume Buffer B (mL)	Free [Ca ²⁺] (nM)
1	0	2.0	
2	0.1	1.9	3.80
3	0.2	1.8	1.80
4	0.3	1.7	1.13
5	0.4	1.6	0.80
6	0.5	1.5	0.60
7	0.6	1.4	0.47
8	0.7	1.3	0.37
9	0.8	1.2	0.30
10	0.9	1.1	0.24
11	1.0	1.0	0.20
12	1.1	0.9	0.16
13	1.2	0.8	0.13
14	1.3	0.7	0.11
15	1.4	0.6	0.09
16	1.5	0.5	0.07
17	1.6	0.4	0.05
18	1.7	0.3	0.04
19	1.8	0.2	0.02
20	1.9	0.1	0.01
21	2.0	0	0.00

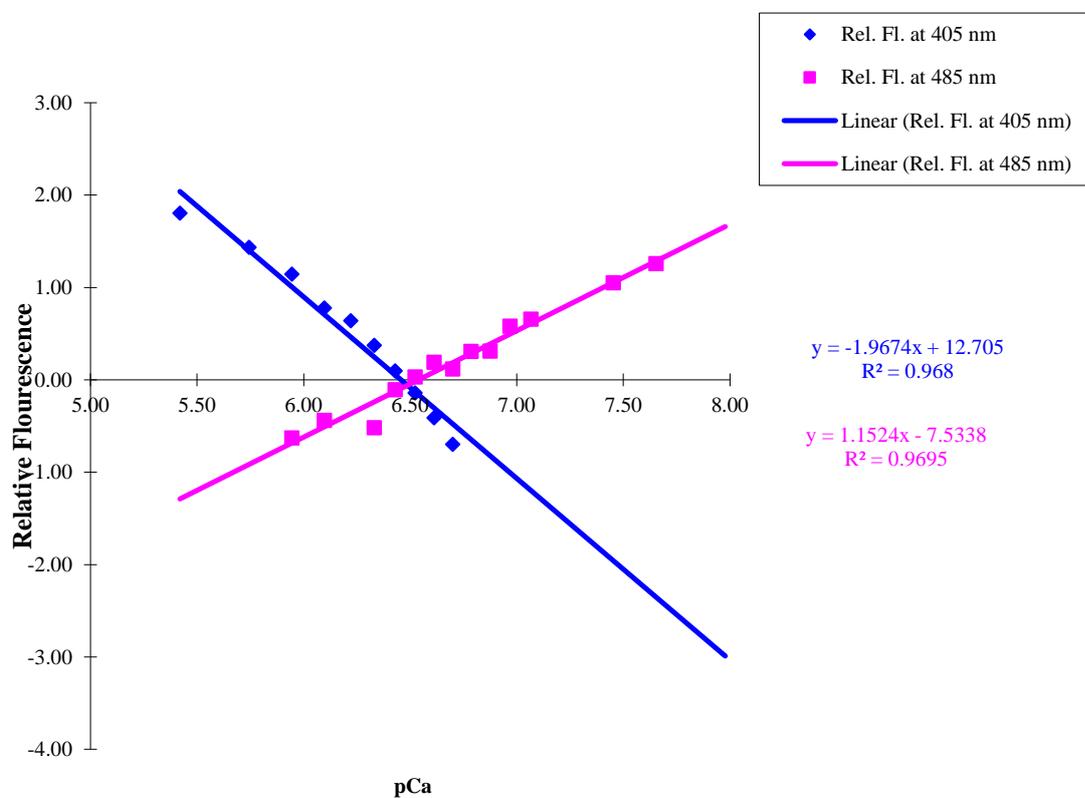


Figure 2.2 Determination of K_d: pCa vs Relative Fluorescence.

2.5.4 SR Assay Procedure

Each cuvette contained 1.9mL of assay solution and was placed in the 37°C-regulated chamber, where it was stirred constantly. The assay solution comprised of the assay buffer (40mM KCl & 40mM sodium HEPES; pH 7.1); substrate (5mM MgATP + 25mM PCr + 0.25mM ADP), and 10µM CaCl₂. The above solutions were added to each cuvette, before the addition of 15µL of 7.5µM Indo-1, which was then allowed to combine for approximately 20 – 30 seconds. Basal fluorescence was recorded for 10 seconds.

The assay was initiated via injection of 100µL homogenate sample into the cuvette, and the decreasing fluorescence ratio signal (indicative of Ca²⁺ uptake) was monitored and recorded continuously for 170 seconds (Refer to figure 2.3). At that point, the specificity of the assay for Ca²⁺-ATPase activity was proven via the addition of 1µM thapsigargin (a Ca²⁺-ATPase pump inhibitor (Xu et al., 1997)). In the presence of thapsigargin, no further Ca²⁺ uptake occurs. It was then possible to measure any Ca²⁺ leakage from the SR. After a further 30 seconds, 20µL of 0.5M 4-Chloro-M-Cresol (4CMC) was added to induce Ca²⁺ release. 4CMC was used because it can directly activate the luminal side of the calcium release channel (Herrmann-Frank et al., 1996), with a 10 – 25-fold higher potency compared with caffeine (Zorzato et al., 1993).

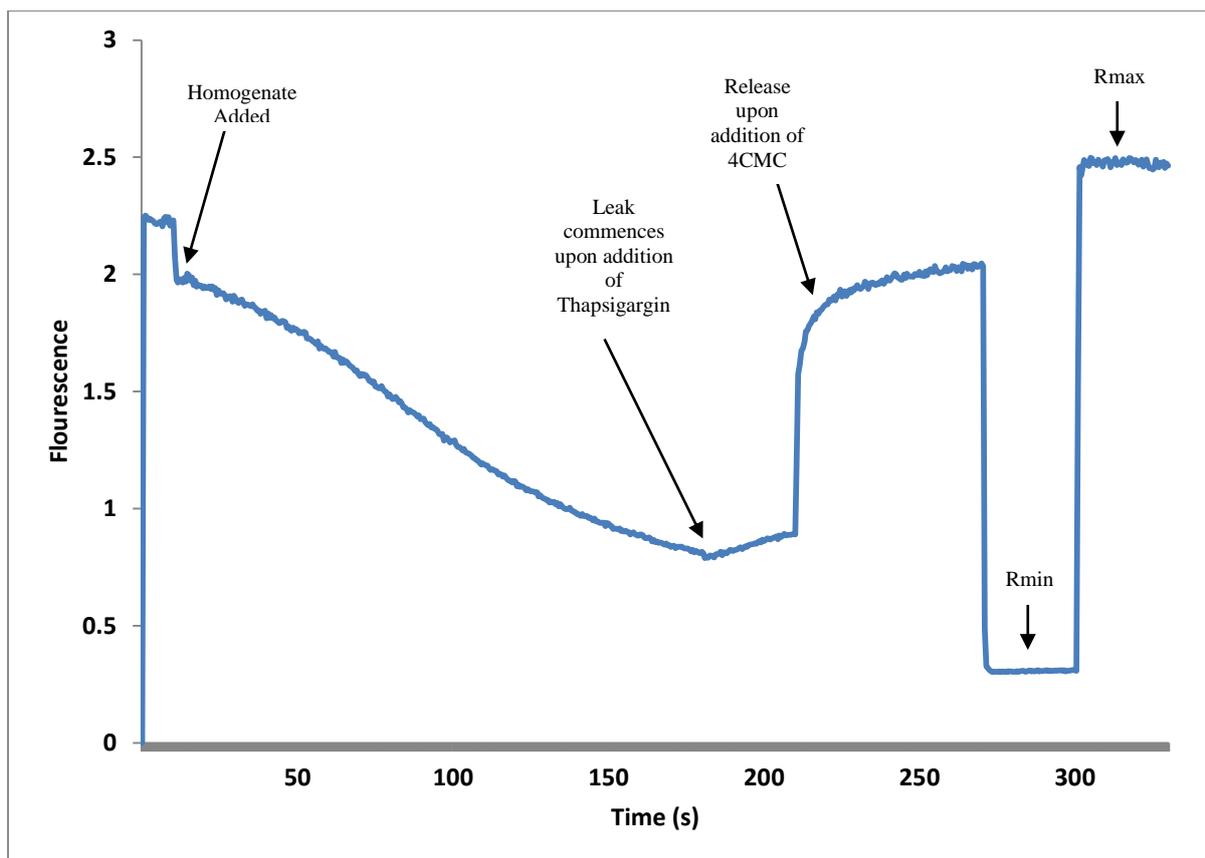


Figure 2.3 Indo-1 Fluorescence Ratio of the SR Ca²⁺ Uptake Assay in Rat Muscle. As Ca²⁺ is sequestered into the SR, extra-vesicular Indo-1 fluorescence ratio decreases. Conversely, the fluorescence ratio increases as Ca²⁺ leaks or is released from the SR.

2.5.5 Estimation of *R_{min}* and *R_{max}*

After 270 seconds, each assay was “calibrated” by adding 20μL of 0.1M EGTA, which gives the minimum fluorescence ratio (*R_{min}*). After a further 30 seconds 20μL of 0.25M CaCl₂ was added to determine the maximum fluorescence ratio (*R_{max}*). These solutions reduce [Ca²⁺] below 1nM allowing the determination of *R_{min}* and increase [Ca²⁺] to millimolar levels allowing the determination of *R_{max}*, respectively. These values were then used to convert the ratio values for individual Ca²⁺ uptake curves to [Ca²⁺].

At the conclusion of the assay, the remaining homogenate sample was snap frozen in liquid nitrogen and stored at -80°C in the ultrafreezer described in section 2.4.2, for later analysis of proteins.

2.5.6 Calculation of Ca²⁺ Uptake Rates

Ca²⁺ uptake rates were calculated for all homogenate samples by converting ratio values derived from the uptake trace after addition of homogenate, to changes in Ca²⁺ concentration using the standard calibration equation described by Grynkiewicz *et al.* (1985):

$$[Ca^{2+}] = [(R - R_{min}) / (R_{max} - R)] \times K_d \times \beta$$

Where: R = the experimental ratio value
R_{min} = the minimum ratio value
R_{max} = the maximum ratio value
K_d = the binding constant for Indo-1 & Ca²⁺; and
β = ratio of (405nm fluorescence R_{min}/405nm fluorescence R_{max})

The rate of [Ca²⁺] change was determined and Ca²⁺ uptake, leak and release calculated for each second of the assay, thereby allowing for the calculation of fastest uptake, leak and release rates in nmol Ca²⁺.min⁻¹. The total change in [Ca²⁺] was determined for the initial 10 seconds of the assay after the homogenate was added, and in the first 10 seconds after addition of 4CMC and thapsigargin so that the fastest possible rate could be calculated for each of uptake, leak and release respectively. Average rates were also determined across the duration of each of uptake, leak and release rate components.

2.6 Mitochondrial Function

2.6.1 Muscle Acquisition and Preparation of Mitochondrial Suspension

As discussed in section 2.5.1, a portion of both RG and WG muscles were used for assessment of mitochondrial function. Approximately 10mg of this muscle portion was weighed into a cryule tube and snap frozen in liquid nitrogen for later analysis of Citrate

Synthase (CS) Total. For both red and white portions, the sample was initially homogenised by finely chopping it with a scalpel, then further mincing it in a glass mortar in which approximately 1mL of homogenising solution was added, herein referred to as Solution A (containing 100mM KCl, 50mM TRIS, 5mM MgCl₂.H₂O, 1.8 mM ATP, and 1.0 mM EDTA, pH 7.2). This homogenate was used to extract the mitochondria for this assay. The homogenate sample was centrifuged for 3 minutes at 650G and 4°C, after which the pellet was discarded and the supernatant recentrifuged for a further 3 minutes at 15000G at 4°C. The new pellet formed, containing the mitochondria, was resuspended in exactly 1mL of Solution A and re-centrifuged at 15000G for 3 minutes at 4°C. The supernatant was again discarded and 200µL of a Solution B (containing 180.0 mM Sucrose, 35.0 mM KH₂PO₄, 5.0 mM MgAcetate, and 1.0 mM EDTA, pH 7.5) was then added to the pellet containing the mitochondria, which was resuspended and placed on ice immediately, and was the mitochondrial suspension (MS) used for the MAPR analysis.

A 20mg sample of muscle from which MAPR was performed (snap frozen for “CS Total” activity) and MS was stored for later CS analysis. A “CS Before” sample tube comprised of 50µL of MS plus 150µL Solution B; “CS After” tube contained 50µL of MS plus 150µL CS Homogenising Buffer containing 1% Triton X. CS measurements (as described in more detail in section 2.6.4) can be used to determine the (percentage) yield of mitochondria from the whole muscle. From the yield of mitochondria it was possible to calculate MAPR per weight of muscle. Mitochondrial yield is determined from the equation:

$$\text{Yield (\%)} = \frac{\text{CS}_{\text{after}} - \text{CS}_{\text{before}}}{\text{CS}_{\text{total}}} \times 100$$

For future protein determinations, 50µL of MS was added to 200µL Solution B.

2.6.2 Preparation of Solutions for Mitochondrial ATP Production Rate (MAPR) Assay

Firstly, various substrate combinations were prepared for analysis of the three metabolic pathways used for ATP production i.e. pyruvate and malate for carbohydrate metabolism, palmitoyl carnitine for lipid metabolism, and α -Ketoglutarate for protein metabolism. In addition, succinate and rotenone was used to test the functions of complex 4 in the electron transport chain. These were prepared in the same concentrations and combinations stipulated in Wibom and Hultman (1990). These were: Pyruvate (1.0mM) + Malate (1.0mM) (P+M); Palmitoyl Carnitine (0.005mM) + Malate (1.0mM) (PC+M); α -Ketoglutarate (10mM) (α -KG); Succinate (20mM) + Rotenone (0.1mM) (S+R); Blank (containing no substrates); and Glutamate(15mM) + Succinate (15mM) (G+S). In addition, a substrate combination of all three major metabolic pathways was also prepared; Pyruvate (1.0mM) + Palmitoyl Carnitine (0.005mM) + α -Ketoglutarate (10mM) + Malate (1.0mM) (PPKM). Each substrate combination also contained 0.04mM ADP. The substrate combinations listed were stored on ice and in the dark until analysis. Other solutions required for the assay were a 20 μ M ATP Standard, AMRS Monitoring Reagent containing 18.4mL Solution C (comprising of 1.0mM $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$, 10.0mM MgAcetate, 1.0mg/ml Bovine Serum Albumin, 180mM Sucrose, 35mM KH_2PO_4 and 1.0mM EDTA, pH 7.5 with KOH) + 1600 μ L ATP Firefly Luciferase (ATP-FL-AAM). The final mitochondrial suspension used for the assay contained 490 μ L AMRS plus 10 μ L from the protein tube which had 50 μ L of MS plus 200 μ L of Solution B.

2.6.3 MAPR Assay Procedure

MAPR was determined with the luminometric method based on firefly luciferase (Wibom and Hultman, 1990; Wibom et al., 1990; Wibom et al., 2002). This involves the emission of light from firefly luciferase where the degree of luminescence by firefly luciferase is proportional to the ATP concentration (Lundin and Thore, 1975). 200 μ L of each substrate

was added to appropriate wells of a 96-well microplate. This was inserted into a fluorimeter (Fluoroskan Ascent FL, Labsystems, Finland) which read the plate after being incubated for five minutes, using Ascent Research Edition Software package (Version 2.2.6). After the first reading was complete, 20 μ L of ATP Standard was added to each well of the plate and the program restarted. Once complete, the final step involved adding 10 μ L of the final MS into the wells. The ATP production was monitored by the fluorescence derived from the firefly luciferase and quantified by comparisons with the fluorescence observed by the addition of 20 μ L of ATP. All substrate combinations were measured in triplicate.

2.6.4 Citrate Synthase Analysis

CS was determined in both muscle tissue and mitochondrial suspension as described by (Rybalka et al., 2014) . This allowed for the determination of mitochondrial yield for correction of MAPR. Mitochondrial yield was calculated using the data from the proportion of CS activity in the “CS Before”, “CS After”, and “CS Total” samples. CS Before activity is that contained in the original MS extract. CS After activity involves fracturing and disintegrating the mitochondrial membranes and thus CS liberation. CS Total activity was measured in a separate snap-frozen sample of the same muscle on which MAPR was performed after mechanical homogenisation and complete disintegration of sub-cellular structures.

The solutions used for each of the CS methods included: Tris Buffer (containing 3.03g Tris[hydroxymethyl] aminomethane plus 3.94g of Tris[hhydroxymethyl]aminomethand hydrochloride per 500mL, pH 8.3); DTNB (containing 3.96mg of Ellman’s reagent per 10mL Tris buffer); 3mM Acetyl-Coenzyme A; 10mM Oxaloacetate; CS Homogenising Reagent (containing 1.305g KCl plus 0.074g EDTA per 100mL, pH 7.4); and 1% vol/vol Triton X.

2.6.5 CS Assay Procedure

The CS Before samples were thawed and used for the procedure, whereas the CS After samples underwent three freeze/thaw cycles (in liquid nitrogen/25°C water bath, respectively). The CS Total samples were defrosted and weighed into a cryule. 50µL of homogenising solution for every mg muscle weight was added to this tube. The muscle sample was homogenised using an OMNI tissue homogeniser (OMNI, Japan) with 3x15 second medium power bursts, followed by 1x15 second full power burst. A spectrophotometer (X-mark microplate reader (Bio-Rad Laboratories, Australia) was set at 412nm for all CS analyses, and the following solutions added to each well of a 96-well plate: 165µL Tris Buffer, 40µL Acetyl-Coenzyme A, 15µL oxaloacetate and 25µL DTNB. The 96-well plate was incubated at 25°C in the spectrophotometer for 2 minutes, then a blank reading recorded for 2 minutes. After the blank reading, 35µL of the CS Before sample was added, whereas for CS After, 10 µL of sample was added and for CS Total, 5µL of sample was added to the wells. The change in CS activity over time was read for a further 3 minutes.

2.7 Protein Determination

The amount of contractile, SR, mitochondrial and total proteins were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Australia, Product #23225). This enabled the conversion of Ca²⁺ uptake rates from nmol/min/mg muscle to nmol/min/µg protein as well as determine the percentage of mitochondrial and contractile protein of the total protein content.

2.7.1 Tissue Preparation for Contractile and Total Protein

Tissue preparation for contractile and total proteins in EDL and Soleus were performed according to the methods described by Beitzel et al. (2004). A 20mg portion of EDL and

Soleus muscle was weighed and all visible fat and connective removed. The muscle was allowed to defrost on a petri dish sitting on ice, and minced with a scalpel blade. The minced muscle was transferred to a glass mortar cooled in ice. 1mL of ice cold buffer containing 50mM KCl, 10mM KH₂PO₄, 2mM MgCl₂.6H₂O, 0.5mM EDTA and 2mM DTT (1:50 wet weight:volume dilution). Muscle tissue was homogenised in the glass mortar and pestle until no visible fragment of muscle remained. The homogenate was transferred to an eppendorf centrifuge tube which was stored on ice. 200uL of crude homogenate was set aside for total protein determination, which was then diluted a further 1:20 to be within the range of the standard curve. The remaining homogenate was centrifuged (Heraeus megafuge 40R centrifuge, Catalogue #75004518, Thermofisher Scientific, Australia) at 1000G for 10 minutes at 5°C. The supernatant containing cytosolic proteins was discarded and the pellet containing the contractile proteins was resuspended in 200uL of the ice-cold buffer using the tip of a glass pasteur pipette until no visible lumps appeared, ensuring no air bubbles were introduced to the sample. The sample was then snap frozen in liquid nitrogen and stored at -80°C for later assessment of contractile proteins.

2.7.2 Tissue Preparation for SR and Total Proteins

To determine the total protein concentration in RG and WG, the frozen homogenates described in section 2.5.1 were thawed on ice and diluted a further 1:6.25 with milli-Q water giving a total 1:50 dilution. 200uL of this crude homogenate was set aside for analysis of total proteins.

The remaining homogenate was used to isolate SR proteins according to the methods of Balagopal et al. (1997). The homogenate was centrifuged at 600G for 3 minutes at 4°C (Heraeus megafuge 40R centrifuge, Cat. #75004518, Thermofisher Scientific, Australia). The

supernatant containing SR proteins was decanted and the remaining pellet discarded. The supernatant was centrifuged again at 7000G for 3 minutes at 4°C, the supernatant collected and pellet discarded. The supernatant was then transferred to an ultra-centrifuge tube and centrifuged at 100,000G for 60 minutes at 4°C (Sorvall MTX150 micro-ultracentrifuge, Model 46963, Asheville, USA). The supernatant once again collected and pellet discarded. 1ml of 1M perchloric acid was added and sample was vortexed. This was recentrifuged at 1500G for 3 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 1ml of 1M perchloric acid. This suspension was centrifuged at 1500G for 3 minutes at 4°C, the supernatant decanted and discarded and the pellet resuspended in 200uL of buffer.

2.7.3 Tissue Preparation for Mitochondrial Proteins

The method according to Wibom et al. (1991) was used to prepare the tissue for mitochondrial proteins in both RG and WG muscle samples as described in section 2.6.2. These frozen samples were thawed on ice and used in the Pierce BCA protein assay.

2.7.4 Pierce BCA Protein Assay Procedure

All isolated and crude samples were thawed and diluted a further 1:8 with Milli-Q water. A series of Albumin standards (BSA) were diluted from a stock solution of 2mg/mL within the range of the standard curve (20-2000ug/mL) using the same diluent as each sample. The BCA working reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). 25uL of each standard or sample replicate were pipetted into a clear 96-well microplate and 200uL of the working reagent added to each well. The plate was mixed thoroughly on a plate shaker for 30 seconds, covered and incubated at 37° for 30 minutes. The plate was allowed to cool to room temperature and absorbance of

samples and standards was read at 562nm on a spectrophotometric plate reader (X-mark microplate reader, Bio-Rad Laboratories, Australia).

A standard curve was prepared using the average spectral reading of each standard solution (refer to Figure 2.4), and the derived slope equation used to determine the protein content of all samples.

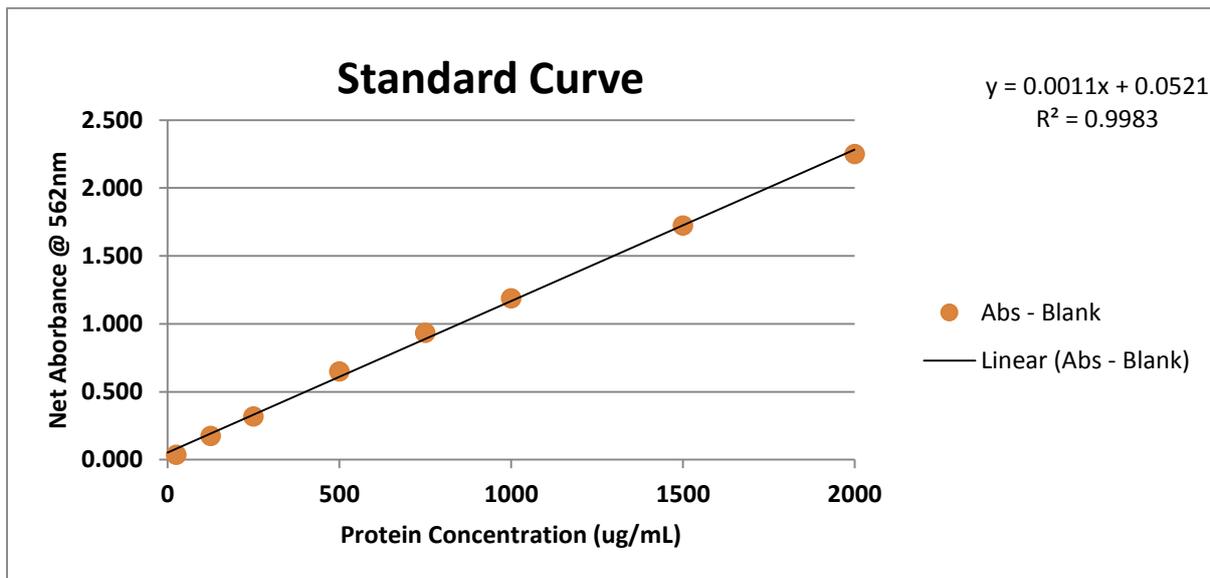


Figure 2.4 Protein Standard Curve.

2.8 Metabolites

Metabolite concentrations (PCr, creatine (Cr), and ATP) of all snap frozen rat muscle samples were analysed to determine creatine loading and/or changes in ATP/PCr/Cr concentrations for all groups.

2.8.1 Freeze Drying Samples

Muscle samples were removed from the ultrafreezer (-80°C) and trimmed in a cooled (-25°C) cryostat using a scalpel. Approximately 5mg of dry muscle is required for the extraction process, and since the typical dry weight: wet weight ratio is 1:4 (23%), approximately 20mg of wet weight muscle was weighed into cryules. Holes were punctured into cryule lids, and

capped tubes were placed into a pre-cooled freeze dryer (-40°C) (Edwards Modulyo, Edwards High Vacuum, Britain, England). Samples were freeze-dried for a minimum of 36 hours.

At the completion of freeze-drying, muscle sample dry weight was determined, then sample was transferred to a desiccator at room temperature (20-23°C). The dry weight: wet weight ratio was then calculated. The benefit of freeze-drying is that it allows samples to be successfully handled at room temperature afterwards.

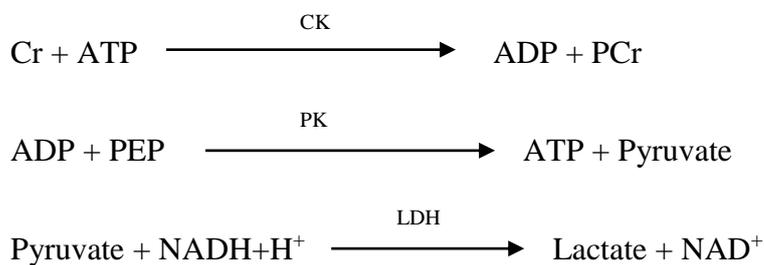
2.8.2 Metabolite Extraction

Samples were removed from the dessicator, and crushed to a fine powder whereby visible blood and connective tissue were removed. 2mg of powdered muscle was weighed into an eppendorf tube and stored in desiccant until metabolite extraction. The extraction protocol was done in accordance to Harris et al. (1974). It was initiated by addition of 250µL of ice cold 0.5M perchloric acid (PCA)/1mM ethylenediaminetetra-acetic acid (EDTA) to powdered samples. The suspension was then vortexed and tapped repeatedly for 10 minutes to ensure appropriate mixing and removal of fibres from the vessel wall. Samples were centrifuged at 28,000rpm at 0°C for 2 minutes. Eppendorf tubes were returned to ice and 200µL of the supernatant was removed without disturbing the pellet, and placed into a second set of eppendorf tubes and the pellet discarded. 50µL of 2.1M ice cold KHCO_3^- was added to each eppendorf containing the supernatant and left to stand on ice for 5 minutes. Samples were re-centrifuged at 28,000rpm for 2 minutes at 0°C. Supernatant was removed with plastic pasteur pipettes, placed into labelled cryules and stored in an ultra-freezer (-80°C) until subsequent metabolite analysis of ATP, PCr and Cr.

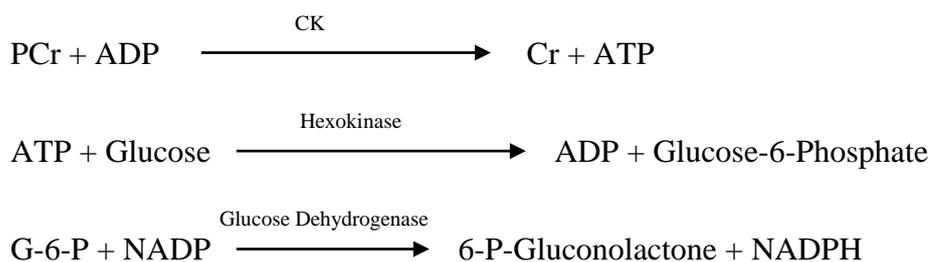
2.8.3 ATP, PCr and Creatine Analysis

Muscle extracts were analysed for ATP, PCr and Creatine content using enzymatic techniques with fluorimetric detection (Fluoroskan Ascent FL, Labsystems, Finland) of a range of standards of known metabolite concentrations as described by Lowry and Passonneau (1972).

Theoretically, muscle Cr is determined using the following equations:



A cocktail containing all of the above reagents (except for CK) was added to all the wells of a white-96-well microplate. Blanks, standards and muscle extracts (in triplicate) were added to the wells of microplate plate. An initial reading (R1) was taken at 340nm, which gave fluorescence due to the starting concentration of NADH in the cocktail. The conversion of creatine to lactate (with the oxidation of NADH to NAD⁺) was then initiated with the addition of CK. The 96-well plate was left to incubate for one hour in the dark at room temperature, and a final fluorescence reading was taken (R2). The difference between R1 and R2 was due to the reduction of NADH+H⁺, and is proportionate to the concentration of creatine in the initial sample. Similarly, ATP and PCr content were determined using the following reactions:



A cocktail containing all of the above reagents (minus the hexokinase and CK) was added to all the wells of a white 96-well microplate. The initial reading (R1) was taken at 340nm, and the reaction converting ATP to 6-Phosphate-Gluconolactone was initiated via addition of a dilute hexokinase solution. The plate was then incubated in the dark at room temperature for 30 minutes, and a second reading (R2) was taken. The difference between R2 and R1 was due to the production of NADPH and was proportional to the concentration of ATP in the initial sample. Subsequently, a solution containing ADP and CK was added to all well of the plate, which were then incubated for 60 minutes, after which a third reading (R3) was taken. The difference between R3 and R2 was due to further production of NADPH+H⁺ and was proportional to the PCr concentration in the initial sample.

2.9 Cytokine Analysis

2.9.1 Skeletal Muscle Cytokine Extraction Protocol

EDL and Soleus muscles were removed as per section 2.4.1, with the “non-contracted” side utilised for cytokine analysis. The cytokine extraction protocol was done in accordance with Cooke et al. (2011). Approximately 20 mg of muscle was cut from the sample, weighed, and subsequently placed in an autoclaved microcentrifuge tube. The skeletal muscle samples were homogenized using a commercial cell extraction buffer (Catalogue #FNN0011, Biosource International, Camarillo, CA) and a hand held Kontes tissue homogenizer. The cell extraction buffer was supplemented with 1mM phenylmethanesulphonylfluoride (PMSF) and a protease inhibitor cocktail (Cat. P2714, Sigma Chemical Company, St. Louis, MO). A 0.3 M PMSF stock solution in dimethyl sulfoxide (DMSO) was produced. 17µL of this stock solution was added to 5mL of cell extraction buffer to create a final concentration of 1mM PMSF. Based on the manufacture, the protease inhibitor cocktail that was utilised was comprised of a

combination of water-soluble protease inhibitors with broad specificity for the inhibition of serine, cysteine, and metallo-proteases. Per the manufacture's recommendations, 500 μ L of protease inhibitor cocktail was added to 5mL of cell extraction buffer. As a result of the unstable nature of PMSF, this solution was both created and added to the cell extraction buffer immediately before the process of muscle homogenization.

Cell extraction buffer was added to each frozen muscle sample at a ratio of 20.8mL per gram of muscle tissue. Muscle tissue was homogenized via a hand held Kontes pestle in an autoclaved microcentrifuge tube, and the mixture was vortexed and incubated on ice for 30 minutes, with occasional vortexing.

Lysates were clarified by centrifugation at 14,000 rpm (13,000 x g) at 4°C for 10 minutes. Clarified cell extracts were transferred to clean microcentrifuge tubes and stored at -80°C until ready for analysis.

2.9.2 Skeletal Muscle and Plasma Cytokine Sample Preparation

Skeletal muscle extracts obtained in Section 2.9.1 and plasma samples obtained in section 2.2 were thawed on ice and 120 μ L aliquoted and diluted 1:2 in Diluent Buffer and Incubation Buffer, respectively, from the Rat TNF- α Elisa kit (Catalogue #KRC3011, Invitrogen Corporation, Camarillo, CA). Samples were mixed thoroughly before loading into the microtiter wells. A further 120 μ L of muscle extract and plasma was aliquoted and diluted 1:2 in Standard Diluent Buffer from the Rat IL-6 Elisa kit (Catalogue #KRC0061, Invitrogen Corporation, Camarillo, CA). The remaining plasma sample was snap frozen and stored at -80°C for later analysis of Insulin and Glucose.

2.9.3 Cytokine Analysis – Il-6

Muscle and plasma Il-6 was determined using a rat Enzyme Linked Immuno-Sorbent Assay (ELISA) kit (Invitrogen catalogue #KRC0061, Camarillo, CA), whereby a monoclonal antibody specific for Rat Il-6 has been coated onto the wells of the microtitre strips.

2.9.4 Preparation of Il-6 ELISA Kit Solutions

The Wash Buffer Concentrate (25X) was allowed to reach room temperature and mixed to ensure that any precipitated salts have redissolved. This was diluted in one volume of the Wash Buffer Concentrate (25X) with 24 volumes of deionized water.

A set of Rat Il-6 standards were prepared by firstly reconstituting a stock standard Il-6 to 7,500pg/ml using the Standard Diluent Buffer. Once mixed and allowed to stand for 10 minutes to ensure complete reconstitution, 0.15mL of the reconstituted standard was added to a tube containing 0.6mL Standard Diluent Buffer to produce 1500 pg/ml Rat Il-6, and mixed well. Serial dilutions (1:2) of the 1500pg/mL standard were made to produce further standards of 750, 375, 187.5, 93.8, 46.9, and 23.5pg/mL Rat Il-6. Standard Diluent Buffer was used as the 0pg/mL blank.

Finally, the enzyme Streptavidin-HRP (100X) (50% glycerol; 3.3mM thymol) was brought to room temperature, gently mixed and 15 minutes prior to use, diluted by adding 10 µl of the 100X concentrated solution with 1 ml of Streptavidin-HRP Diluent for each 8-well strip used in the assay. This was labelled as the Streptavidin-HRP Working Solution.

2.9.5 Il-6 ELISA Assay Protocol

All reagents were brought to room temperature before use, and gently mixed prior to use. The number of 8-well strips needed for the assay was determined and inserted into the frame(s) of the microtitre plate – extra strips were rebagged and stored in the refrigerator for future use. All standards and samples were analysed in duplicates. To the zero standard wells 100µL of the Standard Diluent Buffer was added. Well(s) reserved for chromogen blank were left empty.

To all wells, 50µL of Incubation Buffer (containing 0.05% sodium azide) was added. To appropriate wells, 100µL of pre-diluted standards, plasma and muscle tissue samples were added. Rat Il-6 High and Low Controls (lyophilized Rat recombinant Il-6 containing 0.1% sodium azide and reconstituted as per manufacturer's instructions) were also added to appropriate wells. All wells were mixed on a plate shaker contained in an X-mark microplate reader (Bio-Rad Laboratories, Australia). The plate was covered and incubated for 2 hours at 37°C. This allows the Il-6 to bind to the monoclonal antibody coated on the walls of the wells. After incubation, the solution was aspirated from the wells and discarded. Wells were washed by adding 0.4mL of the diluted wash buffer, allowed to soak for 15-30 seconds, then aspirated and discarded. This was repeated 4 times after which the plate was inverted and tapped dry on absorbent tissue.

To each well except the blanks, 100µL of the secondary antibody (biotinylated Rat Il-6 Biotin Conjugate solution) was added. The plate was covered and incubated for 1.5 hours at room temperature to allow binding of the secondary antibody. This was then aspirated and

discarded from wells. Wells were washed 4 times as mentioned above, after the first incubation period.

The enzyme Streptavidin-HRP Working Solution was made up as per section 2.6.3.1. 100 μ L of this working Solution was added to each well except the chromogen blanks. The plate covered and allowed to incubate for a further 30 minutes at room temperature to allow the enzyme to bind to the secondary antibody. Following incubation, wells were aspirated and washed a further 4 times to remove any unbound enzyme.

Following washing, 100 μ L of a substrate solution referred to as the Stabilized Chromagen (containing tetramethylbenzidine (TMB)) was added to each well. This substrate is acted upon by the bound enzyme to produce a blue colour. This is incubated for 30 minutes at room temperature and in the dark. Finally, 100 μ L of Stop Solution was added to each well. The plate was gently mixed by tapping the sides gently. The solution in the wells then turned yellow. The absorbance of each well was read at 450nm using an X-mark microplate reader (Bio-Rad Laboratories, Australia) which had previously been blanked against a chromogen blank composed of 100 μ L each of Stabilized Chromogen and Stop Solution. The plate was read within 30 minutes of adding the Stop Solution.

Using curve fitting software (MyAssays Analysis Software) a standard curve was generated using a four parameter algorithm to provide the best standard curve fit (see Figure 2.5). The concentrations for unknown samples and controls were determined from the standard curve and multiplied by 2 to correct for the overall 1:2 dilution during sample preparation.

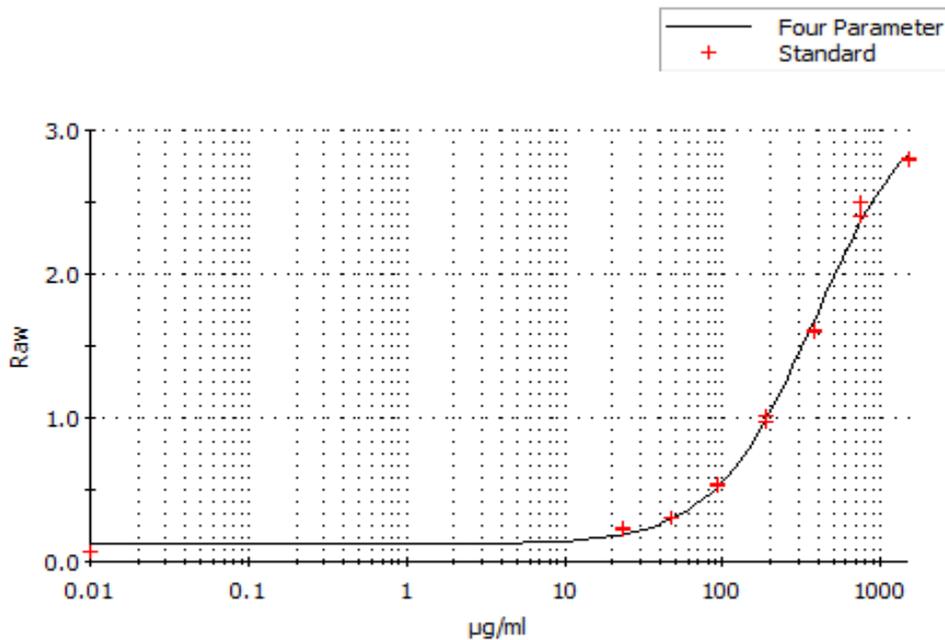


Figure 2.5. Standard Curve for IL-6 ELISA.

2.9.6 Cytokine Analysis – TNF- α

Muscle and plasma TNF- α was determined using a rat Enzyme Linked Immuno-Sorbent Assay (ELISA) kit (Invitrogen catalogue #KRC3011, Camarillo, CA), whereby a monoclonal antibody specific for Rat TNF- α has been coated onto the wells of the microtitre strips.

2.9.7 Preparation of TNF- α ELISA Kit Solutions

Samples were thawed on ice, a portion of plasma (120uL) was diluted 1:2 with Incubation Buffer, whereas muscle samples were diluted 1:2 with Standard Diluent Buffer (0.1% sodium azide) and mixed well prior to analysis.

The Wash Buffer Concentrate (25X) and Streptavidin-HRP Concentrate (100X) was prepared as in section 2.9.4. The standards were also prepared utilising the method outlined in the

latter mentioned section, however a stock standard of TNF- α was reconstituted to 2,000pg/ml with Standard Diluent Buffer as per manufacturer's instructions. Once mixed and allowed to stand for 10 minutes to ensure complete reconstitution, 0.225mL of the reconstituted standard was added to a tube containing 0.375mL Standard Diluent Buffer to produce 750pg/ml Rat TNF- α , and mixed well. Serial dilutions (1:2) of the 750pg/mL standard were made to produce further standards of 375, 187.5, 93.8, 46.9, 23.4, and 11.7pg/mL Rat Il-6. Standard Diluent Buffer was used as the 0pg/mL blank.

2.9.8 TNF- α ELISA Assay Protocol

All reagents were initially treated as per IL-6 protocol in section 2.9.5 paragraph 1. Then rat TNF- α High and Low Controls (lyophilized Rat recombinant TNF- α containing 0.1% sodium azide and reconstituted as per manufacturer's instructions) were also added to appropriate wells. All wells were mixed on a plate shaker built into the X-mark microplate reader (Bio-Rad Laboratories, Australia). The plate was covered and incubated for 2 hours at room temperature. This allows the TNF- α to bind to the monoclonal antibody coated on the walls of the wells. After incubation, the solution was aspirated from the wells and discarded. Wells were washed 4 times as per Il-6 method.

To each well except the chromagen blanks, 100 μ L of the secondary antibody (biotinylated Rat TNF- α Biotin Conjugate solution) was added. The plate was covered and incubated for 1 hour at room temperature to allow binding of the secondary antibody. This was then aspirated from wells, discarded and washed 4 times to remove any unbound antibody.

The enzyme Streptavidin-HRP Working Solution was made up as per section 2.9.4. 100 μ L of this working Solution was added to each well except the chromogen blanks. The plate covered and allowed to incubate for a further 30 minutes at room temperature. Following incubation, wells were aspirated and washed a further 4 times.

As per II-6 method, 100 μ L of the Stabilized Chromagen was added to each well to produce a blue colour. This was incubated for 30 minutes at room temperature and in the dark. Finally, 100 μ L of Stop Solution was added to each well. The plate was gently mixed by tapping the sides gently. The solution in the wells then turned yellow. The absorbance of each well was read spectrophotometrically at 450nm using, having blanked the plate reader against a chromogen blank composed of 100 μ L each of Stabilized Chromogen and Stop Solution. The plate was read within 30 minutes of adding the Stop Solution. TNF- α concentration in samples were determined by producing a standard curve as per II-6 in Figure 2.5 of section 2.9.5.

2.10 Insulin Resistance Using QUICKI

In the studies herein, insulin sensitivity was measured using the quantitative insulin sensitivity check index (QUICKI) as developed by Katz et al. (2000a). This method utilises fasting blood samples and thus QUICKI can be defined as: $QUICKI = 1/[(\text{Log}(I_o) + \log(G_o))]$, where I_o is the fasting plasma insulin level (microunits per mL), and G_o is the fasting blood glucose level (mg per dL). A lower index indicates less sensitivity to insulin.

2.10.1 Plasma Insulin Analysis

Plasma Insulin was determined using a rat/mouse Enzyme Linked Immuno-Sorbent Assay (ELISA) kit (Millipore, catalogue #EZRMI-13K, St Charles, Missouri, USA). The principles of the protocol are the same as for Il-6 and TNF- α in section 2.9.3 and 2.9.6 respectively.

2.10.2 Preparation of Insulin ELISA Kit Solutions

All solutions and standards provided in the kit were ready to use and required no preparation, with the exception of the Wash Buffer Concentrate (containing 50mM Tris Buffered Saline containing Tween-20), which was diluted 10X by mixing the entire content of each bottle of Wash Buffer with 450ml de-ionized water. The solutions and their contents are outlined in Table 2.1).

Table 2.1 Solutions contained within the Millipore Rat/Mouse Insulin ELISA kit

Reagent	Composition
Rat/Mouse Insulin Standards in assay Buffer	0.2, 0.5, 1, 2, 5 and 10ng/ml
Rat/Mouse Insulin Quality Controls 1 & 2	Rat insulin Quality Control buffer
Matrix Solution	Charcoal stripped pooled mouse serum
Assay Buffer	0.05 M phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.08% sodium azide, and 1% BSA
Rat/Mouse Insulin Detection Antibody	Pre-titered biotinylated anti-insulin antibody
Enzyme Solution	Pre-titered streptavidin-horseradish peroxidase conjugate in buffer
Substrate	3, 3', 5, 5'-tetramethylbenzidine in buffer
Stop Solution	0.3M HCl

2.10.3 Rat/Mouse Insulin ELISA Assay Protocol

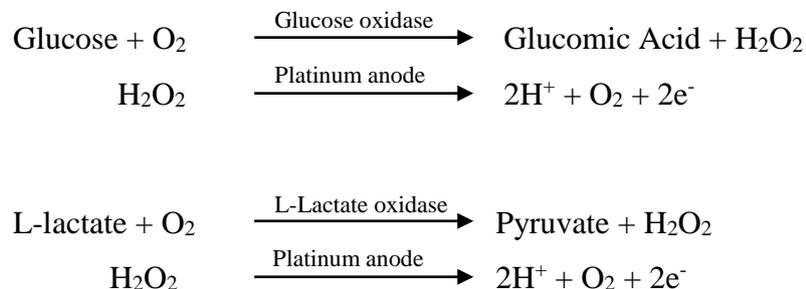
All reagents were brought to room temperature prior to setting up the assay, and plasma samples were thawed on ice. The required number of strips were removed from the Microtiter Assay Plate and assembled in an empty plate holder. Each well was washed 3 times with 300 μ L of diluted Wash Buffer per wash. The Wash Buffer was decanted and any residual buffer removed from all wells by inverting the plate and tapping it onto absorbent towels several times.

To the reagent blank and sample wells, 10 μ L of Assay Buffer was added. Following this, 10 μ L of Matrix Solution was added to the reagent blank, Standards, and Control wells. In duplicate, 10 μ L of all Standards, Controls and Samples were added to appropriate wells. To all wells, 80 μ L of the Detection Antibody was added, the plate was covered, shaken on an orbital plate shaker at moderate speed (400-500rpm), and incubated at room temperature for 2 hours. Following incubation, the solutions were decanted, the plate inverted and tapped to remove residual solution from wells, and washed 3 times with 300 μ L of diluted Wash Buffer per well. Once decanted and all residual buffer removed by tapping as previously mentioned, 100 μ L of Enzyme Solution was added to each well and the plate was incubated with moderate shaking at room temperature for 30 minutes on the microtiter plate shaker. Thereafter, solutions were decanted and washed 6 times with 300 μ L of diluted Wash Buffer per well. After decanting and removal of residual solution, 100 μ L of Substrate Solution was added to each well, and shaken in the plate shaker for approximately 15 minutes causing a blue colour formation in wells containing Insulin Standards, with intensity proportional to increasing concentrations of insulin. Finally 100 μ L of Stop Solution was added to each well, and the plate was shaken, at which point the solution turned yellow and absorbance was read at 450nm and 590nm, with the difference in absorbance units recorded. A standard curve was

produced using a 4-parameter logistic equation as for cytokine analysis, and insulin concentrations were determined from this. The assay was considered acceptable if the quality control values fell within the calculated quality control range as stipulated in the assay kit manual.

2.10.4 Plasma Glucose and Lactate

Plasma glucose and lactate were measured using a Yellow Springs analyser (YSI 2300 STAT; Yellow Springs Instrument, Ohio, USA). These assays were based on glucose and L-lactate oxidase methods for glucose and lactate respectively (Chua and Tan, 1978). The YSI sample chamber collected 25ul of each plasma sample and diffused this into a membrane placed over an electrochemical probe containing both glucose and L-lactate oxidase. Glucose was oxidized into gluconic acid and hydrogen peroxide, whilst L-lactate was oxidized to form pyruvate and hydrogen peroxide (see equation below). Hydrogen peroxide (H₂O₂) was detected amperometrically at the platinum electrode surface, and hence the current flow at this electrode indicated the concentration of hydrogen peroxide. This was directly proportional to the concentration of glucose and L-lactate present in each sample. All samples were analysed in duplicates. The YSI was calibrated every 5 samples.



2.11 Histology

Tissue that had been prepared for histological analysis (refer to section 2.4.2) was removed from the ultra-freezer and kept in a canister of liquid nitrogen. When ready for cutting, each sample was removed from the liquid nitrogen and placed in a microtome cryostat (Leico CM1950 Kryostat, Leica Biosystems, Germany) cooled to -20°C. The muscle sample was mounted onto a chuck with Tissue-Tek and 10µm thick transverse sections were cut and melted onto a glass microscope slide (Menzel-Glasar, Braunschweig, Germany). Details of various staining techniques are outlined in relevant chapters.

2.12 Statistics

The relevant statistical analysis details are provided in each chapter.

CHAPTER 3

Skeletal Muscle Atrophy in Sedentary Zucker Obese Rats is not Caused by Calpain-Mediated Muscle Damage or Lipid Peroxidation Induced by Oxidative Stress⁴

⁴ This chapter has been published as indicated by reference 1 on page iv.

3.1 Introduction

T2D is a chronic lifestyle disease characterised by high plasma free fatty acids (FFA), hyperglycaemia, hyperinsulinaemia and insulin resistance; and which effects multiple organ systems (Scheuermann-Freestone et al., 2003). Notably, the skeletal musculature undergoes significant atrophy (Cotter et al., 1993), which has further adverse impacts on disease progression. Whilst protein synthesis and degradation imbalance likely accounts for this atrophy (Trostler et al., 1981; Durschlag and Layman, 1983), the triggers that lead to changes in the activation of these pathways in T2D and hence alterations in protein turnover are still controversial. Several catalytic pathways have been implicated in atrophic cellular protein degradation including the autophagosome-lysosomal, ubiquitin proteasome, Caspase and Ca^{2+} -dependent calpain pathways (Belcastro et al., 1994; Goll et al., 2008; Murton et al., 2008). While a role for the ubiquitin proteasome (Wang et al., 1998) and autophagosome-lysosomal (He et al., 2012; Yoshizaki et al., 2012) pathways have been established in T2D-related skeletal muscle atrophy, much less is known about the Ca^{2+} -dependent calpain system. Indeed, a potential mechanism that may contribute to T2D induced muscle degradation is via increased susceptibility of the skeletal musculature to damage – in particular, via the flow on effects of Ca^{2+} dysregulation, calpain activation and sustained free radical production, both of which are common features of pathological muscle wasting in a variety of diseases (Bartoli and Richard, 2005).

The calpains are a family of Ca^{2+} -dependent cysteine proteases – skeletal muscle fibres contain both the ubiquitous isoforms μ -calpain, m-calpain, and calpain-10, as well as the muscle-specific form, calpain-3 (Sorimachi et al., 1989). Those calpains activated within a physiologically relevant $[\text{Ca}^{2+}]$ range are calpain-3 and μ -calpain (Murphy et al., 2006a; Murphy and Lamb, 2009). Calpain 3 plays a role in remodelling and maintaining normal

sarcomeric structures, whereas μ -calpain is associated with dismantling sarcomeric structures; and a balance in their activities is important for skeletal muscle integrity (see review (Murphy, 2010)). Intracellular Ca^{2+} concentrations above resting cytosolic levels cause autolysis of μ -Calpain and Calpain-3, thus increasing their proteolytic activity (Murphy et al., 2006b; Murphy and Lamb, 2009). Over-activation of calpains due to Ca^{2+} overload has been implicated in many pathological conditions including, Parkinson's disease and muscular dystrophy (Vanderklish and Bahr, 2000; Allen et al., 2010). As there is substantial evidence suggesting dysregulation of intracellular Ca^{2+} homeostasis in T2D (for review see (Levy, 1999)), it is possible that calpains are being over-activated by excessive intracellular Ca^{2+} accumulation in this disease. Thus far, calpain activity has not been investigated for a role T2D-associated atrophy.

Much like calcium dysregulation induces damage-associated muscle atrophy, increases in FFA content can lead to excessive production of reactive oxygen species (ROS) and reduced antioxidant defences (Johansen et al., 2005) resulting in damage to proteins, lipids and nucleic acids (Turrens, 2003). With respect to T2D, much of the existing literature on heightened ROS production has been restricted to the mitochondria (Bonnard et al., 2008; Herlein et al., 2011; Hey-Mogensen et al., 2012) and the impact on whole muscle has not been addressed. High FFA content is also accompanied by changes in the lipid profile of cells which affects membrane integrity and fluidity, as well as leaving membranes more susceptible to ROS-induced lipid peroxidation further impairing their structural integrity (Maritim et al., 2003; Taniyama and Griendling, 2003). Free radical peroxidation of arachidonic acid, a component of cell membranes, forms a prostaglandin-like end product known as the F_2 -isoprostanes. Mass spectrometry assessment of F_2 -isoprostanes is regarded as the gold standard biomarker of oxidative stress (for review see Kaviarasan et al. (2009))

and plasma F₂-isoprostanes are significantly higher in both diabetic compared to non-diabetic patients (Davi et al., 1999) and in obese versus lean diabetic Zucker rats (Laight et al., 1999) T2D. F₂-isoprostanes is measurable in skeletal muscle (Milne et al., 2007), however no study to date has investigated F₂-isoprostane production in T2D skeletal muscle, and the influence of increased oxidative stress on atrophy and fibre morphology in this disease.

Evans Blue Dye (EBD) has been established as a useful tool to determine cell membrane permeability and can be assessed using different techniques including red auto-fluorescence in tissue sections using fluorescence microscopy (Matsuda et al., 1995). EBD binds to plasma albumin (Hamer et al., 2002) and has been used to identify damaged skeletal muscle fibres which results in them becoming permeable to albumin (Matsuda et al., 1995). As such, EBD would be a useful way to determine membrane permeability and to identify potentially damaged myofibres in T2D – especially given the dysregulation of calcium homeostasis and potential for calpain activation, as well as the effects of ROS and isoprostane production on cell integrity.

Therefore, the aim of this study was to determine if the skeletal muscle atrophy associated with obesity and insulin-resistance in the obese rat animal model of the disease is a result of enhanced membrane fragility mediated by excess ROS and/or increased fibre damage due to excess calpain activity, using EBD fluorescence as a marker. We hypothesised that: (1) the sarcolemma of Obese Zucker skeletal muscle would be more permeable to the extracellular fluid (as evidenced by albumin-conjugated EBD fluorescence within myofibres) and show evidence of ROS-induced lipid peroxidation (as determined by F₂ isoprostane production); and (2) that increased sarcolemmal permeability would result in hyperactivated calpains. Herein, we confirm hyperinsulinaemia, hyperglycaemia, reduced insulin sensitivity and

skeletal muscle atrophy in the T2D Obese Zucker rat, but importantly determine that this atrophy is not caused by sarcolemmal damage, elevated lipid peroxidation and degradative calpain activity.

3.2 Methods

3.2.1 Animals

A total of 42 rats were used in this study: 14-week-old male Obese Zucker and age-matched, non-diabetic Lean Zucker littermates (n=7 for T2D biomarker experiments and n=14 for muscle histology, calpain and F₂-isoprostane) served as the experimental and control groups, respectively (Flinders University, Adelaide, Australia). Rats of the same phenotype were housed in pairs and allowed access to food and water *ad libitum*. All experiments were approved by the Victoria University Animal Ethics Experimentation Committee and conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

3.2.2 Biochemical Measures

While Obese Zucker rats demonstrably develop insulin-resistance by 14 weeks of age (Durham and Truett, 2006), we have quantified plasma glucose and insulin concentrations and calculated insulin sensitivity to confirm T2D in our colony. On a separate group of fasted (overnight) and anaesthetised rats (n=7 for Obese and Lean groups), blood samples were collected via cardiac puncture into eppendorf tubes and centrifuged (10 min at 13,300 RPM). Plasma was decanted and immediately analysed for plasma glucose using a Yellow Springs analyser (Yellow Springs Instruments, Ohio, USA). Insulin concentration was determined in 10 µl of plasma by ELISA according to the protocol (Rat/Mouse Insulin ELISA Kit) provided by the manufacturer (Linco Research). Using the derived plasma glucose and

insulin concentrations, insulin sensitivity was calculated using the quantitative insulin sensitivity check index (QUICKI) method ($QUICKI = 1/[\log(I_0) + \log(G_0)]$) (Katz et al., 2000b).

3.2.3 Muscle Sampling Protocol

Animals were injected with EBD as optimised for skeletal muscle by Hamer et al (Hamer et al., 2002). A 1% EBD solution (Ajax Chemicals, 32688) (w/v) in phosphate-buffered saline (PBS, pH 7.5), was filtered through a Millex-GP 0.22 μm filter and stored at 4°C. Twenty four hours prior to muscle sampling, rats were given an intraperitoneal (I.P.) injection of 1% EBD (v/w). After the injection, animals were returned to their cage and allowed access to food and water *ad libitum* for 24 hours to allow optimal uptake of the dye into any leaky myofibres (Hamer et al., 2002).

On the day of muscle sampling, rats were weighed and anaesthetised with Pentobarbitone Sodium (60 $\text{mg}\cdot\text{kg}^{-1}$ body weight). The Extensor Digitorum Longus (EDL, fast-twitch), and Soleus (slow-twitch) muscles were excised, cleaned of excess fat and connective tissue, including tendons, and weighed on standard laboratory scales. The tissue collected was covered with Tissue-Tek and frozen in isopentane cooled in liquid nitrogen for histological analyses of muscle membrane permeability, damage and atrophy. EDL and soleus muscles from the alternate leg were then removed, and snap frozen in liquid nitrogen for analysis of isoprostane production and calpain activity.

3.2.4 Muscle Membrane Permeability, Damage and Atrophy

Frozen embedded sections were cut (10 μm) at -21°C on a cryostat, dipped in cold acetone (Merck) (-20°C) for 1 min and air dried at room temperature. Sections were then dipped into

xylene (Merck) and mounted with DPx (Fluka Biochemika) and a coverslip. Another slide of consecutive serial sections was stained with Haematoxylin and Eosin (H&E) to determine muscle fibre size. In a single-blinded protocol, 100 fibres per section were manually traced using Analytical Imaging Station (AIS) software and the cross sectional area (μm^2) of each fibre was determined. The area analysed was chosen by placing the section in the centre of the field of vision at low (40x) magnification, and then counting the fibres in view when changing to the higher (100x) magnification.

EBD sections were visualised by fluorescence microscopy using a N2.1 green wavelength filter set (band pass = 515-560nm; (low pass = 590nm). The proportion of cross-sectional area positive for EBD (EBD+) was determined and the fluorescence intensity (relative units) of EBD+ areas was quantified. The location of EBD fluorescence intensity in the frozen sections was assessed semi-quantitatively using a seven-point Likert scale scoring system as per Hamer et al (Hamer et al., 2002). The scale, from 0 to 6, was defined as ranging from 0=no signal; 1=minimal signal; 2=weak signal; 3=good signal; 4=moderate signal; 5=strong signal; 6 very strong signal. A score on this scale for EBD red fluorescence was recorded for both myofibre penetration and the interstitium, viewed at a final magnification of 100x. H&E sections were viewed with light microscopy (Zeiss Axiolab, Carl Zeiss GmbH).

3.2.5 ROS-Induced F₂-Isoprostane Production

Tissue F₂-isoprostanes were measured as previously described (Mori et al., 1999). F₂-isoprostanes were detected by electron-capture negative ionization GC-MS after solid-phase extraction and corrected for total arachidonic acid content. Arachidonic acid was measured in lipid extracts from frozen muscle, after conversion to the methyl esters with 2 mL 4% H₂SO₄ in methanol (90 C, 10 min). The methyl esters of fatty acids were analysed by gas

chromatography as previously described (Croft et al., 1996). Heptadecanoic acid (50 μ L of stock 1 mg/mL) was used as internal standard.

3.2.6 Calpain Analyses

μ -calpain and calpain-3 autolysis was determined in skeletal muscle from Obese (n=6) and Lean (n=6) rats. Muscle samples were homogenised using 10 volumes of ice-cold extraction buffer, comprising 0.4M Tris-Cl, pH 6.8, and 25mM EGTA ($[Ca^{2+}] < 10mM$). SDS was added to a final concentration of 4%. Muscle homogenates were incubated at 4°C for 20-40min, an aliquot was kept for protein concentration assay (Quant-iT fluorescence assay, Invitrogen), and the remaining homogenate was diluted 1:5 v/v with extraction buffer. This was added to SDS loading buffer (2:1 v/v) comprising of 0.125M Tris HCl, 10% glycerol, 4% SDS, 4 M urea, 10% mercaptoethanol, and 0.001% bromophenol blue, pH 6.8 which had been diluted (2:1 v/v) in physiological based solution. Samples were stored at -20°C until analysis. Samples were analysed by Western blotting as previously described (Murphy and Lamb, 2009). Total protein from muscle samples were separated on an 8% SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes were probed with antibodies against μ -Calpain (1:1,000 mouse monoclonal, Sigma monoclonal, clone 15C10), and calpain-3 (1:200 mouse monoclonal, Novocastra monoclonal 12A2), and goat anti-mouse horseradish peroxidase (HRP) (1:50,000 Bio-Rad) was then added to the membranes. Bands were visualised with West Pico chemiluminescent substrate (Pierce), and densitometry was completed using Quantity One software (Bio-Rad). Once transferred, gels were stained with BioSafe Coomassie blue (Bio-Rad), and myosin heavy chain (MHC) in the post-transferred gel as well as membranes probed with actin (Sigma A-2066) were used as an indicator of sample loaded (Murphy et al., 2006b). Full-length μ -calpain was visualised as an 80kDa protein, and its activation was confirmed by its autolysis to 78- and 76-kDa proteins. Calpain-

3 was observed as a 94-kDa protein with activation confirmed by autolysis to proteins of approximately 60-, 58-, and 55-kDa (Murphy et al., 2006a). Data for the Western blots are presented as the density of the bands corresponding to the autolysed products relative to the density of the total bands representing μ -calpain or calpain-3 for a given sample. This indicated the proportion of μ -calpain or calpain-3 that was autolysed in a particular sample, irrespective of any minor differences in protein loading.

3.2.7 Statistical Analysis

Results are expressed as means \pm Standard Error of Mean (SEM) and compared by two-way ANOVA, with animal strain and muscle type as factors. No significant interactions were detected. In all cases a $P < 0.05$ was considered statistically significant.

3.3 Results

3.3.1 Weight and Metabolic Parameters

As expected, Obese Zucker rats had significantly higher body weights ($569.0\text{g} \pm 10.8\text{g}$ versus $369.7\text{g} \pm 8.0\text{g}$ than Lean controls; $p < 0.01$). We have also confirmed hyperglycaemia and hyperinsulinemia in the Obese Zucker model comparative to Lean controls, with plasma glucose and insulin concentrations of 271.2 ± 36.2 mg/dL versus 110.4 ± 18.2 mg/dL ($p < 0.005$) and 11.8 ± 0.3 $\mu\text{Units/mL}$ versus 3.8 ± 0.7 $\mu\text{Units/mL}$ ($p < 0.001$), respectively (Figure 3.1). Insulin resistance as determined by the QUICKI method, was also confirmed in the Obese Zucker model with an index of 0.29 ± 0.004 compared to 0.40 ± 0.03 in Lean controls ($p < 0.05$; Figure 3.1).

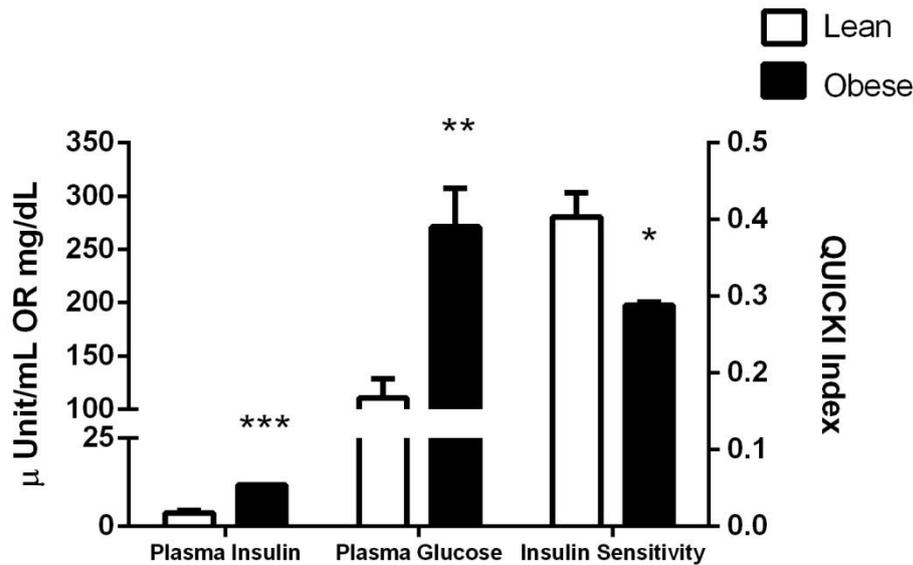


Figure 3.1 Plasma insulin and glucose concentration (normalised to body weight) and relative insulin sensitivity as determined by the quantitative insulin sensitivity check index (QUICKI) in Lean versus Obese Zucker rats (n=7). ***p<0.001; **p<0.005; *p<0.05 Lean versus Obese Zucker.

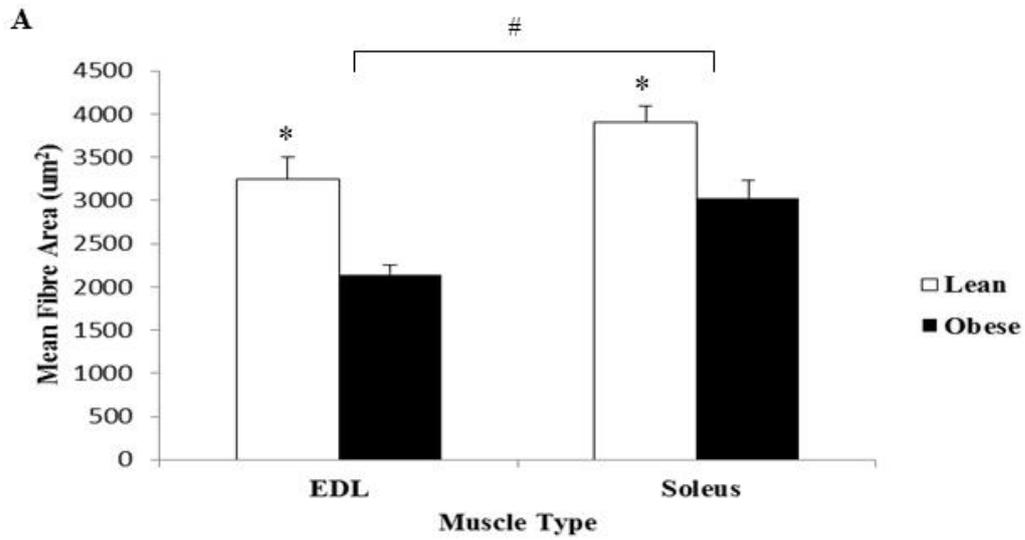
3.3.2 Muscle Atrophy

Despite their higher body weights, Obese rats displayed significantly lower skeletal muscle weights ($0.132\text{g} \pm 0.003\text{g}$ versus $0.167\text{g} \pm 0.004\text{g}$ for Lean control EDL and $0.160\text{g} \pm 0.007\text{g}$ versus $0.184\text{g} \pm 0.005\text{g}$ for Lean control soleus; $p < 0.01$) compared to Lean rats. The lower muscle weights in the Obese rats corresponded with a 35% smaller mean fibre area in EDL ($p < 0.01$) and 23% smaller fibre area in soleus ($p < 0.01$) compared to Lean controls (Figure 3.2A-C). Furthermore, fast-twitch EDL fibres were smaller than the slow-twitch soleus muscle fibres ($p < 0.01$) irrespective of animal group.

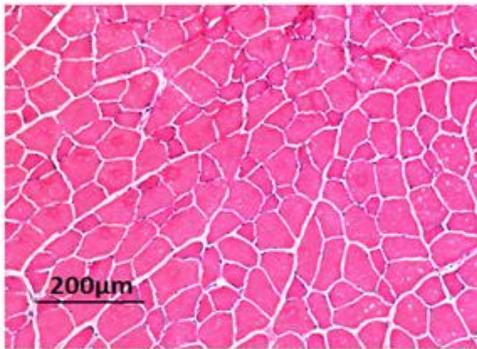
3.3.3 Muscle Membrane Permeability and Damage

Obese rats displayed a ~4-fold increase in the % of EBD+ to total cross sectional area in both EDL and soleus sections, compared to Lean rats (Figure 3.3A). In addition, the EBD+ cross sectional area was significantly greater in soleus compared to EDL ($p < 0.05$). Of the fibre area that stained EBD+, fluorescence intensity (arbitrary fluorescence units) was significantly

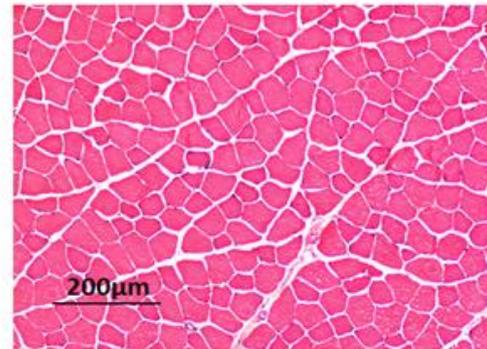
higher in Obese compared to Lean rats for both EDL and soleus ($p < 0.05$) (Figure 3.3B). The muscle sections from Obese rats clearly showed, however, that while exhibiting a higher proportion of EBD fluorescence compared to Lean controls, the dye is localised to the interstitium and not penetrating the sarcolemma (as indicated by the solid arrows Figures 3.3D and 3.3E) . As such, the location (intramyofibre or interstitium) of EBD fluorescence within the the total muscle section was semi-quantified using a seven-point Likert scale where 0 indicates no signal and 6 indicates very strong signal (Figure 3.3C). There was no difference in the intramyofibre EBD fluorescence intensity between Lean or Obese muscle, nor was there any difference between EDL and soleus ($p > 0.05$). However, the EBD fluorescence intensity was significantly greater in the interstitium of Obese compared to Lean EDL ($p < 0.001$) and soleus ($p < 0.005$).



B

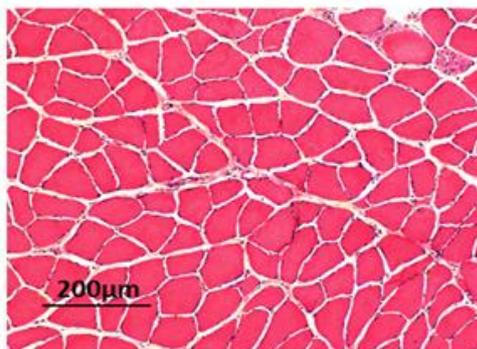


EDL Lean

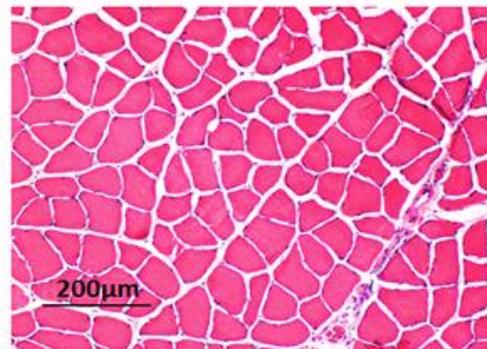


EDL Obese

C



Soleus Lean



Soleus Obese

Figure 3.2 Average fibre area of skeletal muscle tissue (um²) (A). H&E stained skeletal muscle sections of EDL (B), and Soleus (C) of Obese and Lean rats. Magnification x100. *p<0.01 vs Obese. #p<0.01 vs EDL. n=14 for each group.

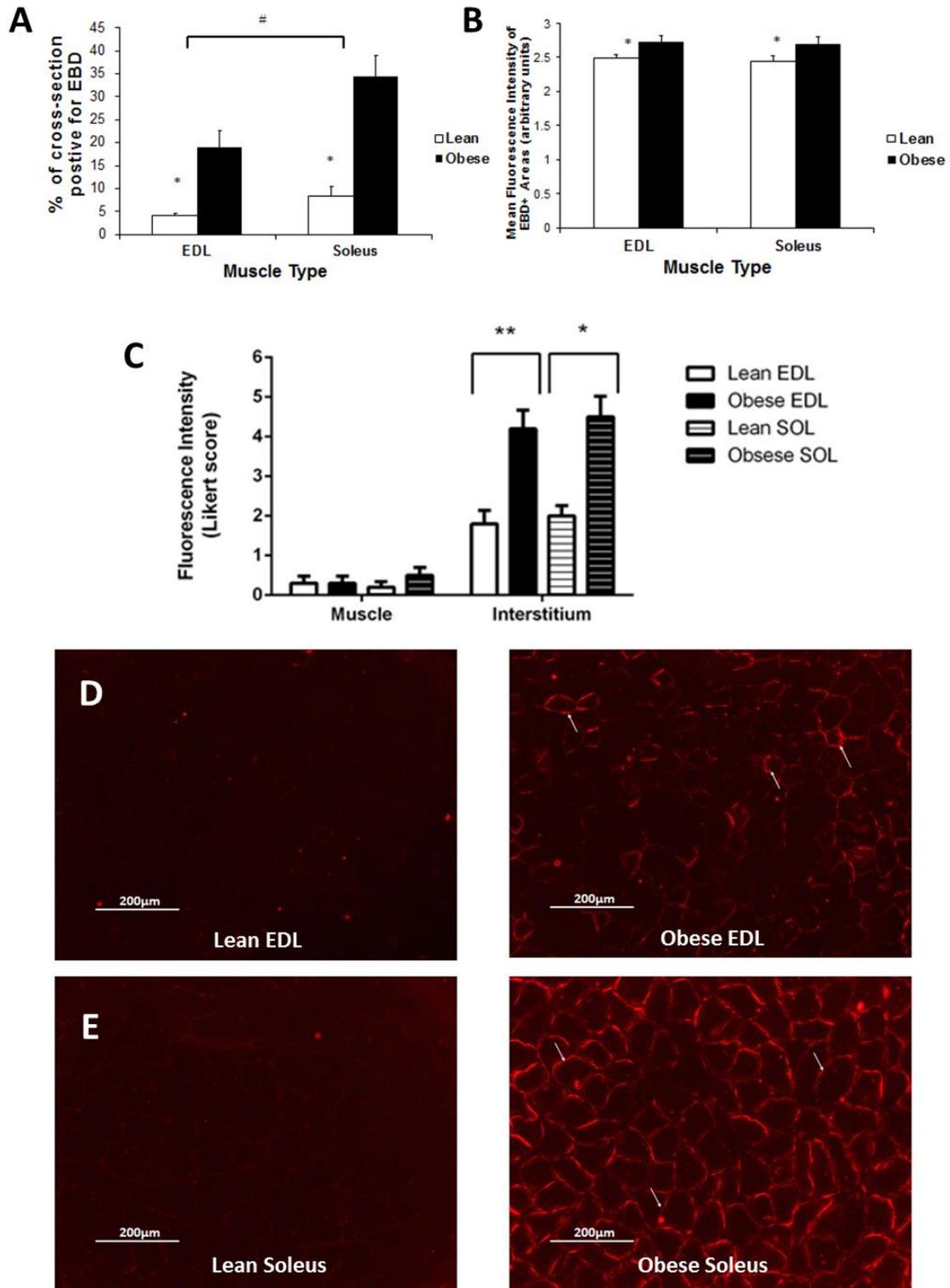


Figure 3.3 EBD staining in muscle fibres of Zucker rats. Proportion (%) of cross-sectional area that is EBD+ (A). Average Fluorescence intensity (arbitrary units) of EBD+ areas (B). Mean strength of EBD signal in myofibres and the interstitium as scored by a semi-quantitative Likert scale (C). Muscle sections of EDL (D), and Soleus (E) of Obese and Lean rats showing EBD accumulation around the skeletal muscle membranes of Obese rats (solid arrows), with minimal penetration of the dye in Lean rats. Magnification x100. * $p < 0.05$ vs Obese. # $p < 0.05$ vs EDL. $n = 14$ for each animal group.

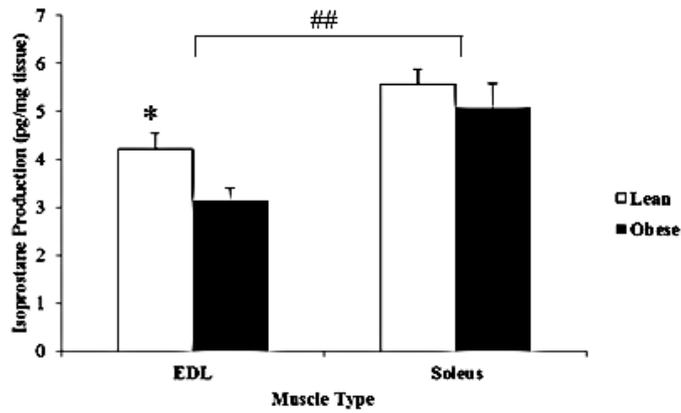
3.3.4 ROS-Induced F₂-Isoprostane Production

F₂-isoprostane production was measured in EDL and soleus muscles. Lean Zucker rats contained 27% more F₂-isoprostanes compared to Obese littermates in EDL (p<0.05) (Figure 3.4A), and a similar trend in soleus (p=0.07). However the lower total arachidonic acid content in both EDL (p<0.01) and soleus (p<0.05) muscles in Obese compared with Lean samples (Figure 3.4B) indicated comparable F₂-isoprostane production when corrected for arachidonic acid content (Figure 3.4C). Soleus muscle produced significantly more F₂-isoprostanes compared to EDL (p<0.05) for both groups of animals.

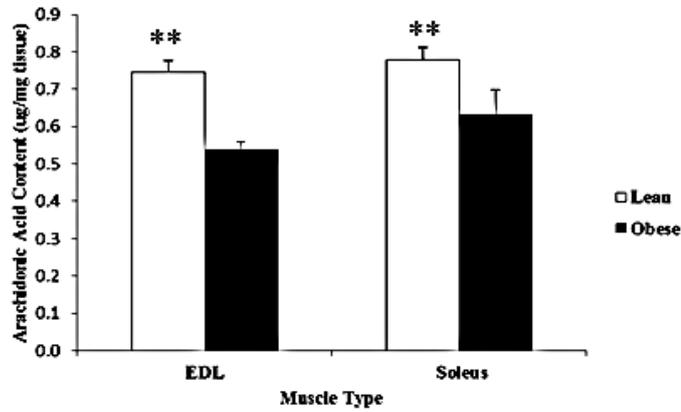
3.3.5 Calpain Analyses

To determine if activation of Ca²⁺-mediated proteolytic pathways are a mechanism involved in the muscle atrophy detected in Obese Zucker rats, both μ -calpain and calpain-3 autolysis were measured in EDL and soleus (Figure 3.5). As shown by Western blot analyses, neither μ -calpain nor calpain-3 autolysis, and hence activation, were increased in Obese rats compared to Lean rats in either EDL or soleus muscle.

A



B



C

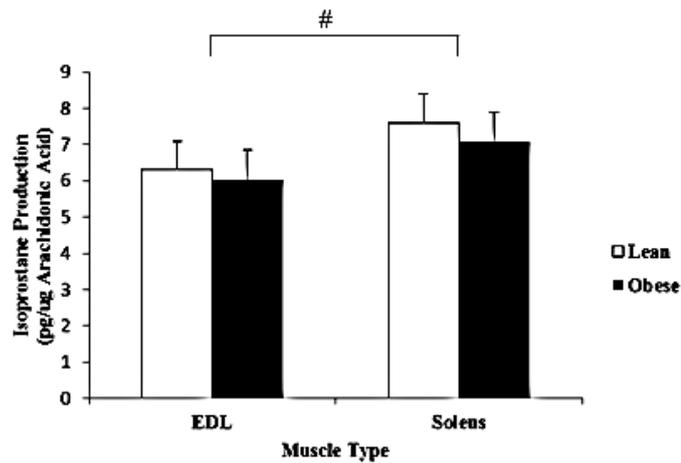


Figure 3.4 F₂-isoprostane production per mg of tissue in Zucker rats (A). Total muscle arachidonic acid content (B). F₂-isoprostane production corrected for arachidonic acid content (C). *p<0.05 vs Obese. **p<0.01 vs Obese. #p<0.05 vs EDL. ##p<0.01 vs EDL. n=13 for each group.

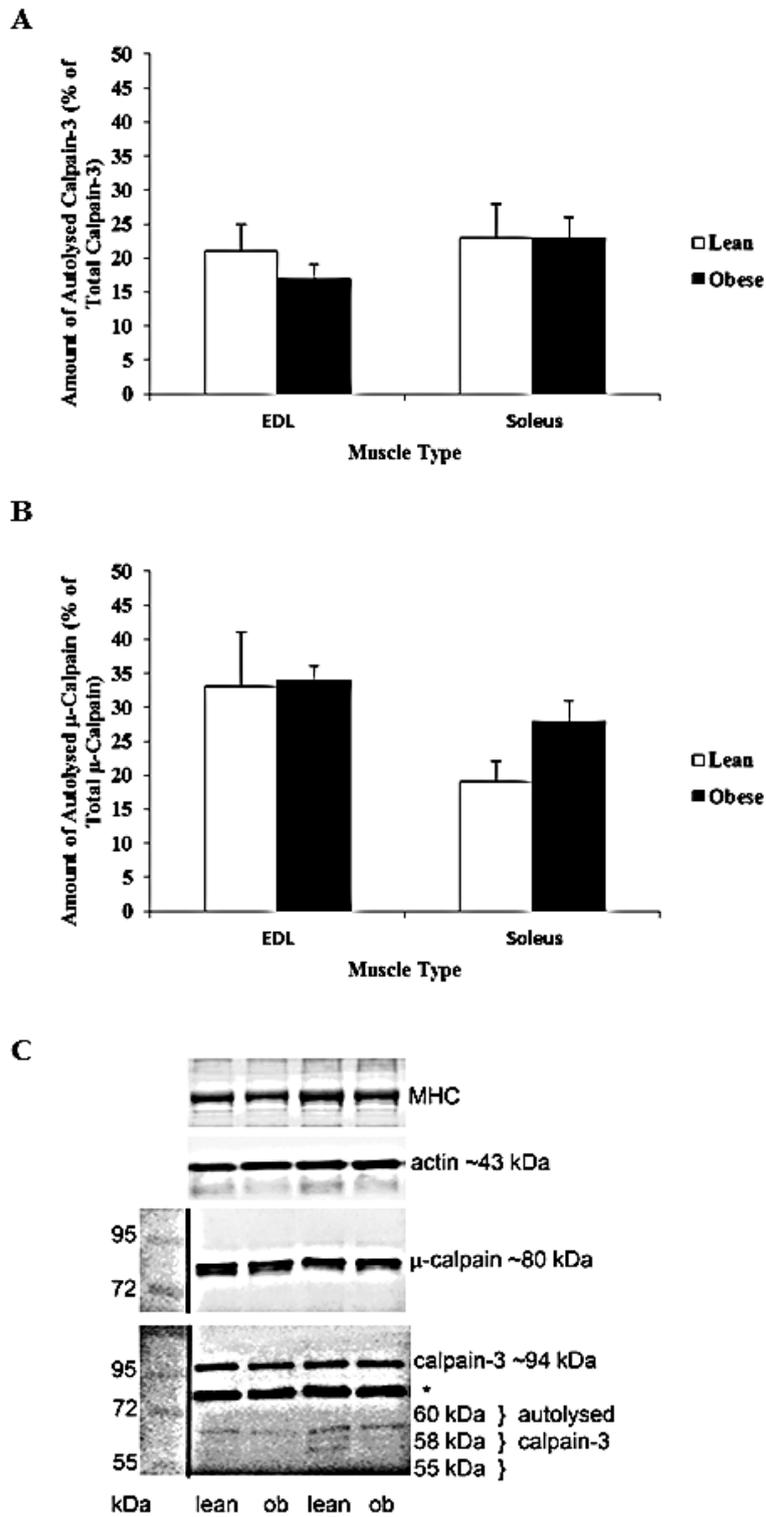


Figure 3.5 Percentage of activated Calpain-3 (A) and μ -Calpain (B) in skeletal muscle. Western blots show either μ -Calpain or Calpain-3 in Obese and Lean rats (C), with molecular mass markers, taken as a white light image prior to chemiluminescence and then images superimposed, indicated on the left. n=6 for all groups. * indicates the non-specific band seen in rodent skeletal muscle with this antibody.

3.4 Discussion

The Obese Zucker rat is an established model of T2D mellitus and displays the hallmark biochemical characteristics of the disease including hyperinsulinemia, hyperglycaemia and hyperlipidemia, in addition to insulin resistance, as confirmed in this study and by others (Durham and Truett, 2006). At the skeletal muscular level, atrophy is a well-documented consequence of T2D (Croft et al., 1996; Cederholm et al., 2000). This has been confirmed in the current study, with a significant reduction in the muscle weights of Obese Zucker rats compared to Lean controls, accompanied by decreased fibre area in both fast- and slow-twitch muscles. Muscular atrophy was observed despite significantly increased body weights in the Obese group, in which the increased load-bearing of the musculature should theoretically induce compensatory hypertrophic adaptation to support the body weight. We have investigated the hypothesis that diabetes-associated muscular atrophy is resultant of an increased susceptibility to myocellular damage – specifically due to increased ROS-activated lipid peroxidation of the sarcolemma, permeability of the sarcolemma to the extracellular fluid and hyperactivation of the cytosolic protease calpain. This study highlights that skeletal muscle atrophy of fast and slow twitch fibres from the Obese Zucker rat is not directly associated with heightened protein degradation due to physiological $[Ca^{2+}]$ -range calpain activation, nor is it resultant of ROS-mediated lipid peroxidation.

Impaired Ca^{2+} regulation has been reported to be an influencing factor in the impaired muscle function and morphology observed in T2D. Given this, $[Ca^{2+}]$ -activated calpain autolysis was thought to be a likely mechanism inducing the atrophy in Obese muscle. While the direct quantification of intracellular $[Ca^{2+}]$ and the Ca^{2+} -handling capacity of Zucker skeletal muscle is outside the scope of this study, we have confirmed that any Ca^{2+} dysregulation present is insufficient to induce autolysis of calpain-3 and/or μ -calpain, both of which

activate in the presence of heightened $[Ca^{2+}]_i$ within physiological levels. It has been documented that for calpain-3 to become activated, $[Ca^{2+}]_i$ of 200 nM for at least 60 min is required (Murphy and Lamb, 2009), whereas for u-calpain, 3-50uM of Ca^{2+} is required (Murphy et al., 2006b). Our data suggests that neither of these $[Ca^{2+}]_i$ ranges are being maintained for any sufficient period of time to induce sustained calpain-activation and protein degradation to warrant the observed muscle atrophy.

A common cause of persistently elevated $[Ca^{2+}]_i$, as observed in many muscle pathologies, is sarcolemmal instability which results in microtears within the phospholipid structure and/or hyperactive stretch-induced leak channel activity (Guharay and Sachs, 1984; McNeil and Khakee, 1992). Using EBD as a marker of muscle damage, we have demonstrated that sarcolemmal integrity is maintained in skeletal muscle from Obese rats, suggesting that (1) hyperglycaemia and/or hyperinsulinemia do not directly cause instability and/or increased permeability of the sarcolemma; and (2) that muscle damage is not a cause of the observed atrophy. When the sarcolemma is porous, albumin-conjugated EBD can freely move into the muscle where it becomes trapped, resulting in muscle fibres that fluoresce red when viewed microscopically. While this study has demonstrated a higher percentage of EBD fluorescence in skeletal muscle cross-sections of Obese rats, dye accumulation was interestingly confined to the external membrane/interstitium rather than being evenly distributed throughout the sarcoplasm as is evidenced in EBD+ sections of damaged skeletal muscle from the *mdx* mouse model of Duchenne Muscular Dystrophy (Hamer et al., 2002) – a severe muscle wasting disease of which a feature is sarcolemmal instability and hyperactivation of Ca^{2+} /calpain-induced damage pathways. This demonstrates comparable sarcolemmal integrity between sedentary Obese Zucker rats and Lean controls, with no obvious signs of elevated muscle damage to the diabetic condition. To our knowledge, accumulation of EBD-

conjugated albumin at the extracellular sarcolemmal surface has not been reported previously in diabetic skeletal muscle. We speculate that this is reflective of increased extravasation of EBD-bound albumin from associated capillaries (St-Pierre et al., 2006) and subsequent binding of albumin to membrane glycoproteins (Schnitzer et al., 1988), which are notably overexpressed on the extracellular surface of membranes in response to chronic exposure to a hyperglycaemic environment (Stehno-Bittel et al., 2003). Excessive albumin accumulation demonstrably promotes modification of the size and compilation of the interstitium (Scalia et al., 2007), reduces membrane fluidity (Beck et al., 1998) and binds Ca^{2+}_e (Kragh-Hansen and Vorum, 1993). While such an effect may serve as a protective mechanism to hyperglycaemia, how this would impact upon normal skeletal muscle preservation and function is currently unknown, although impaired nutrient diffusion and reduced nutrient availability to ATP synthesis could be a likely outcome.

ROS-induced F_2 -isoprostane production (measured as a marker of lipid peroxidation) was hypothesised as another potential mechanism responsible for the atrophy found in the Obese Zucker model of diabetes. Previous research has demonstrated increased F_2 -isoprostanes in plasma of patients with T2D (Davi et al., 1999). However, our results indicate that F_2 -isoprostane production was comparable in both EDL and soleus muscle from Obese compared to Lean rats. Isoprostane formation was significantly lower (data not shown) in muscle from Obese rats compared to Lean controls, however, so too was total arachidonic acid content (data not shown). Thus, when corrected for total arachidonic acid content, F_2 -isoprostane production was comparable between Obese Zucker and control skeletal muscle. This correction is essential given the reliance on arachidonic acid availability for isoprostane production. Whether this decrease in arachidonic acid content (and thus isoprostane production) is reflective of prior sarcolemmal damage, or simply less capacity for production,

is unknown. As albumin has a tendency to bind to, and stimulate, the release of arachidonic acid from the membrane (Beck et al., 1998) and our EBD data demonstrates EBD-conjugated albumin accumulation in the interstitial space proximal to the extracellular sarcolemmal surface, we speculate that albumin is having a direct effect on the arachidonic acid content of the sarcolemma in Obese Zucker muscle. Low membrane arachidonic acid content, as has been demonstrated in the current study, also reportedly reduces membrane fluidity (Beck et al., 1998). How changes to sarcolemmal fluidity effect normal skeletal muscle preservation and function is currently unknown. A notable limitation of our study is that we have included only F₂-isoprostane measurement as a marker of lipid peroxidation – future research would benefit from parallel analysis of lipid peroxidation byproducts such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) to enable complete assessment of membrane lipid damage.

Another interesting observation was that the predominantly slow-twitch soleus muscle displayed a higher rate of F₂-isoprostane production than predominantly fast-twitch EDL muscle. Although more glycolytic in nature, Type II fibres are demonstrably more susceptible to oxidative damage than Type I fibres. Since our soleus muscle sections also had higher albumin-conjugated EBD fluorescence in the interstitium (likely due to their higher capillary density (Murakami, 2010) comparative to glycolytic EDL sections, we speculate that this albumin is actively binding to arachidonic acid and releasing it from the sarcolemma. This highlights that increased vascular permeability, and in particular extravasation of albumin into the interstitium, renders oxidative muscle fibres more susceptible to membrane changes that may subsequently affect muscle function.

Skeletal muscle mass is highly regulated by a variety of molecular pathways that promote or inhibit protein synthesis and degradation. While our data importantly demonstrates that atrophy induced by the diabetic phenotype is not linked to sarcolemmal porosity and the ensuing protein degradative pathways controlled by $[Ca^{2+}]_i$ -induced calpains, there are many other signaling mechanisms that may be contributing to the atrogenic environment. Indeed, the protein synthetic phosphatidylinositol 3-kinase (PI3K)/phosphorylated Akt (pAkt) (Lee et al., 2004) and the protein degradative ubiquitin-proteasome proteolytic pathway (Wang et al., 2006) have both been linked to T2D. The triggers that lead to activation of these pathways and subsequent alterations in protein turnover in T2D are still controversial, however our data alongside others (Durschlag and Layman, 1983) suggests that inhibition of protein synthesis may be the overriding regulator. In addition to impaired glucose uptake into skeletal muscle, insulin resistance also reduces amino acid uptake (King and Betts, 1994) which may explain the slower skeletal muscle protein synthesis rates observed in this disease (Durschlag and Layman, 1983). Paturi et al. (2010) have confirmed reduced mTOR activation in skeletal muscle of Obese rats, which highlights that specifically targeting protein synthesis may be an effective way of reversing/preventing the muscle atrophy observed in diabetic skeletal muscles.

3.5 Conclusion

We have provided further evidence that Obese Zucker rat skeletal muscles are atrophic. The mechanisms are demonstrably not related to sarcolemmal damage, protein degradation induced by heightened calpain activation or ROS-mediated lipid peroxidation. Additionally, we have shown accumulation of albumin (as evidenced by EBD fluorescence) on the extracellular surface of the sarcolemma and interstitium which may be indicative of capillary

macromolecule extravasation – what effect this has on skeletal muscle function and mass preservation is unknown.

CHAPTER 4

Effects of Creatine, Whey and Aspirin on Skeletal Muscle Contractile Function and Recovery from Fatigue in Type 2 Diabetes

4.1 Introduction

People suffering from obesity and T2D have been shown to have an increase in FFA (Scheuermann-Freestone et al., 2003), with some of the detrimental effects to skeletal muscle outlined in Chapter 1. Namely, the resultant insulin resistance that occurs due to interference with insulin receptor signalling, causes a decrease in GluT-4 translocation to the cell membrane, and hence reduced glucose transport and substrate available for metabolism (Boden and Chen, 1995). The reduction in substrate availability potentially hinders ATP production, and hence energy available to muscle function. This leads to overall exercise intolerance found in Obese and Type 2 Diabetic patients (Green et al., 2007).

High FFA levels also impair mitochondrial function. This may be due to the increase in oxidative stress caused by lower levels of the antioxidants, MnSOD and GSH, leading to inefficient buffering against ROS (Johansen et al., 2005). It may also be a result of the decrease in size and number of mitochondria (He et al., 2001), as well as impairments found in the structure of the inner mitochondrial matrix (Kelley et al., 2002). The high FFA further reduces the fluidity of complex V of the respiratory chain (Lamson and Plaza, 2002) and hence inhibits its function. Overall this may result in a lower ATP production which could potentially impair normal cell function; in particular the contraction relaxation cycle which relies on sufficient ATP for optimal contractile function and recovery. Other organelles utilising ATP may also be affected.

The sarcoplasmic reticulum utilises ATP to help regulate intracellular calcium concentrations, and allows for normal contraction/relaxation processes to occur (Berchtold et al., 2000). Normally, the process of SR Ca^{2+} resequestration occurs via the Ca-ATPase pump. Once ATP stores are depleted however, they may be resynthesised via the CK reaction,

which utilises PCr and ADP to do so (Chapter 1.3.4). However, studies have found that calcium regulation is disrupted in T2D, although there has been debate as to whether Ca^{2+} regulation is increased or decreased (Eibschutz et al., 1984; Ganguly et al., 1986). This controversy may be due to differences in the stages of the disease that were examined. However it is perhaps likely that SR function would be inhibited given the reduced energy available to it, as well as studies which have found that an increase in FFA may also reduce the fluidity of Ca^{2+} pump, hence slowing its efficiency.

Finally, the high FFA as well as superoxide production has been shown to activate an inflammatory response, increasing cytokine levels of Il-6 and TNF- α (Mohamed et al., 1999; Dandona et al., 2004), which ultimately causes reduced expression of insulin receptors, as well as impairing insulin signal transduction, resulting in insulin resistance. The inflammatory response also increases ROS production causing further inflammation. Inflammation has been shown to cause β -cell destruction in the pancreas & ultimately T2D (Dandona et al., 2004). Cytokines have been shown to down-regulate SERCA pumps and in heart failure patients, Il-6 can lead to skeletal and cardiac muscle atrophy.

Therefore it is essential to understand in finer detail some of the impairments pertaining to skeletal muscle function in terms of contractile, SR, and mitochondrial function as well as the effects of inflammation on these processes in T2D, and how the supplements discussed in chapter 1.4 (Creatine, Whey, and Aspirin) may be able to reverse some of these impairments, to improve muscle function in this disease. As such chapters 4 to 7 aim to address each of these processes separately, however due to the lack of availability of ZDF rats, these experiments were all performed on the same group of animals, although they will be discussed separately.

As discussed in chapter 1, earlier studies have demonstrated changes in muscle morphology and function in T2D (Sayer et al., 2005). However there is much controversy pertaining to exactly how various skeletal muscle groups are affected, which also varies according to the model of T2D employed. As found in chapter 3, the mechanisms don't involve muscle damage, thus examining other mechanisms of skeletal muscle dysfunction is necessary. Whilst the best model of T2D would be humans, the nature of the experiments conducted in this chapter requires the use of appropriate animal models, with the Zucker Diabetic Fatty rat the most appropriate model to use. No studies to date have looked at the combination of creatine, whey and aspirin supplementation in improving some of the skeletal muscle changes that take place in insulin resistant states such as T2D.

The aim of chapter 4 was to ascertain any perturbations in contractile function, fatigability, and recovery from fatigue in Obese and Lean ZDF rats, as well as whether the combination of Creatine, Whey and Aspirin treatment could alter these parameters. It is hypothesised that ZDF rats will have impaired contractile function and resistance to fatigue, with reductions in the ability to recover from a fatigue protocol, and the supplementation will reverse/prevent this.

4.2 Methods

The methods used to determine contractile function are detailed in chapter 2. Details regarding animals, anaesthesia, tissue acquisition and supplementation regime are outlined in sections 2.1 to 2.3, respectively. Briefly, Obese and Lean ZDF rats were obtained from Monash Animal Services (Monash University Melbourne, Australia) at 8 weeks of age and divided into four groups being Lean (n=10), Obese (n=12), Lean supplemented (LSupp; n=11), and Obese supplemented (ObSupp; n=13). All experiments in chapters 4-7 were

approved by the Animal Ethics Experimentation Committee, Victoria University (AEETH 10/10). Animals were supplemented for a period of 6 weeks with Creatine Monohydrate (0.09g/kg/day), Whey Protein Isolate (1.5g/kg/day), and Aspirin (100mg/kg/day). Adjustments were made to the amount of supplement added to the diets of Lean and Obese animals based on the phenotype of the animal. This was determined from an earlier pilot study revealing that the average food intake of Obese rats was approximately 20g/day versus 15g/day for Lean animals. To prevent the Obese animals from having higher amounts of supplementation due to higher food intake, they were given less supplement per kg of chow. Likewise, the amount of aspirin was also adjusted based on the higher water intake of Obese animals compared to Lean littermates. Table 4.0 summarises the dosage each animal group received.

Contractile function was determined as detailed in chapter 2.4. Contractile proteins and total protein determination is outlined in chapter 2.7.1 and 2.7.2 respectively. Metabolites were measured as outlined in chapter 2.8. Histological sections were prepared as detailed in chapter 2.11. Specifically, the Myosin ATPase and Capillary Density stains were performed for the purposes of this chapter, as outlined in the following two sub-sections.

4.2.1 Myosin ATPase Stain

In order to determine any fibre type alterations, the myosin ATPase staining method according to (Brooke and Kaiser, 1970; Hamalainen and Pette, 1993) was used. Muscle sections were cut as described in section 2.11. They were placed in the fridge at 4°C to allow muscle to properly adhere to slide. Sections were incubated for 7 minutes in an acid preincubation solution containing 54.3mM sodium acetate and 32.6mM sodium barbital adjusted to a pH of 4.60. This pH alters the lability of Type II fibres, staining them lighter

compared to Type I fibres. Further, this incubation also allows for separation of type II fibres into their subset types IIA and IIB. However, for the purposes of this chapter and due to a lack of consistency and shortage in sample availability, fibres were only differentiated into Type I and Type II. Sections were then washed in a solution containing 18mM CaCl₂ and 100mM TRIS - HCl (pH 7.8), followed by incubation in ATP incubation solution containing 4.5mM ATP, 19.5 CaCl₂, and 116mM 221 Alkaline Buffer Solution, pH 9.4 for 45 minutes at room temperature, being agitated at regular intervals. Sections were then incubated for 3 minutes in 11mM CaCl₂ three times over, followed by incubation for 1.5 minutes in 2% CoCl₂ twice over. An incubation of 30 seconds in 10mM Sodium Barbital was followed five times over, with a final dip in 1% ammonium sulphide (NH₄)₂S. Samples were rinsed in tap water, followed by a rinse in distilled water five times over. Tissue sections were then dehydrated in solutions of ethyl alcohol of increasing concentrations (70, 80, 90, 95 and 100%) for two minutes at each concentration. These were then rinsed in xylene for five minutes, and mounted with coverslips using Permount medium. The number of Type I and Type II fibres in each section were counted and a percentage fibre type determined for each sample. An example of a myosin ATPase stain can be seen in Figure 4.0.

4.2.2 Capillary Density Stain

Capillary density was assessed according to the methods of Andersen and Henriksson (1977). Tissue sections were cut to a thickness of 16µM, and fixed in cold (4°C) Carnoy's fixative (comprising of ethanol/chloroform/glacial acetic acid, at a ratio of 6:3:1) for 15 minutes immediately after sectioning, and rinsed in distilled water 3-5 times. Sections were incubated in 1% α-amylase (Sigma A-2771) for 60 minutes at 37°C, with regular intervals of mixing. These were rinsed in distilled water 3-5 times, followed by a 15 minute incubation in 1% periodic acid (Sigma 395B) at room temperature. A further rinse in distilled water 3-5 times

was carried out, before a 15 minute incubation in Schiff's reagent (Sigma, periodic acid-Schiff stain (PAS) kit, 395B) at room temperature. A final wash under running tap water for 10 minutes was carried out after which sections were dehydrated in ascending alcohol concentrations (80%, 90%, 100%) for approximately 30 seconds in each of the alcohol concentrations. Finally, sections were dipped in Xylene for 1min 30 secs and mounted with coverslips with permount medium. Sections were viewed at x200 magnification, where each capillary was counted in the section and expressed per mm². An example of one of the cross-sections stained for capillaries can be seen in Figure 4.1.

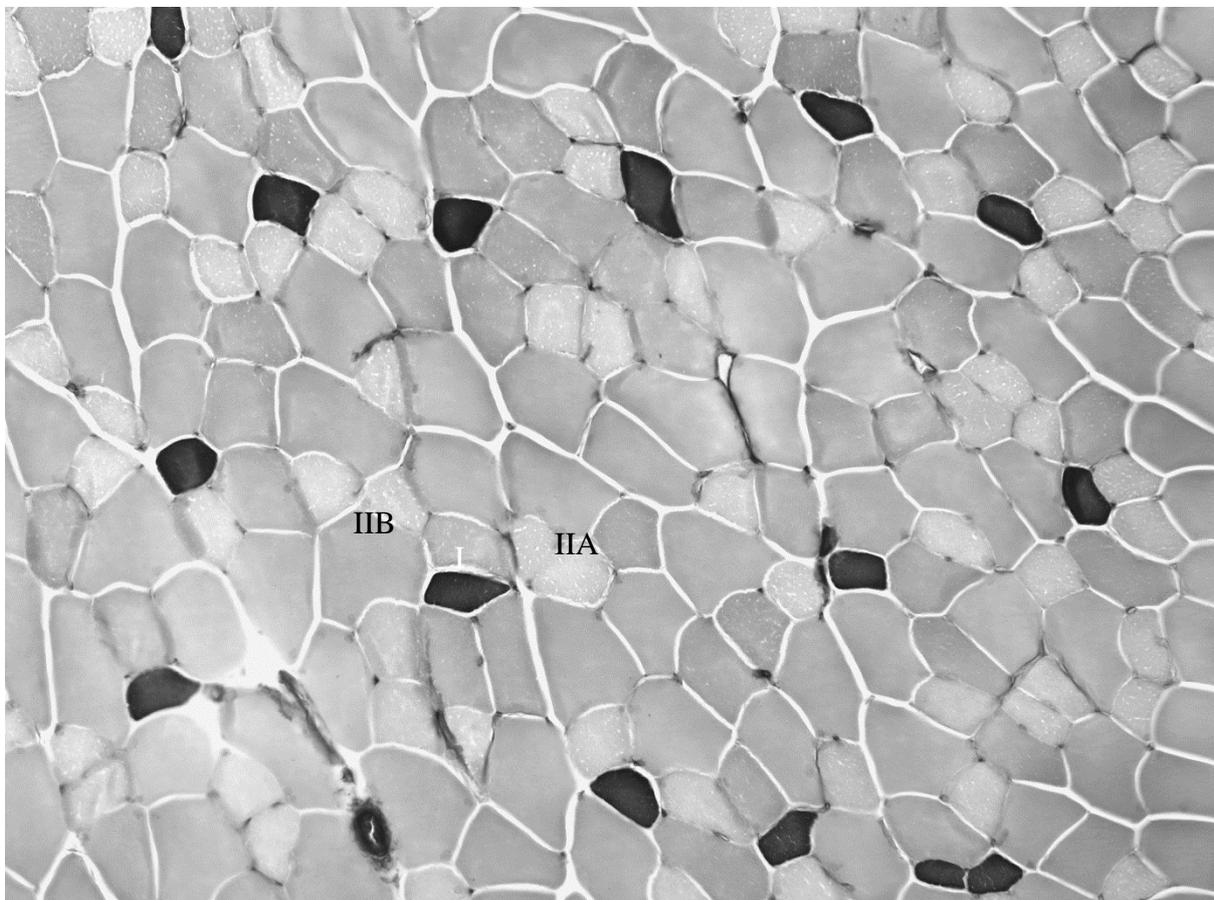


Figure 4.0 Example of Myosin ATPase Stain in EDL Skeletal Muscle of a Supplemented Obese ZDF Rat x200 magnification. Black fibres indicate Type I (Slow twitch) fibres, lighter fibres indicate Type IIA (fast twitch) and intermediate stains indicate IIB (fast twitch) fibres.

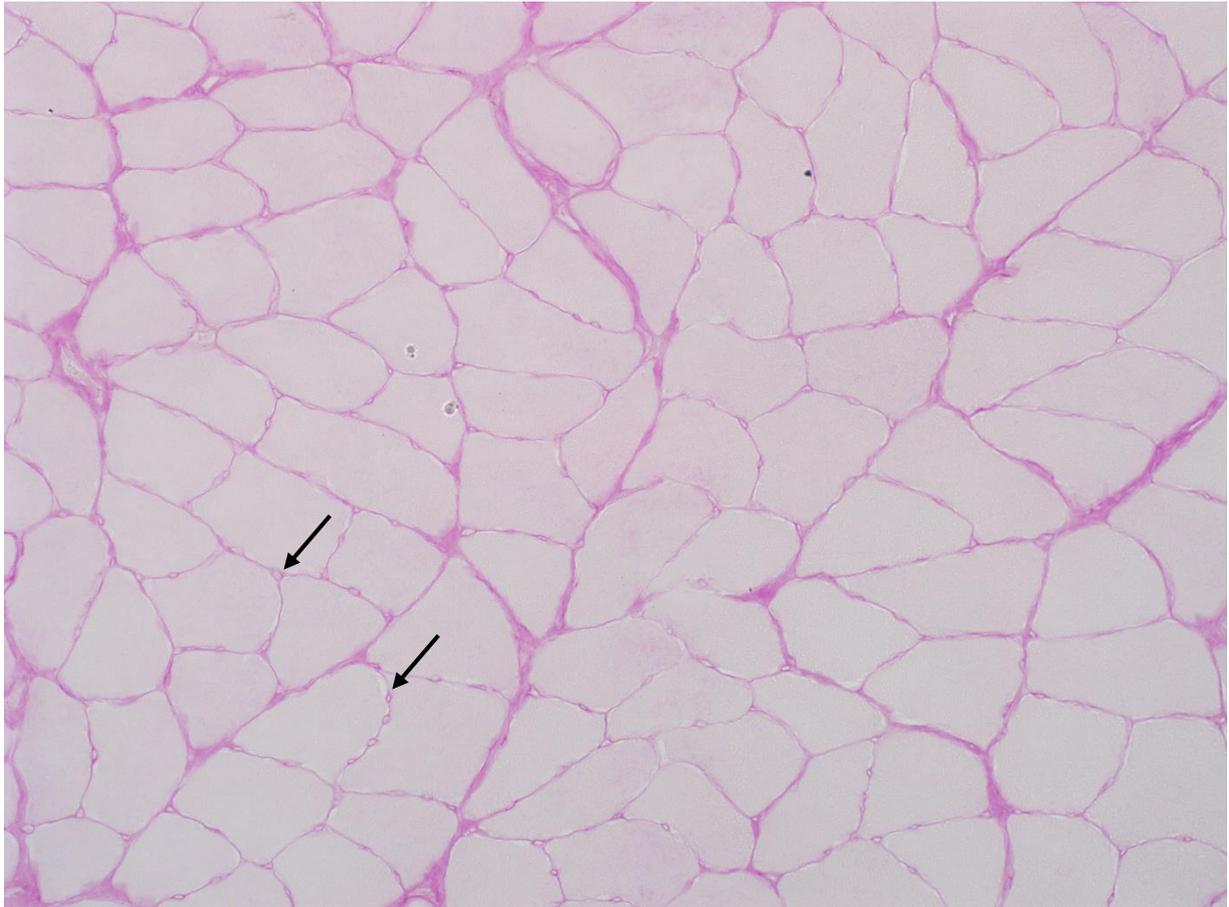


Figure 4.1 Example of Capillary Density Stain in Soleus Skeletal Muscle of a Lean ZDF Rat x200 magnification.

4.2.3 Statistical Analysis

For muscle and plasma samples, a two-way ANOVA, with group and supplement the two between group factors, was used to detect differences. Tukey's post hoc test was done to determine the location of differences. Significance was set at $p < 0.05$. All results are expressed as means \pm SEM.

4.3 Results

4.3.1 Supplementation Rates

Supplementation rates were targeted at 0.1g/kg/day and 1.5g/kg/day for creatine and whey, respectively, and 100mg/kg/day for Aspirin. These targets were all met, with actual supplementations rates of 0.12 ± 0.01 g (Lean) and 0.15 ± 0.02 g (Obese) creatine/kg/day; 1.71

$\pm 0.18\text{g}$ (Lean) and $2.21 \pm 0.35\text{g}$ (Obese) whey/kg/day; and $120 \pm 0.01\text{mg}$ (Lean and Obese) aspirin/kg/day, with no significant differences between animals groups for each supplement. Whilst an attempt was made to prevent animals from consuming excessive amounts of supplement based on their normal average intake, the animals in this study actually ate 5-10g/day more than originally anticipated and tested for (See table 4.0). Hence they received slightly more creatine and whey in both animal groups, although as indicated above, there was no significant difference between the groups ($p=0.2$ for creatine and whey protein). Water intake was as anticipated in lean (25 – 35mls/rat/day) and obese (30 – 40mls/rat/day) across all groups, so on average they obtained their targeted dose of aspirin of 0.1mg/kg/day. In addition, large groups of these animals were purchased at the same time due to a lack of availability of them. Due to the nature of the experiments conducted, they could not all be tested on the same day, as such animals were not all the exact same age at testing, with differences mainly between the LSupp and ObSupp animals (15.7 ± 0.10 versus 14.9 ± 0.20 weeks, respectively; $p<0.05$), and a strong trend between the Lean and LSupp animals (14.9 ± 0.4 versus 15.7 ± 0.1 weeks, respectively; $p=0.07$) (see Figure 4.2). However, given that rats are considered “young adult” at this age, it is unlikely to have impacted the results.

Table 4.0 Actual supplementation rates for Lean and Obese animals for Creatine, Whey and Aspirin.

	LSupp (n=11)	ObSupp (n=13)
g Cr/kg/day	0.12 ± 0.01	0.15 ± 0.02
g Whey/kg/day	1.71 ± 0.12	2.21 ± 0.23
g Aspirin/kg/day	0.12 ± 0.004	0.12 ± 0.01

Results are expressed as means \pm SEM.

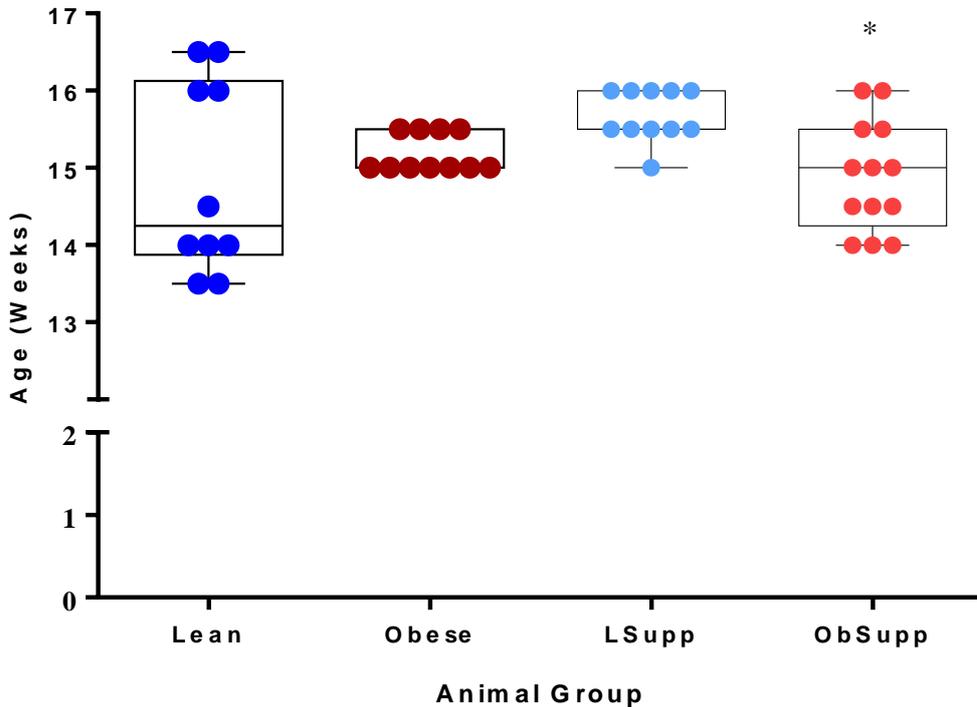


Figure 4.2 Box plot of age differences between rats of Lean (n=10), Obese (n=11), LSupp (n=11) and ObSupp (n=13) ZDF rats. *Significant difference compared to LSupp animals (p<0.05).

4.3.2 Total Weight Gain

The average body weight and total weight gain for each group is shown in Table 4.1. Data for the average weight gain is expressed in g/week as the animals were obtained at different ages, and thus the weekly or daily averages for each group for the duration they were in this study are reported. Lean animals weighed significantly less than all other animal groups, with no significant differences detected between the Obese and ObSupp groups. The ObSupp group gained the most weight and ate the most compared to all other groups (p<0.05), however their water consumption was only significantly greater than the Lean group (p<0.05). Interestingly the Obese animals gained significantly less weight than all other animal groups, despite consuming more food compared to Lean and LSupp animals.

Table 4.1 Animal and muscle weights, body weight gain, food/water consumption and average age for experimental period.

	Lean (n=10)	Obese (n=11)	LSupp (n=11)	ObSupp (n=13)
Mean Body Weight Post Fast (g)	296.3 ± 5.9	430.3 ± 8.8*#	332.6 ± 6.1*	453.8 ± 9.6* #
Mean Weight Gain Pre Fast (g/week)	21.0 ± 2.19	16.2 ± 1.02	18.4 ± 0.42	26.6 ± 1.21^
Mean Weight Gain Post Fast (g/week)	18.0 ± 1.81	11.2 ± 1.09*	17.2 ± 0.50	24.8 ± 1.18^*#
Food Consumption (g/rat/day)	20.1 ± 0.46	28.2 ± 0.60*	21.11 ± 0.55	32.7 ± 0.74^* #
Water Consumption (mLs/rat/day)	23.2 ± 0.5	37.9 ± 2.39*	25.0 ± 0.80	33.3 ± 1.44*
Mean Age (weeks)	14.9 ± 0.40	15.2 ± 0.08	15.7 ± 0.10	14.9 ± 0.20#
EDL Muscle Weights (g)	0.153 ± 0.002	0.122 ± 0.002*	0.165 ± 0.003*	0.125 ± 0.002*
Soleus Muscle Weight (g)	0.121 ± 0.004	0.125 ± 0.006	0.136 ± 0.004*	0.118 ± 0.004
EDL Muscle Mass : Body Mass Ratio (mg/g)	0.518 ± 0.034	0.284 ± 0.017*	0.495 ± 0.017	0.274 ± 0.020#
Soleus Muscle Mass : Body Mass Ratio (mg/g)	0.409 ± 0.045	0.285 ± 0.030*	0.398 ± 0.022	0.259 ± 0.025#

Results are expressed as means ± SEM. * Significant difference from Lean animals (p<0.05). # Significant difference from LSupp animals (p<0.01). ^ Significant difference from Obese groups (p<0.05).

4.3.3 Twitch and Tetanic Force Properties

Absolute and specific (force per muscle cross-sectional area) twitch and tetanic forces are shown in Figure 4.3). Absolute forces were lower in the Obese groups than the Lean groups regardless of supplement status in both slow- and fast-twitch muscles. Similarly, the supplement appeared to increase absolute forces regardless of group, although none of these differences were significant. Interestingly, this resulted in EDL twitch and tetanic force production being significantly higher (p<0.05) in the LSupp compared to the Obese group, although the same difference was not seen in the soleus.

Overall, no differences between any of the groups for specific twitch and tetanic forces were detected, despite similar trends observed in Obese animals and after supplementation. No

differences were observed in the Time-to-Peak tension (TTP), half relaxation time ($1/2RT$), or Pt/Po ratio between the groups in either EDL or Soleus as shown in Table 4.2.

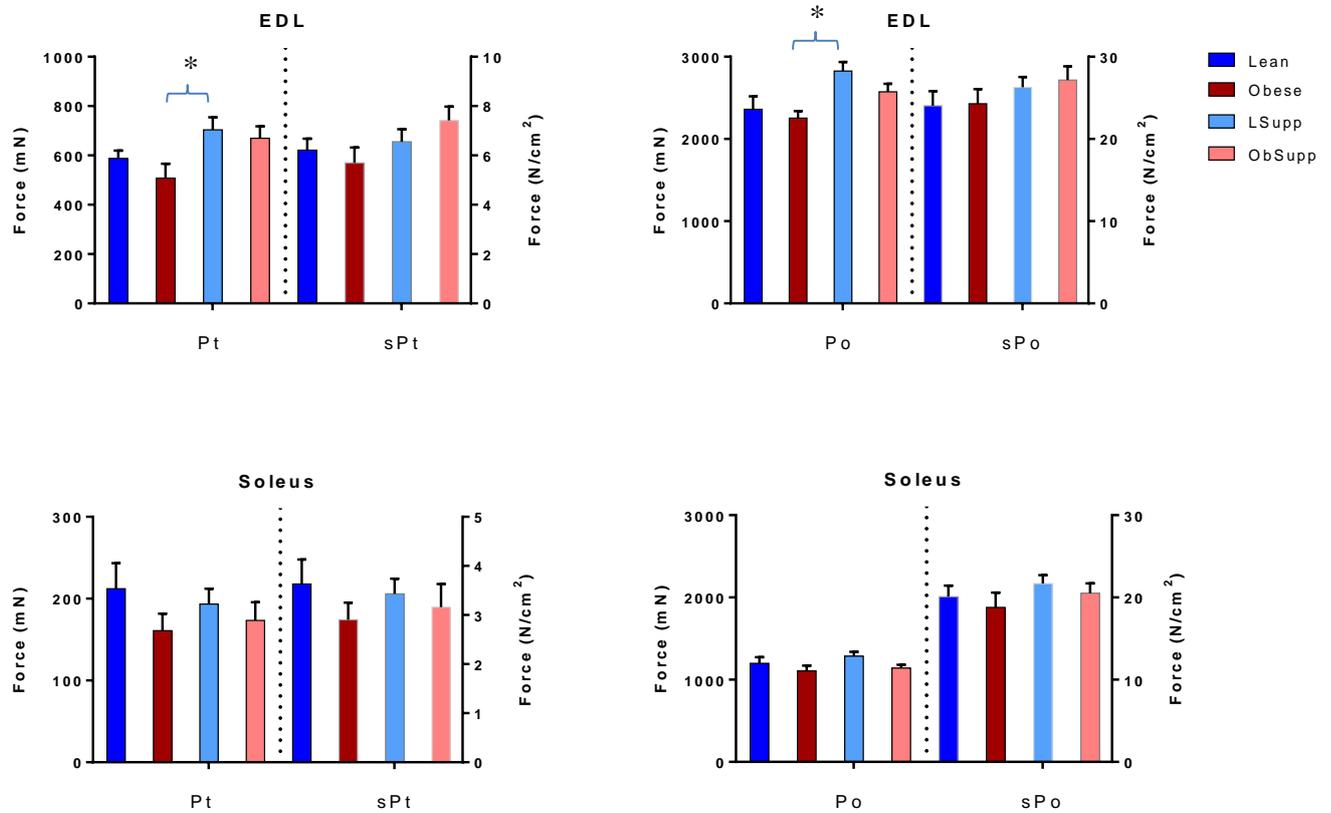


Figure 4.3 Absolute and specific peak twitch and tetanic force production in EDL and Soleus muscle of Lean (n=11), Obese(n=9), LSupp (n=10) and ObSupp (n=11) ZDF rats. Results are expressed as means \pm SEM. *Significant difference between LSupp and Obese animals (p<0.05).

Table 4.2 Isometric Contractile Properties of EDL and Soleus Muscle of Lean, Obese and supplemented ZDF rats.

	Lean (n=11)	Obese (n=9)	LSupp (n=10)	ObSupp (n=11)
<i>EDL</i>				
TTP (ms)	42.37 \pm 1.42	42.91 \pm 1.32	41.52 \pm 0.37	40.35 \pm 0.66
1/2RT (ms)	25.11 \pm 1.88	27.41 \pm 1.65	28.31 \pm 0.66	24.01 \pm 1.05
Pt/Po	0.23 \pm 0.02	0.24 \pm 0.01	0.25 \pm 0.01	0.25 \pm 0.01
<i>Soleus</i>				
TTP (ms)	87.17 \pm 3.59	89.23 \pm 2.83	87.53 \pm 1.55	89.89 \pm 2.79
1/2RT (ms)	95.88 \pm 5.60	95.4 \pm 5.06	100.90 \pm 2.80	101.5 \pm 8.09
Pt/Po	0.13 \pm 0.01	0.13 \pm 0.02	0.15 \pm 0.02	0.16 \pm 0.01

Results are expressed as means \pm SEM.

The effects of increasing stimulus frequencies were examined on force production (Figure 4.4). For EDL, at 50Hz and 80Hz, there was a significant left shift in the force frequency curve in Lean compared to the Obese animals, such that relative force production (as a percentage of maximum force) was significantly higher in Lean animals ($p < 0.05$). LSupp animals showed the same pattern compared to Obese and ObSupp animals, with significantly higher percent maximum force ($p < 0.05$) at the same 50 and 80Hz frequencies. In soleus muscle, the only significant difference was found at 100Hz where the Obese group had significantly lower percentage of maximum forces compared to all other groups ($p < 0.05$).

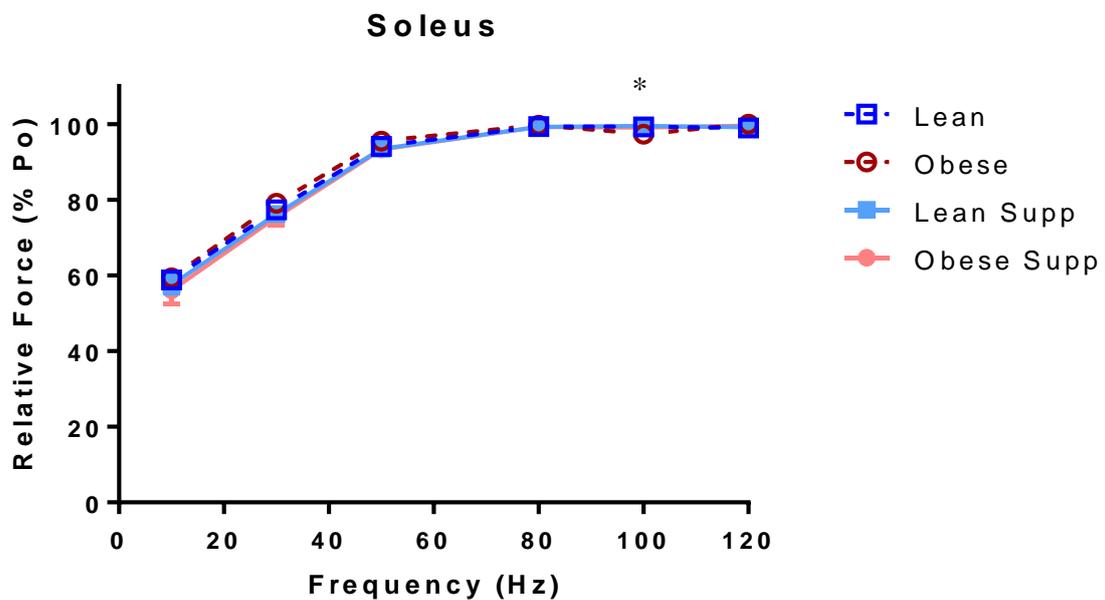
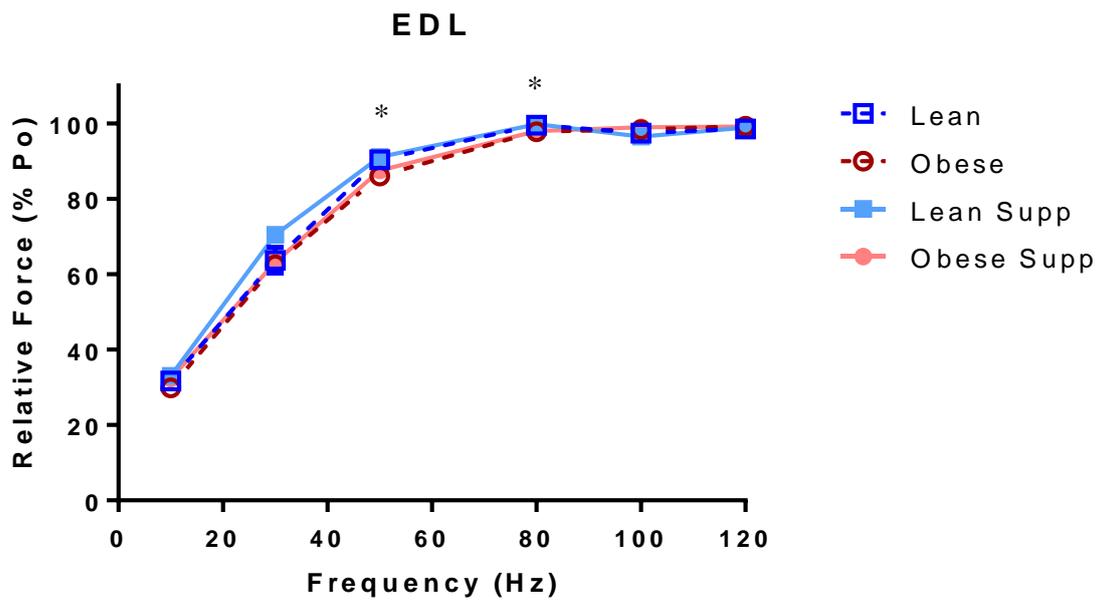


Figure 4.4 Force Frequency Curve in EDL and Soleus muscle of Lean (n=11), Obese (n=9), LSupp (n=10), and ObSupp (n=11) ZDF rats. Results are expressed as mean \pm SEM. *Significant difference between Lean and Obese animals; LSupp animals significantly different to Obese and ObSupp groups ($p < 0.05$).

4.3.4 Fatigue and Recovery Protocol

Fatigue following the 2.5 minute continuous stimulation protocol is shown in Figure 4.5 No significant differences were detected between groups for EDL, although the Obese group show a trend towards having approximately 5% more fatigue than Lean animals ($p=0.3$) with a trend to being reversed in the supplemented Obese group ($p=0.14$ compared to Obese).

In Soleus, Obese animals demonstrated significantly more fatigue than Lean animals ($p<0.01$). The supplements prevented this effect, such that ObSupp animals showed significantly less fatigue than the Obese group ($p<0.01$), to be comparable with Lean animals. In comparing fast- and slow-twitch muscles, EDL muscles in all animal groups fatigued significantly more than soleus muscles ($p<0.05$), which was to be expected.

Figure 4.6 shows the force production at set intervals during a recovery period after the fatigue protocol. In EDL muscle, recovery in ObSupp animals was approximately 10% greater than in Lean animals from 5 minutes to 30 minutes post recovery ($p<0.05$), and almost 20% higher than the LSupp group 45 minutes post recovery ($p<0.05$).

In Soleus muscle, Obese animals showed approximately a 30% reduction in force production throughout the recovery period compared to Lean animals ($p<0.05$). Supplementation significantly improved recovery by about 30% in soleus muscles ($p<0.05$). As such no differences were found between the Lean and ObSupp groups. The same improvements in recovery did not occur in supplemented Lean animals.

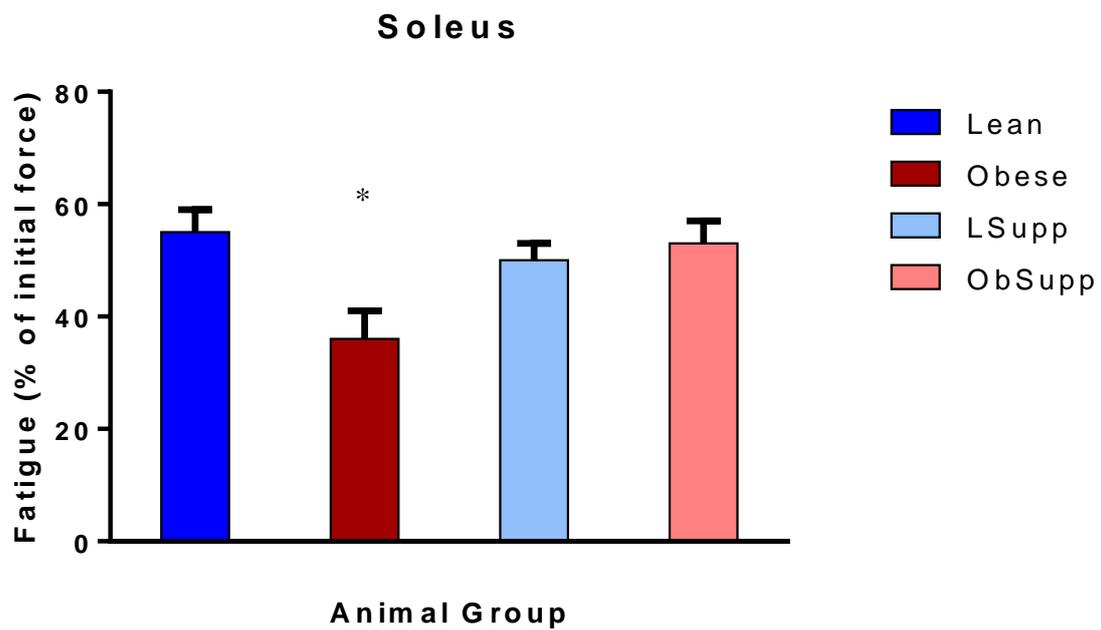
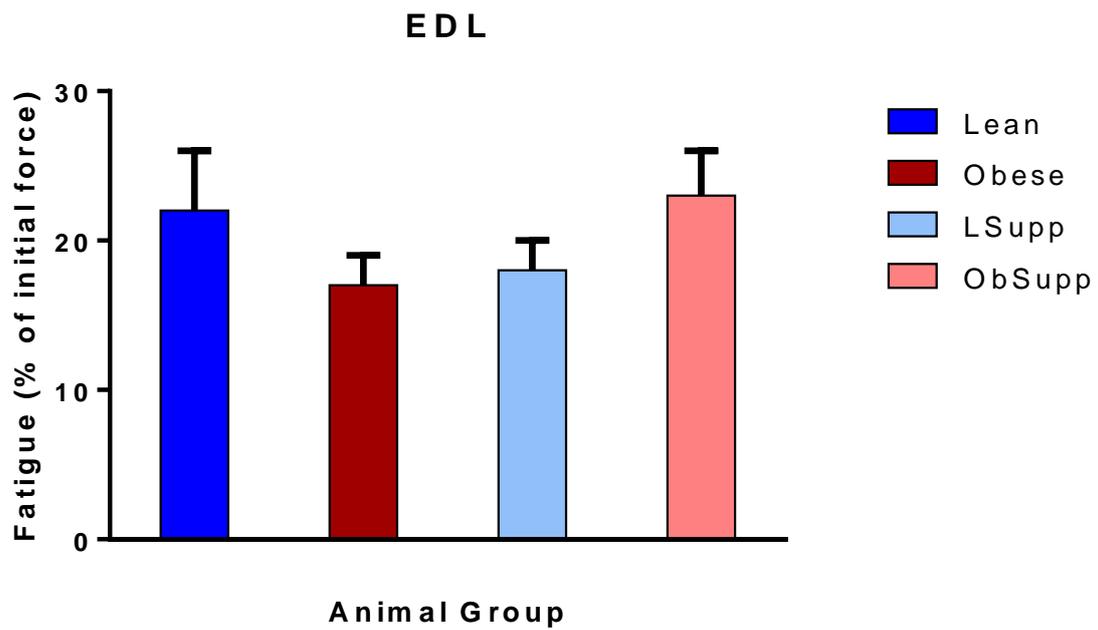


Figure 4.5 Skeletal Muscle Fatigue in Lean (n=12), Obese (n=10), LSupp (n=10) and ObSupp (n=11) ZDF rats in EDL and Soleus. Results are expressed as mean \pm SEM. * Significant difference compared to Lean and ObSupp animals ($p < 0.01$).

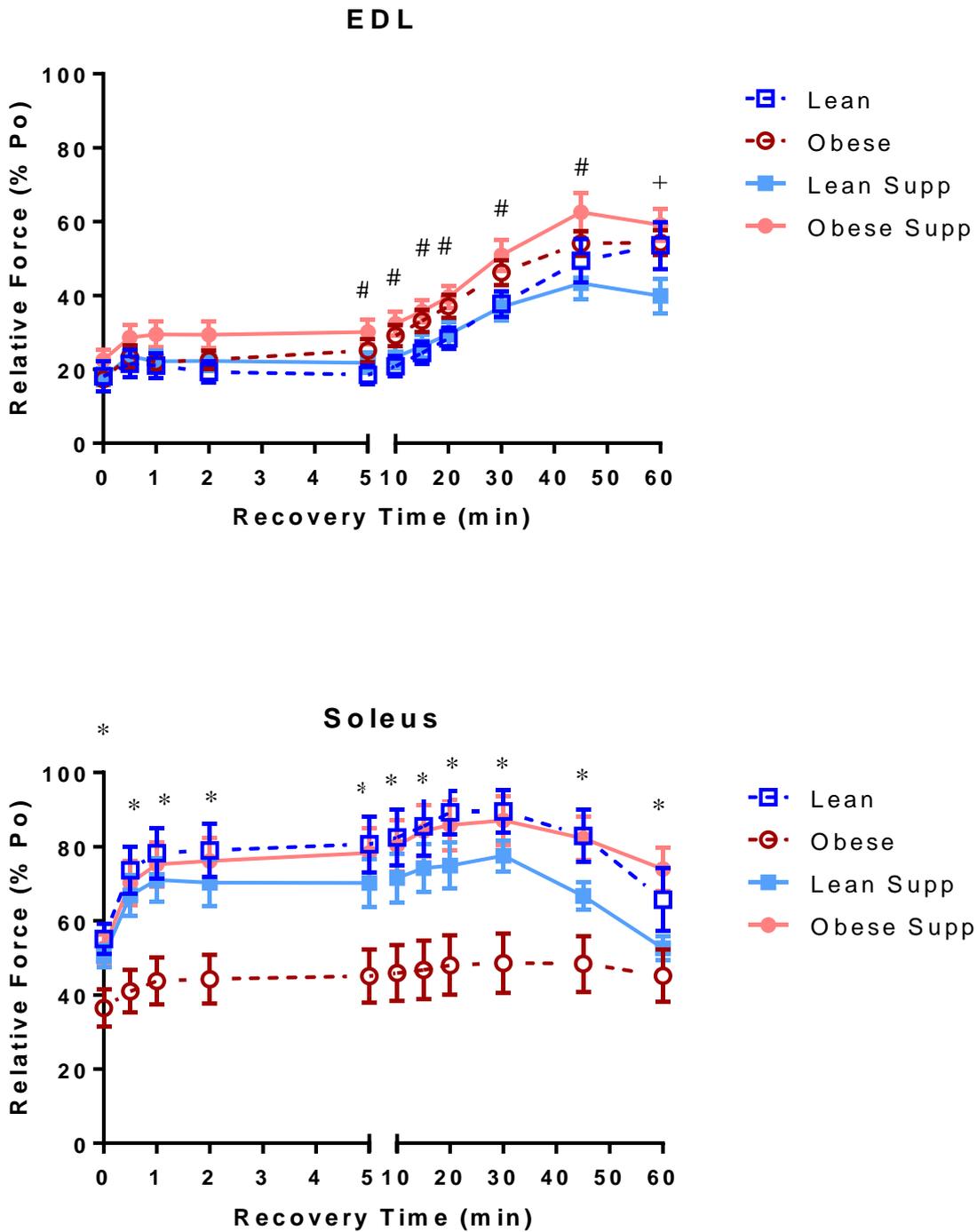


Figure 4.6 Recovery of EDL and Soleus muscle after a fatigue protocol in Lean (n=12), Obese (n=10), LSupp (n=10) and ObSupp (n=11) ZDF rats. Results are expressed as mean \pm SEM. * Significant difference between Obese vs Lean, and Obese vs ObSupp ($p < 0.05$) at the indicated frequencies. # Significant difference between ObSupp vs Lean only ($p < 0.05$). + Significant difference between LSupp vs ObSupp only ($p < 0.05$).

4.3.5 Muscle Metabolites

Table 4.3 below shows muscle metabolite content including ATP, PCr and Total Cr in EDL and Soleus muscles. In EDL, no differences were detected in PCr or Total Cr content. However with ATP, the ObSupp group had significantly higher ATP content than both the Lean ($P<0.05$) and Obese groups ($p<0.01$).

Cr content was lower in ObSupp soleus muscles compared to Lean ($p<0.01$) and LSupp ($p<0.05$) groups. No other differences between the groups were detected in the soleus muscles.

Table 4.3 Muscle ATP, PCr, and Cr Content in EDL and Soleus Muscle of Lean (n=8), Obese (n=8), LSupp (n=10) and ObSupp (n=10) ZDF Rats.

	Lean (n=11)	Obese (n=9)	LSupp (n=10)	ObSupp (n=11)
<i>EDL</i>	(n=9)	(n=9)	(n=10)	(n=10)
ATP (mmol/kg dw)	32.5 ± 0.36	29.0 ± 0.27	33.8 ± 0.27	34.5 ± 0.37*#
PCr (mmol/kg dw)	46.2 ± 0.44	52.4 ± 0.42	54.8 ± 0.29	48.7 ± 0.37
Cr (mmol/kg dw)	57.8 ± 0.44	58.4 ± 0.39	65.2 ± 0.25	61.4 ± 0.38
<i>Soleus</i>	(n=7)	(n=7)	(n=9)	(n=7)
ATP (mmol/kg dw)	17.9 ± 0.23	17.6 ± 0.16	19.2 ± 0.20	18.9 ± 0.37
PCr (mmol/kg dw)	35.4 ± 0.47	31.8 ± 0.44	39.6 ± 0.26	29.2 ± 0.40
Cr (mmol/kg dw)	54.3 ± 0.40	44.5 ± 0.44	48.7 ± 0.33	35.4 ± 0.32*##

Results are expressed as means ± SEM. *Significant difference compared to Lean ($p<0.05$). #Significant difference compared to Obese ($p<0.01$). **Significant difference compared to Lean ($p<0.01$). ##Significant difference compared to LSupp ($p<0.05$).

4.3.6 Contractile Proteins

Contractile and total proteins were determined in both EDL and Soleus (Table 4.4). Contractile proteins in the obese group were significantly lower compared to Lean ($p<0.05$). No other differences were found between any other groups, including supplemented animals. The percentage of contractile proteins over total proteins in EDL only was significantly lower

in the Obese compared to Lean group ($p < 0.05$), with supplementation significantly increasing this in the ObSupp compared to Obese group ($p < 0.05$).

Table 4.4 Contractile Proteins in EDL and Soleus Muscles of ZDF rats.

	Lean	Obese	LSupp	ObSupp
Contractile Protein EDL (ug/mg muscle)	5038 ± 378.8 (n=9)	3289 ± 656.3* (n=6)	4165 ± 314.7 (n=9)	4480 ± 520.2 (n=11)
Contractile Protein Soleus (ug/mg muscle)	2978 ± 425.7 (n=7)	3368 ± 343.5 (n=8)	3347 ± 432.5 (n=10)	3845 ± 421.4 (n=13)
Total Protein EDL (mg/mg muscle)	169 ± 17.2 (n=9)	138 ± 31.6 (n=6)	155 ± 25.1 (n=9)	154 ± 32.3 (n=11)
Total Protein Soleus (mg/mg muscle)	149 ± 28.4 (n=7)	130 ± 15.8 (n=8)	205 ± 33.7 (n=10)	174 ± 10.4 (n=13)
Contractile/Total Protein (%) EDL	17 ± 0.3 (9)	5.7 ± 0.2* (n=6)	14 ± 0.4 (n=9)	10 ± 0.2# (n=11)
Contractile/Total Protein (%) Soleus	2.7 ± 0.2 (n=7)	3.0 ± 0.2 (n=8)	2.1 ± 0.2 (n=10)	2.5 ± 0.1 (n=13)

Results are expressed as means ± SEM. * Significant difference compared to Lean ($p < 0.05$). # Significant difference compared to Obese ($p < 0.05$).

4.3.7 Fibre Type Proportions in EDL and Soleus

Since not all sections were able to clearly differentiate the subset of type II fibres, Table 4.5 shows the proportion of Type I and Type II muscle fibres determined in EDL and soleus muscles.

Table 4.5 Proportion of Type I and II Fibres in EDL and Soleus of ZDF Rats.

	EDL			Soleus		
	Type I Fibres (%)		Type II Fibres (%)	Type I Fibres (%)		Type II Fibres (%)
Lean	4.22 ± 0.99	n=5	95.78 ± 0.99	96.04 ± 1.4	n=4	5.28 ± 0.68
Obese	4.72 ± 1.01	n=4	95.28 ± 1.01	90.55 ± 1.86*	n=5	8.57 ± 0.79*
LSupp	8.80 ± 1.08*	n=3	91.20 ± 1.08*	92.92 ± 3.84	n=4	15.81 ± 3.94
ObSupp	6.47 ± 0.46	n=4	93.53 ± 0.46	86.06 ± 2.54	n=4	13.94 ± 2.54

Results are expressed as means ± SEM. *Significant difference compared to Lean ($p < 0.05$).

Obese rats showed no differences in fibre type proportions in EDL compared to Lean littermates. Supplementation significantly increased the proportion of Type I in EDL of Lean animals ($p < 0.05$), whilst decreasing the proportion of Type II fibres. A similar trend was observed in the ObSupp animals compared to Obese, although this was not significant. In soleus, Obese rats had a significantly lower proportion of Type I fibres compared to Lean animals ($p < 0.05$), and a concomitant higher proportion of Type II fibres. No significant differences were found following supplementation.

4.3.8 Muscle Atrophy

As indicated in Table 4.1, EDL muscle weights were significantly lower in the Obese and ObSupp compared to Lean animals ($p < 0.01$) with supplementation significantly increasing muscle weights in the LSupp versus Lean groups ($p < 0.01$). Correspondingly, EDL type II fibre area was significantly smaller in the Obese and ObSupp groups compared to Lean littermates ($p < 0.01$), however fibre area was not increased with supplementation across any of the groups (see Table 4.6). Indeed the EDL muscle mass to body mass ratio, showed that Obese muscles are significantly smaller compared to Lean animals ($0.284 \pm 0.017\text{mg/g}$ versus $0.518 \pm 0.034\text{mg/g}$ respectively, $p < 0.01$), however, the supplements had no effect on muscle mass to body mass ratio (Refer to Table 4.1).

Soleus muscle weights were only significantly higher in the LSupp group compared to the Lean animals ($p < 0.01$). Fibre areas were not different across any of the animal groups despite slight reductions in Obese rats. When comparing the soleus muscle mass to body mass ratios, Obese soleus muscles were significantly smaller compared to Lean animals ($0.285 \pm 0.030\text{mg/g}$ versus $0.409 \pm 0.045\text{mg/g}$, respectively, $p < 0.01$), however, the supplements had no effect on muscle mass to body mass ratio (Refer to Table 4.1). Finally, both type I and

type II fibres in the EDL were significantly smaller than those in Soleus, which has also recently been shown in chemically-induced diabetes (Nonaka et al., 2014).

Table 4.6 Skeletal Muscle Fibre Area of ZDF Rats.

		Lean	Obese	LSupp	ObSupp
EDL Fibre Area (μm^2)	Type I	1028 \pm 174	871 \pm 101	1160 \pm 167	821 \pm 16
	Type II	2513 \pm 83	2012 \pm 153*	2453 \pm 146	1897 \pm 187*
Soleus Fibre Area (μm^2)	Type I^a	2788 \pm 118	2597 \pm 93	2843 \pm 252	2965 \pm 231
	Type II^a	6987 \pm 1004	6486 \pm 1060	4237 \pm 345	5307 \pm 381

Results are expressed as mean \pm SEM. *Significant difference compared to Lean animals ($p < 0.01$). ^a Significant difference compared to EDL ($p < 0.05$).

4.3.9 Plasma Lactate Concentrations and Capillary Density

Plasma lactate concentration was obtained simultaneously to glucose concentration as discussed in chapter 3. From Table 4.7 it can be seen that ZDF rats had almost double the amount of lactic acid compared to their Lean littermates ($p < 0.05$). Supplementation did not have any effect on plasma lactic acid concentrations in Lean animals, however the ObSupp group had approximately 1.5 times more lactate compared to the Obese group ($p < 0.05$), and almost 3 times more lactate compared to LSupp group ($p < 0.01$).

Table 4.7 Plasma Lactate Concentrations and Skeletal Muscle Capillary Density in ZDF Rats.

	Lean	Obese	LSupp	ObSupp
Lactate (mmol/L)	4.6 \pm 1.6 (n=6)	7.8 \pm 1.0(n=8)*	4.3 \pm 1.1 (n=9)	12.8 \pm 1.9 (n=11)#
Capillary Density EDL (No. per mm^2)	0.5 \pm 0.04 (n=6)	0.3 \pm 0.08 (n=3)*	0.49 \pm 0.06 (n=6)	0.44 \pm 0.01 (n=3)
Capillary Density Soleus (No. per mm^2)	0.53 \pm 0.04 (n=6)	0.39 \pm 0.04 (n=3)**	0.47 \pm 0.04 (n=5)	0.38 \pm 0.06 (n=3)

Results are expressed as mean \pm SEM. * Significant difference compared to Lean, LSupp, and ObSupp animals ($p < 0.05$). # Significant difference compared to Lean and LSupp animals ($p < 0.01$). ** Significant different compared Lean ($p < 0.05$).

Obese ZDF rats had a significantly lower capillary density compared to Lean ZDF rats in both EDL and Soleus, ($p < 0.05$). Creatine, whey and aspirin increased the number of capillaries found in EDL muscles of the ObSupp group ($p < 0.05$), such that they were comparable to their Lean littermates. The supplements however, had no effect on soleus muscles

4.4 Discussion

This study aimed to characterise some of the contractile properties of Obese ZDF rats compared to their Lean littermates, and explore skeletal muscle fatigue and recovery indices in this strain of rat, which is commonly used as an animal model of T2D (Peterson et al., 1990; Peterson, 2001; Srinivasan and Ramarao, 2007). It was further determined if Creatine, Whey and Aspirin could attenuate any negative impact of insulin resistance and T2D on skeletal muscle contractile function. Overall, few significant differences in contractile properties were found between Obese ZDF and Lean ZDF rats in EDL or Soleus muscles, and as such supplementation did little to alter these properties. Soleus muscle from Obese ZDF rats however did fatigue significantly more than Lean ZDF rats, and the supplementation significantly attenuated this such that there were no differences compared to Lean littermates. Supplementation also showed improvements to the fatigue index in the EDL muscle of Obese ZDF animals, albeit this was not significant, however no effect was seen in Lean animals for either muscle group. Overall, muscle recovery was significantly better in EDL ObSupp compared to Lean animals, whereas in Soleus, Obese animal recovery was significantly lower compared to Lean animals and Obese supplemented animals.

Skeletal muscle relative P_t , P_o , and P_t/P_o ratios were not observed to be different between Obese and Lean ZDF rats, despite the atrophic changes to muscle morphology observed in

Chapter 3. This finding is consistent with a previous study in Ob/Ob mice (Warmington et al., 2000) and C57BL/6J mice fed a 60% high fat diet (Shortreed et al., 2009). When looking at relative force production, a trend for lower forces were observed in Obese compared to Lean rats. This may indicate the start of muscle dysfunction in these young adults, and as such, investigating these properties as the disease progresses would be of interest to determine if progressive deterioration is continuous. Indeed it has been shown that progressive reductions in tetanic force production does occur with increasing diabetes duration (Cotter et al., 1993). TTP and $\frac{1}{2}$ RT in this study were not statistically different between animal groups in either muscle, albeit, there was a trend towards slower times in Obese compared to Lean animals. This was not consistent with Warmington et al. (2000) who demonstrated slower TTP and $\frac{1}{2}$ RT in Ob/Ob mice in both EDL and Soleus, which suggests potential problems with SR function. Animals were approximately 5 months of age in that study and so disease progression may have impacted muscle function to a greater degree compared to rats of this study who were approximately 14 weeks of age. This might indicate that SR function early in the disease may either be normal, or starting to decrease in function, as contraction and relaxation of muscle fibres is dependent on optimal the Ca^{2+} uptake and release from SERCA pumps. However, others have shown changes in TTP, $\frac{1}{2}$ RT and tetanic tension in Soleus but not EDL (Cameron et al., 1990; McGuire and MacDermott, 1999) after two months of chemically-induced diabetes.

Supplementation with Creatine, Whey and Aspirin showed some promising results where both relative and absolute Pt and Po were showing a trend to beginning improved, but once again not statistically significant. No other studies to date have looked at utilising these supplements to treat muscle function in T2D. In EDL but not soleus, TTP and $\frac{1}{2}$ RT in ObSupp animals also showed slight improvements albeit non-significant. This may be due to

creatine supplementation allowing greater production of ATP to fuel the Ca^{2+} -ATPase driven pump which speed the process of Ca^{2+} uptake into the sarcoplasmic reticulum and hence quicker $\frac{1}{2}\text{RT}$ times. However, a few possible explanations for the non-significant findings include dosage. The dosage used in this study is often used in healthy adults, and as such, a higher dose may be required in disease states that could benefit from them. In addition, these supplements are also used in conjunction with exercise for better uptake into muscle and greater effect (Cribb and Hayes, 2006a; Pasiakos et al., 2015). This study aimed to look at the effects of the supplement regime alone and so future studies including a combination of exercise training and supplementation may increase any benefit observed, as it does in athletes undergoing training regimes (Sundell et al., 2011). Of course, given there was no significant reduction in contractile function of Obese animals, supplementation may be unlikely to show an effect. However given that trained healthy athletes benefit from supplementation with creatine and whey, it may be possible to show improvements in trained diabetic patients. Once again, given the young age of these animals, future research to look at the time course of the progression of muscle dysfunction in this disease would be beneficial, as well to determine if supplementation could attenuate disease progression.

At two sub-optimal frequencies in EDL, Obese animals produced a lower percentage of their maximal force compared to lean animals. These results are inconsistent with that of Warmington et al. (2000), who found that lean mice in fact produced lower P_0 at sub-optimal frequencies compared to Ob/Ob mice in both EDL and Soleus. Those results are consistent with the slowed contraction times seen in Ob/Ob mice resulting in higher levels of fused tetanic contractions at lower frequencies resulting in more force. Given that there was no difference in contraction times in the current study, the higher forces in the Lean EDL muscles could occur due to more calcium released in the lean muscles, suggesting higher SR

calcium and thus higher release. SR function is explored further in Chapter 5. Alternatively, there may be a higher sensitivity to calcium, however analysis of single muscle fibres would be needed to elucidate whether this is the case.

The fatigue protocol indicated that for EDL, whilst no differences were seen in fatigue resistance across animal groups, there was a tendency for Obese animals to reach a slightly lower force percentage compared to Lean animals, and this was attenuated with supplementation. A similar trend was found in ATP concentrations pre-fatigue in EDL muscles, where animals commenced with slightly lower concentrations in Obese compared to Lean and significantly more in ObSupp compared to Obese. This coincided with the ObSupp also demonstrating ~5% higher fatigue resistance. It is likely that whey protein influenced the higher ATP concentrations by improving mitochondrial function and ATP production. This also highlights the role of initial ATP concentrations in fatigue rate of the Obese animals, and may also reflect the ability of the Obese animals to regenerate ATP, via the mitochondria in sufficient quantities to match functional demand. The ability of the mitochondria to produce ATP in these animals will be discussed further in Chapter 6.

The tendency for greater fatigue in the Obese animals observed in this study is consistent with higher susceptibility to fatigue reported in diabetic sufferers (Tomas-Carus et al., 2015). In an intact system, supply of nutrients to the muscle via optimal blood supply may be an important factor influencing fatigue. Indeed in this study it was found that Obese animals had significantly fewer capillaries in EDL compared to all other animal groups. This may indicate a less than sufficient blood supply to skeletal muscles and hence less nutrients available to muscle metabolism to regenerate ATP. Reductions in O₂ delivery in EDL muscles of prediabetic ZDF rats has been reported (Ellis et al., 2010) and furthermore, insulin attenuates

the O₂-dependent release of ATP from RBCs (Hanson et al., 2009) potentially explaining the slight impairments in fatigue. The supplementation, whilst having no effect on Lean animal capillary density, showed significant increases in the Obese muscles. This would lead to improved O₂ delivery and would be a contributor to any improvements in the fatigue index and recovery seen in the ObSupp group. To the author's knowledge, this is the first study to test the effects of creatine, whey and aspirin on skeletal muscle capillary density in T2D and how this relates to fatigue and recovery. As such, further studies to determine the role of each individual supplement on these variables would be required, as well as measuring these parameters in an intact system. However it could be speculated that creatine may have been partially responsible for the increase in capillary density, given that (Moraes et al., 2014) found that creatine supplementation increased skin capillary density and recruitment in healthy, moderately physical young adults. Aspirin has also been shown to increase both capillary density and the capillary-to-muscle ratio in rat hearts that have endured a myocardial infarct (Van Kerckhoven et al., 2004), and hence also partially responsible for the changes to capillary density seen above. Interestingly, the improved recovery from fatigue in the current study was obtained in isolated *ex vivo* muscle contraction experiments. Thus, O₂ delivery would not be a relevant mechanism, suggesting that intramuscular storage of O₂ by myoglobin may have subsequently been increased. Additionally, improved oxidative function may have also played a role in the fatigue recovery process. Indeed, plasma lactate was significantly higher in the Obese group compared to the Lean groups, suggestive of a reduced O₂ supply and/or use that would result in increased need for anaerobic glycolysis to fuel ATP regeneration. However, there was, if anything, further increases in lactate concentrations in the ObSupp animals, demonstrating the complex interplay between whole body metabolism and O₂ delivery in the intact system and potential improvements at the organ and organelle level.

Whilst, as expected, we showed that EDL muscle in all animal groups fatigued to a greater extent than soleus muscle due to the higher proportion of fast-fatiguable type II fibres in EDL, we expected EDL to show more stark results pertaining to fatigue in Obese compared to Lean rats. However this was not the case and interestingly, soleus showed significantly more fatigue in the Obese group compared to the Lean, with the supplements significantly improving this (possibly through the direct actions of creatine providing more substrate for the creatine kinase reaction to produce ATP) such that there were no differences compared to the Lean group. Once again, the supplements appeared to have no effect on fatigue in the Lean animals. ATP levels in soleus were not significantly different across the animal groups and this may be explained again by the utilisation of Cr and PCr to maintain ATP, particularly in the ObSupp group. Once again, capillary density has been shown to be significantly reduced in Obese soleus muscle, as such another plausible explanation for the higher fatigue index in T2D. However unlike EDL, the supplementation did not increase the number of capillaries for slow twitch muscles, indicating a fibre type specific action of the supplements. Unfortunately, Ellis et al. (2010) did not study the effects on O₂ delivery in soleus muscles and thus comparison cannot be made as they were for the EDL. Furthermore, the ObSupp group had double the lactate, so one might expect no improvements in fatigue, however in this study the fatigue index was improved in soleus perhaps indicating that the muscles were capable of working harder. Alternatively, this can be explained by the fibre type switch found in soleus where there was an increase in the proportion of Type II muscle fibres. This fibre switch has also occurred in other animal and human studies (Torgan et al., 1989; Wade et al., 1990; He et al., 1995; Kristensen et al., 2014) deeming muscles less oxidative and more prone to fatigue. Adachi et al. (2007), further showed ZDF rats to have a decrease in Type IIa fibres yet higher proportion of type IIc fibres in soleus, with an overall

decrease in SDH activity of Type I and II fibres of soleus muscle further explaining the fatigue found in this strain of rats. Due to inconsistency of ATPase staining procedure and limitations in samples, we could not determine the breakdown of Type IIa and IIb fibers in this group of animals. Nevertheless, the fibre switches are not surprising given that during long periods of inactivity, slow twitch fibres have been found to transform to fast twitch fibres (Haggmark and Thorstensson, 1979; Howald, 1982). However, there seems to be different findings across different muscles and different models of obesity and T2D. For example, Yasuda et al. (2001); (2002; 2006) report that the fibre type distribution of soleus in OLETF and GK rats to have a lower percentage of type IIa fibre and higher percentage of IIb fibres. Warmington et al. (2000) showed the opposite effect where Obese muscles were in fact more fatigue resistant than Lean muscle, and fibre proportions were of a more slow type profile and hence less fatigable. Specifically that EDL had a lower percentage of Type IIb fibres and more Type IIa in Obese, whereas soleus had a lower percentage of Type IIb fibres.

EDL muscle recovery was not different between Obese and Lean animals at any of the time points, however given that Obese animals were slightly more fatigued (albeit not significant), their force production in the first 30 seconds of recovery indicated a better recovery rate as they reached the same levels as Lean animals. This is the opposite of what occurred in the Ob/Ob mice of the study by Warmington et al. (2000). Supplementation significantly improved recovery in the ObSupp animals but not in LSupp animals and once again may be a result of the higher ATP content in the group of animals or it may be that there were an increase in the number of capillaries of the ObSupp group allow better clearance of muscle metabolites.

In contrast, Obese soleus muscle recovery was significantly lower compared to Lean animals which again can be explained by the fibre switch that occurred in this muscle. Supplementation significantly improved recovery in Obese animals whereby the main plausible explanation for this improvement is enhanced creatine kinase production of ATP, given the significant reduction in PCr and Cr found in soleus ObSupp animals.

4.5 Conclusion

In conclusion, muscle peak twitch and tetanic forces were not found to be affected by the insulin resistant state, with no significant alterations to TTP and $\frac{1}{2}RT$ detected in either EDL or Soleus, and supplementation with Creatine, Whey and Aspirin having little effect on these parameters. Obese compared to Lean animals were more affected by the fatigue protocol, with concurrent reductions in recovery. Supplementation appears to have had some beneficial effects on reducing the fatigue index in skeletal muscle of Obese animals as well as improving muscle recovery from fatigue. Whilst the mechanisms and specific modes of action of each supplement require further investigation, these findings have important implications in improving the exercise intolerance found in T2D patients, which may impact on developing lifestyle changes required to treat this condition and improve the deleterious effects of the insulin resistant, hyperglycaemic state for this cohort of people.

CHAPTER 5

Effects of Creatine, Whey and Aspirin on Skeletal Muscle SR Function in Type 2 Diabetes.

5.1 Introduction

Ca^{2+} is an important ion required for the normal function of muscle cells. It regulates muscle contraction and relaxation, as well as modulates other cellular functions (Shamoo, 1985). Thus, regulating intracellular Ca^{2+} is important as high concentrations has been found to trigger the cycle of cell damage, resulting in muscle degradation and proteolysis (Berchtold et al., 2000). The SR is one of the main membrane transport systems involved in the regulation of intracellular Ca^{2+} , releasing and re-uptaking Ca^{2+} to allow muscle contraction and relaxation to take place. The process of Ca^{2+} resequestration into the SR occurs via the SERCA pump, and the efficiency of the pump has been found to be maintained through the phosphocreatine (PCr) system by converting adenosine diphosphate (ADP) and hydrogen ion (H^+) to ATP, which fuels pumping activity.

If Ca^{2+} is not sequestered efficiently into the SR, as has been shown in cardiac tissue of patients with T2D (Belke et al., 2004), it may result in muscle degradation as well as impaired contractile function. In fact, depletion of PCr stores has been found to impair SR Ca^{2+} uptake in patients with T2D (Scheuermann-Freestone et al., 2003), which contributes to poor muscle function. Hence the potential of increasing PCr as a means to improve muscle function in patients suffering from various myopathies and exercise intolerance, such as T2D, is enormous.

Whilst it is well established that SR activity is impaired in cardiac tissue of Type 2 Diabetics, controversy exists in the literature pertaining to SR function in skeletal muscle. Eibschutz et al. (1984) have observed reductions in Ca^{2+} uptake by the SR ATPase pump into fast twitch muscle fibres of chemically-induced diabetic rats. On the other hand, Ganguly et al. (1986) demonstrated enhanced SR Ca^{2+} pump activity and hyperfunction of hamstring muscle,

which contain approximately 50% fast twitch fibres (Dahmane et al., 2006), in STZ-induced diabetic rats. The methodological inconsistencies pertaining to muscle type, fibre-type, and disease stage make drawing conclusions difficult.

Thus, while it is not clear whether there are changes in SR function in T2D, improving SR function would be beneficial for contractile function and improving the atrophy observed in this animal model as a result of activation of the ubiquitin proteasome pathway. Therefore the aim of this study was to determine if ZDF rats impaired skeletal muscle SR function is a characteristic of ZDF skeletal muscle, and, if such changes are fibre-type specific. A secondary aim was to determine if Creatine, Whey and Aspirin treatment could improve SR Ca^{2+} -ATPase function in T2D.

5.2 Methods

Information about animal groups and supplementation are found in chapters 2.1 to 2.3. The methods used to determine SR Ca^{2+} -ATPase function in RG and WG muscle are detailed in chapter 2.5, SR protein determination is detailed in chapter 2.7.2, and metabolites are detailed in chapter 2.8. In addition, a methodological pilot study was conducted to determine the best substrate combination to provide SR vesicles to achieve optimal assay function. The first substrate utilised was 5mM ATP as described in the original methodology (Warmington et al., 1996). Previous studies from our lab (unpublished data), showed that a combination of ATP, PCr and ADP provided the optimal combination for SR studies as these were the CK intermediates used for the SR Ca^{2+} ATPase pump (Rybalka and Hayes, 2007). Therefore, the second substrate combination utilised in this study included these CK intermediates in concentrations of 7mM ATP + 40mM PCr + 12 μ M ADP. These were based on *in vivo* concentrations found in Zucker Lean and Obese rats (Klein et al., 1994; De Feyter et al.,

2008). Two additional substrate combinations were utilised to determine if providing Obese animals with Lean substrate conditions would improve SR function (substrate 3), or if providing the Lean animals with Obese conditions, would impair SR function in Lean animals (substrate 4). Therefore substrate 3 contained a mixture of substrate 2 plus 3mM inorganic phosphate (Pi), and substrate 4 contained substrate 2 plus 2mM Pi.

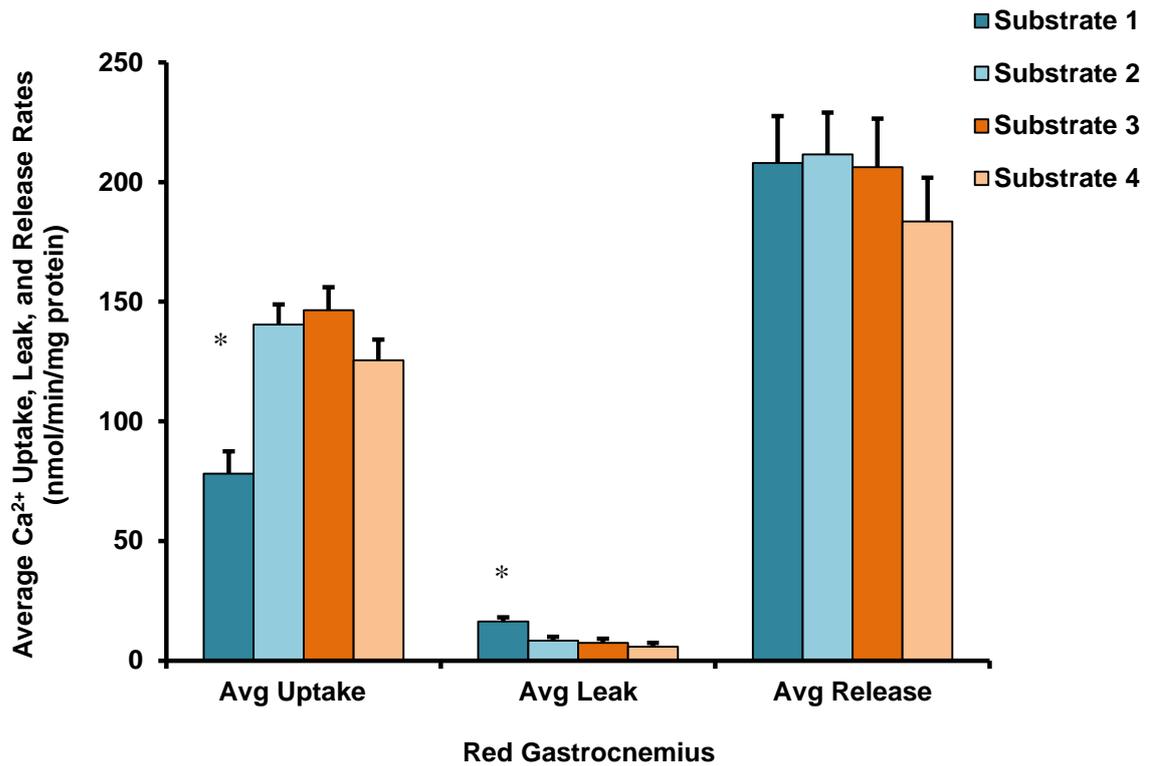
5.2.1 Statistical Analysis

Multivariate analysis was used to determine differences between substrates for each of RG and WG, irrespective of animal group. Similarly, multivariate analysis was used to detect differences between animal groups irrespective of substrate used. A further one-way ANOVA was used to detect differences in SR function between animal groups using the optimum substrate found in the pilot study. This was performed for RG and WG separately. A post hoc Tukey test was performed to determine where the differences were. Significance was set at $p < 0.05$. All results are expressed as means \pm SEM.

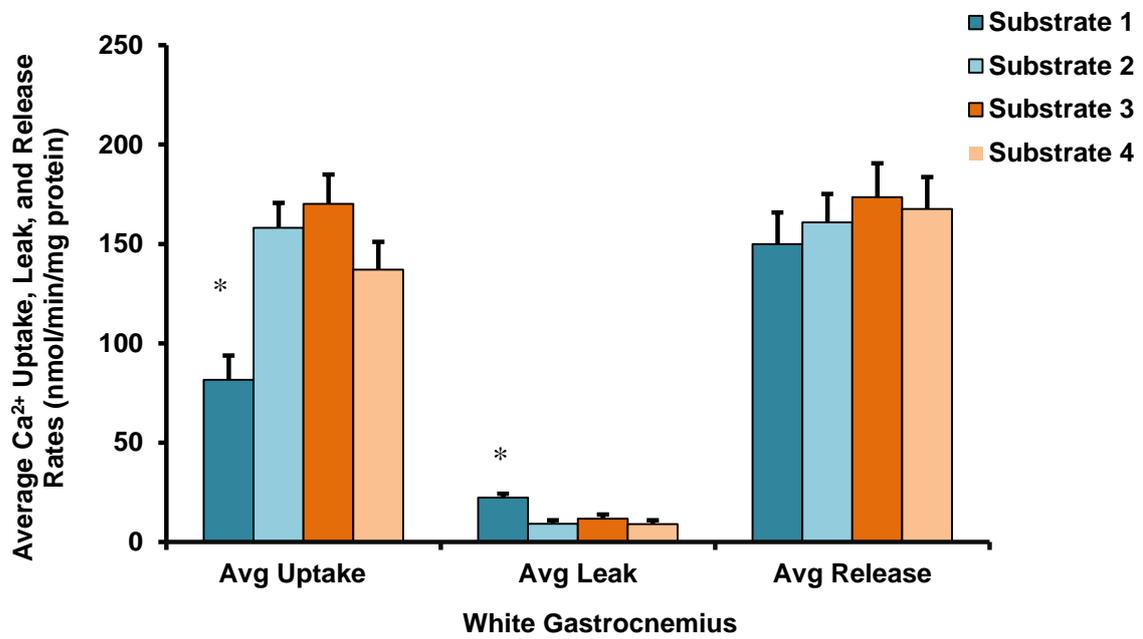
5.3 Results

5.3.1 Methodological Pilot Study

A pilot study was done in order to determine if there were differences in SR function depending on the energy substrate available to the Ca^{2+} -ATPase pump. Overall it was found that in both red and white muscles, substrate 1 (containing only MgATP) produced significantly slower Ca^{2+} uptake rates compared to all other substrates tested ($p < 0.01$) (Figure 5.0). No differences were observed between all other substrates. Leak rates were significantly higher for substrate 1 compared to all other groups ($p < 0.01$), whereas no differences were found for release rates across all substrates.



A.



B.

Figure 5.0 Average Ca^{2+} Uptake, Leak and Release Rates for each substrate in (A) Red Gastrocnemius (slow twitch), and (B) White Gastrocnemius (fast twitch) muscle. (n=25, 29, 20, 23 for substrates 1 – 4 respectively) Results are expressed as means \pm SEM and are pooled samples from all animal groups. * Significant difference to all other substrates ($p < 0.01$).

Analysis of animal groups across all substrates indicated that Obese animals had significantly slower SR Ca^{2+} uptake and release rates for both red and white muscles ($p < 0.01$). Leak rates were significantly slower in white muscles of Obese animals ($p < 0.01$), with a similar trend observed in red muscle ($p = 0.07$) (Figure 5.1).

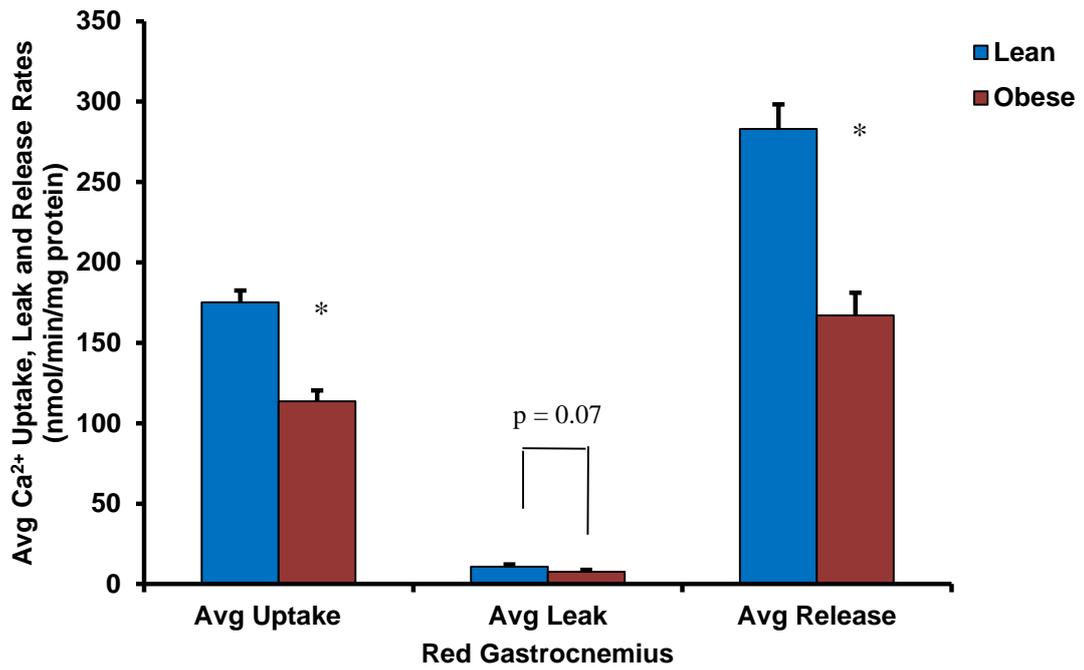
Taken together, results from the substrate pilot study indicated that substrate 2 (containing MgATP, PCr, and ADP) would be best utilised to determine Ca^{2+} transients for final analyses of uptake, leak and release rates. Therefore in order to determine if SR function is impaired if given optimal substrate conditions, Substrate 2 assays were analysed with a two-way ANOVA to determine differences between animal groups and muscle groups and presented in this chapter.

5.3.2 Average SR Calcium Uptake, Leak, Release Rates

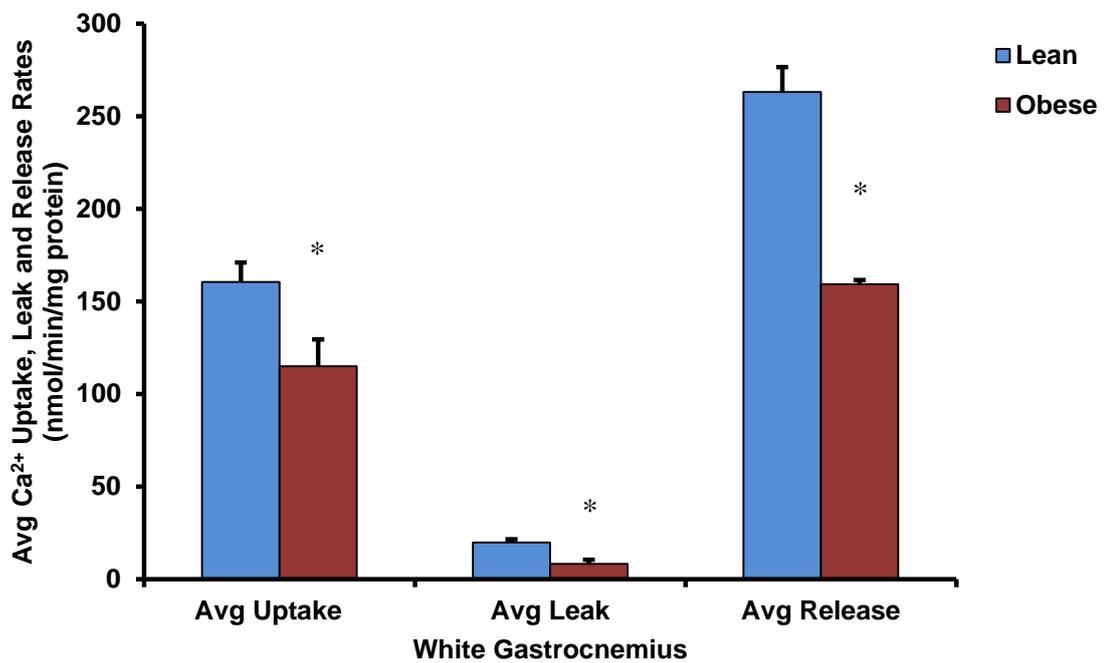
Average Ca^{2+} uptake, leak and release rates were determined for RG and WG muscle as seen in Figure 5.2 – 5.4. Uptake rates showed a strong trend ($p = 0.06$) to being slower in Obese RG muscles compared to littermates. Supplementation had no effect on calcium uptake rates in either Lean or Obese muscles of either muscle group.

RG SR vesicles showed no differences in leak rates between animal groups, except they were 75% lower in the ObSupp compared to LSupp group ($p < 0.01$). WG muscles showed a trend toward slower leak rates in Obese rats compared to Lean ($p = 0.06$). No other differences were observed.

Finally, release rates showed Obese animals to be almost half that of their Lean littermates ($p < 0.01$), again with supplementation having no effect.

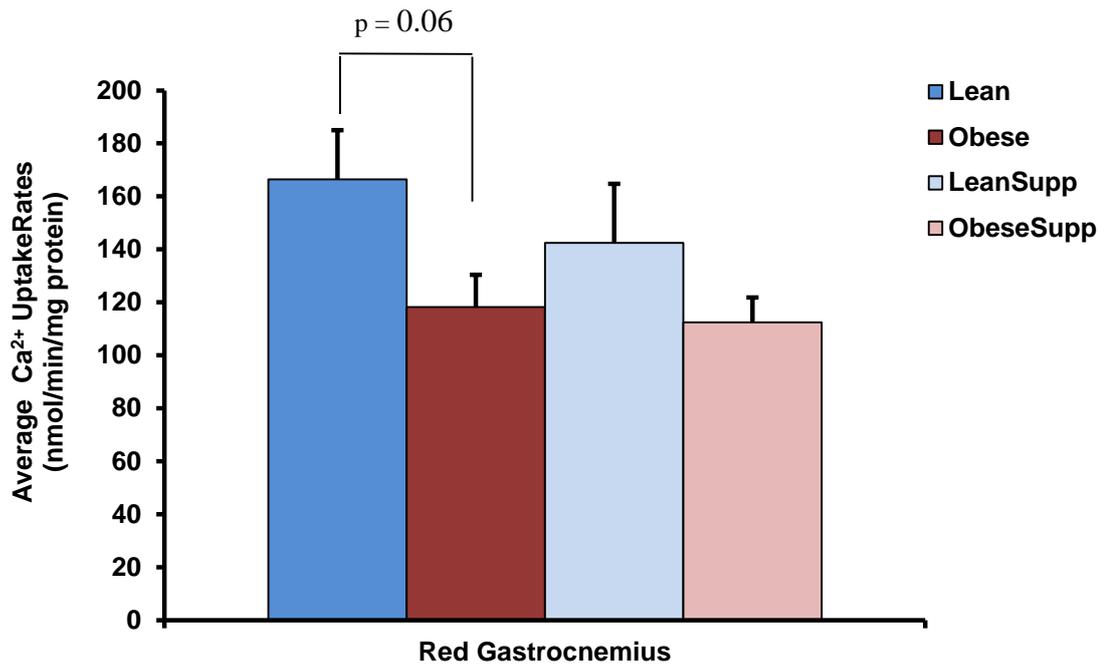


A.

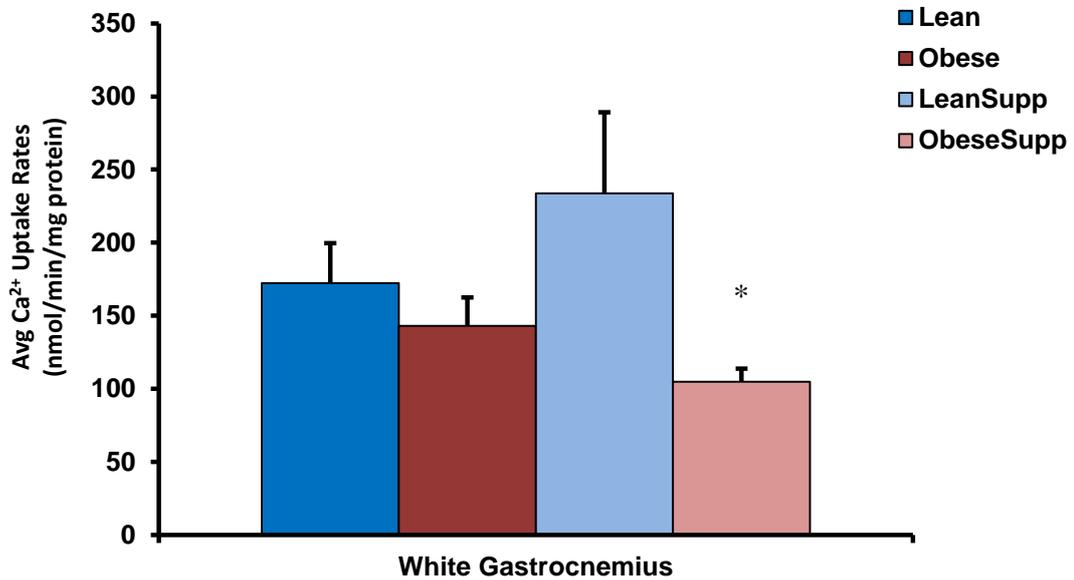


B.

Figure 5.1 Average Ca^{2+} Uptake, Leak and Release Rates for Lean ($n=33$) and Obese ($n=35$) ZDF rats irrespective of Substrate. Results are expressed as means \pm SEM, and are a pooled sample from all substrate groups. * Significant difference to Lean animals ($p<0.01$).

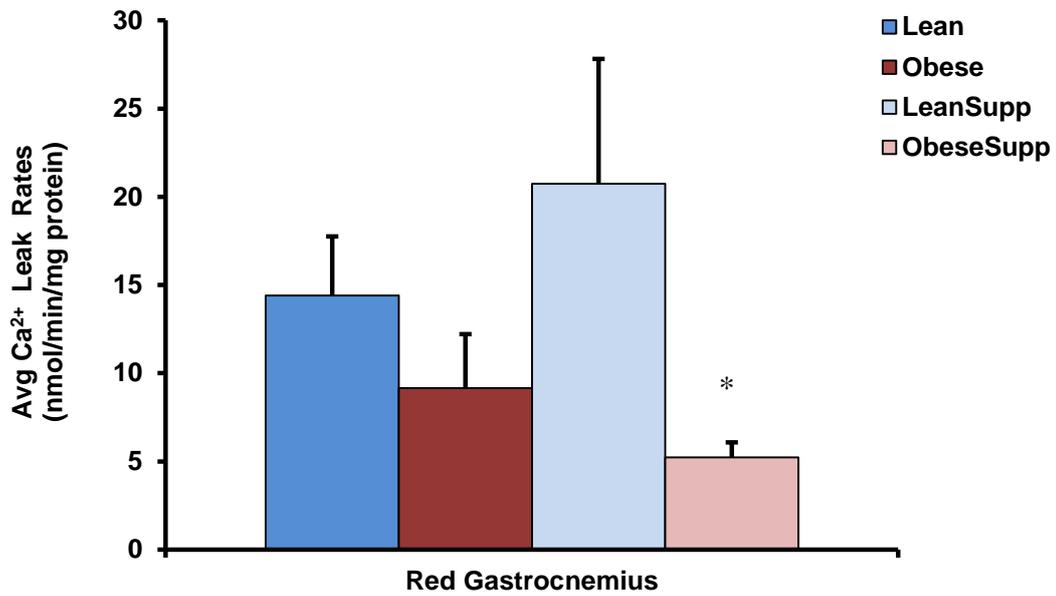


A.

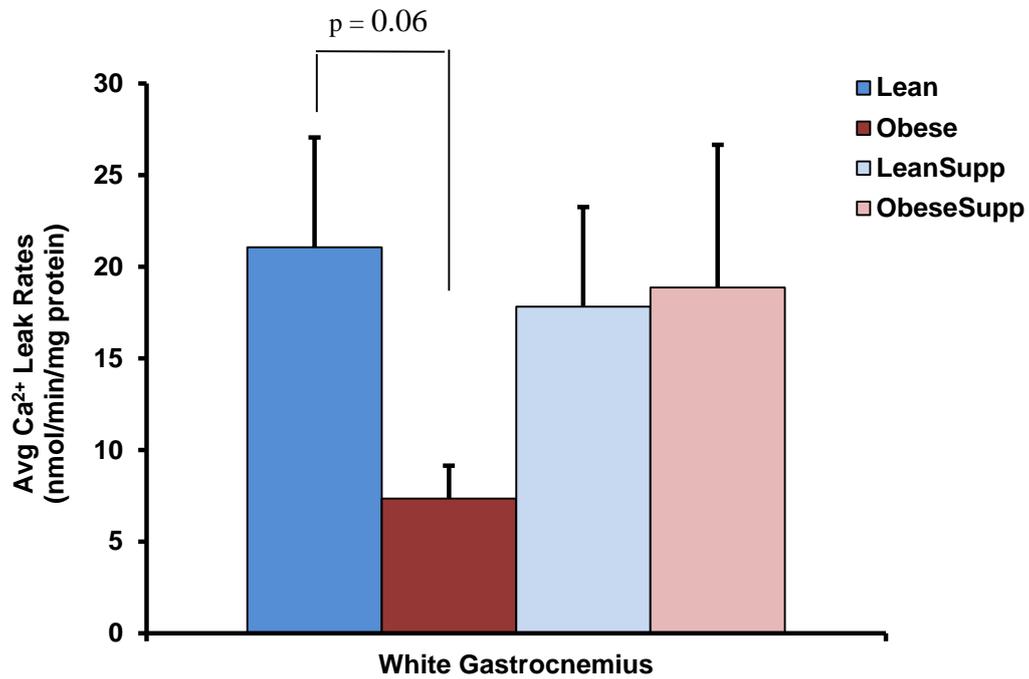


B.

Figure 5.2 Average Ca²⁺ uptake rates into SR vesicles in (A) Red Gastrocnemius (slow twitch), and (B) White Gastrocnemius (fast twitch) muscle of Lean (n=9), Obese (n=11), LSupp (n=9) and ObSupp (n=12) ZDF rats. Results are expressed as means ± SEM. * Significant difference to LSupp animals (p<0.01).

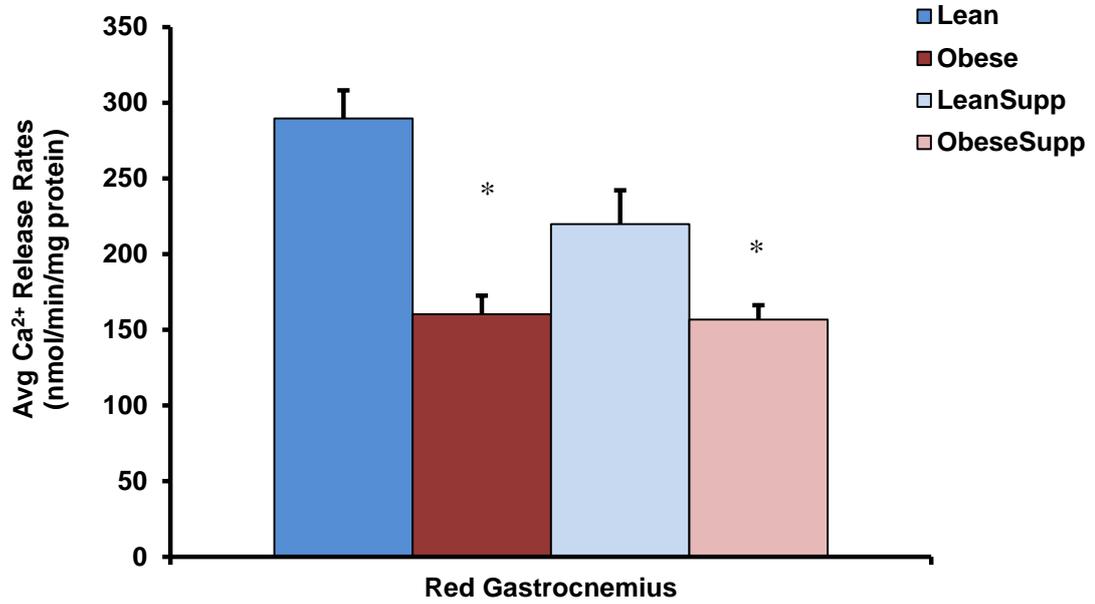


A.

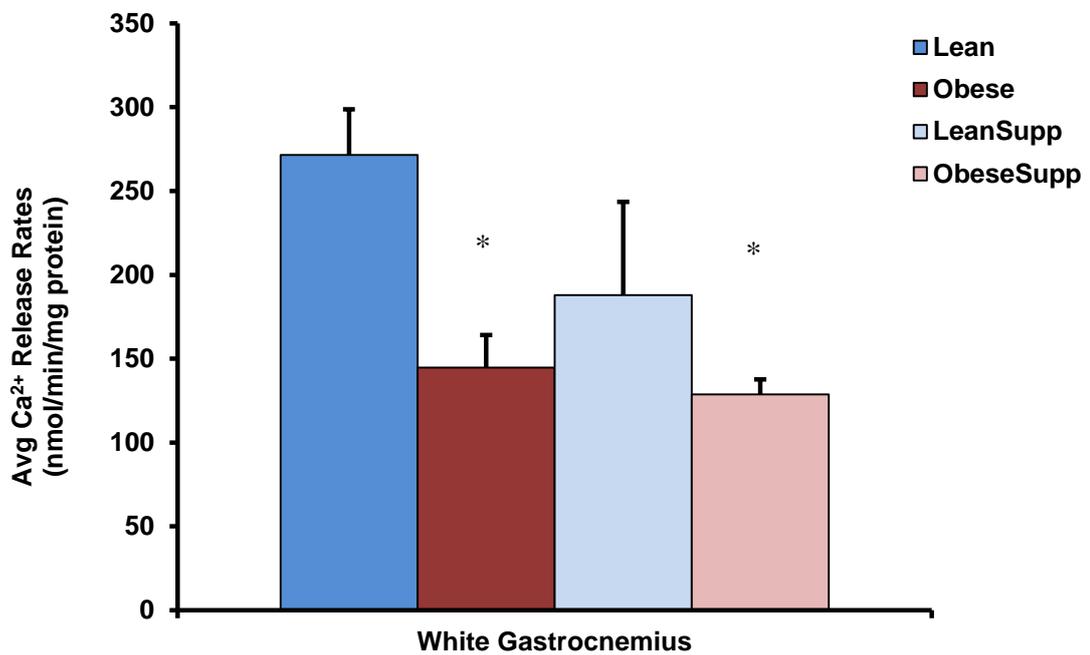


B.

Figure 5.3 Average Ca²⁺ leak rates into SR vesicles in (A) Red Gastrocnemius (slow twitch), and (B) White Gastrocnemius (fast twitch) muscle of Lean (n=9), Obese (n=11), LSupp (n=9) and ObSupp (n=12) ZDF rats. Results are expressed as means ± SEM. * Significant difference compared to LSupp animals (p<0.01).



A.



B.

Figure 5.4 Average Ca²⁺ release rates into SR vesicles in (A) Red Gastrocnemius (slow twitch), and (B) White Gastrocnemius (fast twitch) muscle of Lean (n=9), Obese (n=11), LSupp (n=9) and ObSupp (n=12) ZDF rats. Results are expressed as means ± SEM. * Significant difference to Lean animals (p<0.01).

5.3.3 SR Proteins

SR proteins were determined in each muscle group and graphed in Figure 5.5. Obese animals had significantly more SR proteins compared to Lean littermates ($p < 0.01$) in both RG and WG muscle. Supplementation significantly increased SR proteins in both RG and WG muscle of LSupp compared to Lean, but only in WG muscle of Obese animals ($p < 0.01$).

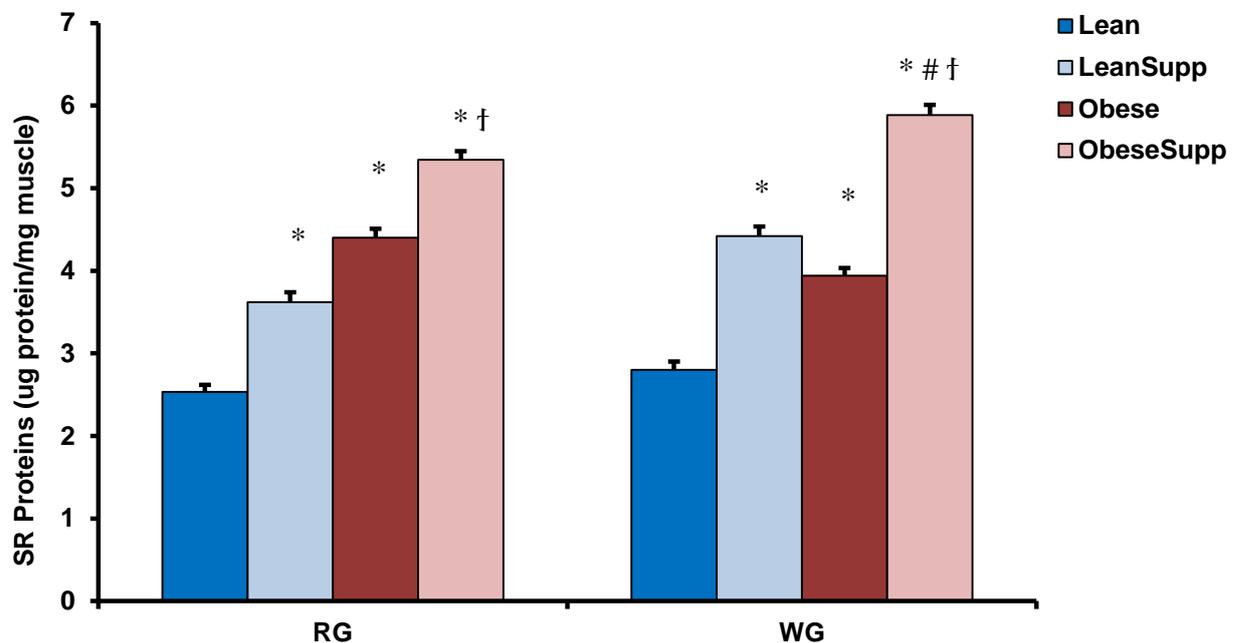


Figure 5.5 SR Proteins in Red Gastrocnemius (RG) and White Gastrocnemius (WG) of Lean (n=9), Obese (n=11), LSupp (n=11) and ObSupp (n=13) ZDF rats. Results are expressed as means \pm SEM. * Significant difference compared to Lean animals ($p < 0.01$). # Significant difference compared to Obese ($p < 0.05$). † Significant difference compared to LSupp ($p < 0.05$).

5.4 Discussion

This study aimed to determine the best energy substrate combination to maximise Ca^{2+} ATPase pump activity in a pilot study, to help provide insights into how SR function might be altered under different energy substrate conditions, and if the experimental protocol is at all limited by these. This study also aimed to examine differences in skeletal muscle SR function in ZDF rats compared to Lean ZDF rats, and whether creatine, whey and aspirin supplementation could ameliorate any of these effects. The major findings of the pilot study were that MgATP alone (substrate 1) resulted in the slowest Ca^{2+} uptake rates and fastest leak rates in both white (predominantly fast twitch) and red (predominantly slow twitch) muscles, but had no effect on the average release rates compared to all other substrates tested (containing varying concentrations of MgATP + PCr + ADP). With regards to SR function, this study found a trend toward slower uptake rates in SR vesicles isolated from red muscle, yet no differences in white muscle of ZDF animals compared to their Lean littermates. Supplementation with a combination of creatine monohydrate, whey protein, and aspirin did not alter this in either the LSupp or ObSupp groups. Leak rates were only significantly reduced in fast twitch muscle, and supplementation did not alter these. Finally, release rates were shown to be significantly slower in both red and white muscles from ZDF animals, once again without any effect of supplementation.

5.4.1 Pilot Study Findings

SR function (Ca^{2+} uptake in particular) has been shown to be dependent on substrate availability, and more effective when combining ATP with PCr and ADP as opposed to ATP alone, given the proximity of CK to the ATPase pump (Korge and Campbell, 1994; Williams et al., 2008). Similarly, the pilot study conducted in this chapter demonstrated faster uptake rates with substrate 2 compared to substrate 1 in utilising the methods by Warmington et al.

(1996). To examine the influence of substrates further, this study looked at substrate concentrations resembling that of the typical intracellular environment of Lean and Obese ZDF rats, namely the different concentrations of inorganic phosphate (Pi) being higher in Obese ZDF rats (Klein et al., 1994; De Feyter et al., 2008), to determine if substrate availability was the rate limiting factor in SR function. We also sought to determine if Lean animals were given Obese substrate conditions, could SR function be altered and vice versa. The results of these additional substrate combinations were similar and hence not discussed individually herein. It can be said that the variations in substrate concentrations were insufficient to alter SR function between animal groups. This suggests that acutely exposing SR vesicles to substrate combinations mimicking the typical diabetic intracellular environment does not impede/impair SR function provided sufficient ATP is present (or can be regenerated). Similarly, placing SR vesicles from diabetic muscles into a typical “Lean” environment does not immediately restore SR function. Thus, changes to SR function in diabetic muscles are likely a long-term maladaptation that may not be easily reversed.

An interesting finding of this pilot study was the significantly higher leak rates observed with substrate 1 compared to all other substrates. In some instances it could be argued that with lower uptake rates, it might be expected to incur lower leak rates due to a reduced Ca^{2+} concentration gradient across the SR membrane. However, in this case, faster leak rates occurred despite the slower uptake rates. This may indicate that the energy substrate available to the system has an influence on leak rates by immediately reuptaking any Ca^{2+} that has leaked. However, in this experiment, Ca^{2+} uptake was inhibited by the addition of thapsigargin, which indicates an alternative mechanism at hand. Chopra et al. (2007) tested whether calsequestrin regulates SR Ca^{2+} release and/or leak directly or indirectly by buffering SR luminal Ca^{2+} . They found that Ca^{2+} leak rates were more closely regulated by

calsequestrin in cardiac myocytes, and that leak more likely occurred via the release channels, irrespective of luminal $[Ca^{2+}]$. However, calsequestrin was not manipulated and/or tested in either of substrate conditions in this study as they were by Chopra et al. (2007). Therefore we can only conclude that some component of the creatine kinase system may have a role in the higher leak rates observed, given that ADP and PCr concentrations were altered in this study. MacDonald and Stephenson (2001) conducted a study in which they tested the rate of SR Ca^{2+} uptake and leak in skinned rat skeletal muscle fibres at different ADP concentrations. They found that as the $[ADP]$ increased, so too did the rate of leak, and furthermore, it did not occur through the RyR. Launikonis and Stephenson (1997) showed that Ruthenium Red (a RyR blocker) had no effect on the SR Ca^{2+} leak, which is also consistent with the Ca^{2+} leak occurring predominantly via an alternative channel or pathway from the RyR. The application of the SR Ca^{2+} pump blocker TBQ was shown to reduce Ca^{2+} leak from the SR (Inesi and Sagara, 1994). Since TBQ reduces the activity of the SR Ca^{2+} pump by binding to the myoplasmic side of the pump like other types of SR Ca^{2+} pump blocking agents (Inesi and Sagara, 1994), a possible explanation for the results in this study is that the SR Ca^{2+} pump is at least partially responsible for the SR Ca^{2+} leak.

In the presence of P_i and ADP, and in the absence of ATP and PCr, it is possible for the SR Ca^{2+} pump to act as a Ca^{2+} - Ca^{2+} exchanger (Chiesi and Wen, 1983; Soler et al., 1990; Dalton et al., 1999). Since ATP resynthesis is unable to meet demand under these conditions, the pump undergoes slippage (Dalton et al., 1999), where the SERCA pump acts as a Ca^{2+} - Ca^{2+} exchanger, transferring luminal Ca^{2+} from a higher $[Ca^{2+}]$ to the myoplasmic compartment with lower $[Ca^{2+}]$. Such a mechanism can explain the results in this pilot study, especially given that there was no PCr in substrate 1, preventing the creatine kinase reaction from

buffering the rising ADP concentrations. Therefore, it is likely that the increase in [ADP], increased slippage of the pump and hence Ca^{2+} leak.

In comparing the release rates across the different substrates, no differences were found contrary to what was hypothesised i.e. greater uptake with substrate 2 would result in a higher SR $[\text{Ca}^{2+}]$ and hence more Ca^{2+} release. This indicates that Ca^{2+} release occurs independently of differences in ADP and PCr availability to the system.

In comparing the two muscle types it was found that there were no differences in either Ca^{2+} uptake or release rates. Although this was expected for the release rates, because all skeletal muscles express the same isoforms of RyR (RyR-1 and -3) (Berchtold et al., 2000; Lanner et al., 2010), this was not expected for the uptake rates. This is because fast- and slow-twitch muscles contain different isoforms of SERCA pumps, which determine the different uptake rates: 1) SERCA1a and -1b are expressed in fast-twitch skeletal muscle; and 2) SERCA2a is expressed in cardiac and slow-twitch skeletal muscle (MacLennan et al., 1997). A plausible explanation is that perhaps the higher fat content found in fast twitch fibres reduced the fluidity of the membrane (as discussed previously), thereby reducing Ca^{2+} movement across the pump.

Overall, this pilot study provided evidence that substrate 2 was the better energy combination to provide to SR vesicles in this type of assay to ensure that the system was not rate limited by to insufficient fuel availability. In addition, uptake, leak and release rates were determined irrespective of substrate utilisation to determine if any differences occurred between animal groups. Given that significant differences were found between animal groups, in order to ensure analysis of results was not impeded by variances in substrate components, subsequent

analyses of SR function across animal groups was conducted with the effects of substrate 2 only.

5.4.2 SR Ca²⁺ Uptake, Leak and Release Findings

Average Ca²⁺ uptake rates were not found to be significantly lower in ZDF animals compared to the Lean animals for both the fast and slow twitch muscles (although there was a strong trend to be lower in RG, p=0.06). Indeed, with a higher sample size to decrease variability (by incorporating the other substrates), we report significant impairments as shown in Figure 5.1, where Obese muscles have slower uptake, leak and release rates compared to Lean animals. This is consistent with the pattern observed with substrate 2 only. Interestingly, although these changes are observed in isolated vesicles exposed to the same assay conditions, the results conform well to the intracellular environment commonly observed in diabetic muscles as well as metabolites measured in this thesis. The high lipid content in obesity has been found to reduce SR Ca²⁺ transport, and this progressively worsens as obesity progresses to T2D (Eibschutz et al., 1984). Further, Ganguly et al. (1983) found that there was an increased cholesterol-to-phospholipid ratio in diabetic rats which may affect membrane fluidity and therefore SR function. Indeed the Obese ZDF rats utilised in this study showed significantly higher fat content, and moreso in slow-twitch muscles compared to fast-twitch muscles, indicating that fat content does impact on SR function.

Given that increased leak through RyRs is often a significant component of altered excitation-contraction coupling (de Groof et al., 2002), it is important to find ways to buffer against high [ADP] and Pi to improve muscle function in obesity and T2D. This may have important implications in appropriately timing the administration of supplements such as creatine, given that it relies on insulin to be transported into the muscle cell; i.e. insulin

resistance must be reduced before administering creatine, so as to obtain the maximal benefits from it. However, it was found in this study that Obese rats had lower leak rates compared to Lean animals particularly in fast twitch fibres. A potential explanation for this is that calsequestrin (CSQ), a high-capacity Ca^{2+} -binding protein in the SR has been shown to be upregulated in chemically-induced diabetes, which has been said to help buffer against the elevated cytosolic Ca^{2+} levels and prevent Ca^{2+} -dependent myonecrosis (Howarth et al., 2002; Chopra et al., 2007). Indeed this would help explain the lack of calpain activation observed in chapter 3.

Release of Ca^{2+} from the SR has been shown to be significantly lower in Obese animals compared to Lean and so it would seem that again, protective mechanisms may be involved to hinder the movement of Ca^{2+} to the muscle cytoplasm and prevent muscle damage. Some of this could be attributed to higher CSQ levels. However, inorganic phosphate has been shown to alter SR function, and there appears to be more Pi in Type 2 Diabetic rats (De Feyter et al., 2008). Inorganic phosphate can enter the SR and the subsequent Ca^{2+} -Pi precipitation in the SR decreases Ca^{2+} availability for release. This has been demonstrated as a mechanism of fatigue following high intensity exercise in healthy humans, and may contribute to the enhanced fatigue observed in the Obese Soleus, at least, described in chapter 4. Once again, the high fat content may also impede ion flow across membranes as discussed earlier.

The Ca^{2+} uptake results in this study are supported by the same patterns in ATP concentrations observed in both fast and slow twitch muscles of both animals groups (see chapter 4.3.5, Table 4.3). Similarly, lower levels of PCr are also observed in the ZDF animals of slow twitch muscles, compared to Lean animals (with the strongest trend towards reduced

Ca²⁺ uptake observed in RG muscles). Uptake of Ca²⁺ into the SR has been shown to preferentially derive its energy from the breakdown of PCr via the creatine kinase reaction (Rossi et al., 1990). Thus, the lower levels of PCr would suggest reduced capacity for the SR to remove Ca²⁺ from the cytosol – this is consistent with comparable Ca²⁺ uptake rates found in this study. Certainly in slow twitch muscle, the lower PCr levels in Obese animals compared to Lean would indicate the initiation of increased energy demand in an attempt to maintain function, given the commencement of impaired SR functions. Indeed, it is interesting that this trend towards lower Ca²⁺ uptake rates is observed on the backdrop of significantly higher SR protein concentrations in the Obese animals, suggesting that altered SR function is driving changes in the expression of proteins controlling calcium regulation. Unfortunately, no separate analysis of the different proteins within the SR were performed, so it is not clear where these changes may be occurring. Given that Ca²⁺ uptake rates were, if anything, lower in the Obese animals, it appears unlikely that SERCA proteins were increased, although there may have been shifts in the isoforms expressed. As already suggested, CSQ is one protein that may have been increased. Two relatively novel proteins that could possibly have been affected are phospholamban and sarcolipin, both of which associate with the Ca²⁺ ATPase pumps (Fajardo et al., 2015a; Bidwell and Kranias, 2016). Sarcolipin in particular is highly expressed in both fast and slow twitch fibres (Fajardo et al., 2015b) and has been shown to provide resistance against diet-induced diabetes (Maurya and Periasamy, 2015). Sarcolipin causes the uncoupling of Ca²⁺ transport from ATP hydrolysis (Gamou et al., 2015), producing a futile cycling of the Ca²⁺ ATPase pump. While this has been suggested to be a major mechanism of thermogenesis in muscles, it is exciting to consider that this could also be a mechanism to increase ATP utilisation (and hence energy expenditure) in an attempt to reduce the effects of obesity. Clearly, further research is required in this area.

It was anticipated that supplementation would help improve SR function, particularly as a direct result from creatine, however it was observed that the combination of creatine, whey and aspirin in this study had no effect on SR Ca²⁺ uptake, leak or release in either Lean or Obese animals. Perhaps this could be attributed again to the fat content, because despite studies showing that whey protein can reduce fat content (Burke et al., 2001; Sousa et al., 2012), supplemented Lean and Obese animals in slow twitch fibres showed higher intramuscular fat content compared to non-supplemented animals and as such no improvements seen (refer to chapter 6.3.3 and Figure 6.2 for results). Interestingly, fast twitch muscle of supplemented Lean and Obese animals showed no changes in fat content compared to non-supplemented animals. SR Ca²⁺ uptake in LSupp supplemented animals showed slight improvements to SR (and these became significantly improved with greater sample size seen in Figure 5.1), whereas no improvements at all were seen in ObSupp animals, which given the already doubled fat content, would be expected.

Altered SR function would be most likely attributed to the creatine contained within the supplement combination given that it is the primary source of energy for SR function. However, no significant increase in PCr of the supplemented rats was observed, except for slight, improvements in LSupp animals. In fact, the expected increase in total muscle creatine due to creatine supplementation was not seen. This can be explained by the fact that firstly, carbohydrate ingestion substantially augments muscle creatine accumulation during creatine supplementation in humans (Green et al., 1996; Casey and Greenhaff, 2000), and in this study, carbohydrate was not added to the supplemented diet (although it would be a significant component of the normal rat chow). Furthermore, Steenge et al. (1998) demonstrated that insulin can enhance muscle creatine accumulation in humans but only when present at physiologically high or supraphysiological concentrations and that this

response may be the result of an insulin-mediated increase in muscle creatine transport rather than creatine delivery. Since the Obese ZDF rats used in this study were insulin resistant and hyperinsulinaemic, any insulin present would be unable to mediate creatine uptake into the muscle cells. In addition, if carbohydrate were added to the creatine supplementation in this study, the amounts of glucose required to evoke a strong enough release of insulin would have been excessively high, thereby compounding the effects of obesity in promoting the state of insulin-resistance, and speeding up that development of T2D. Indeed, down-regulation of the creatine transporters has been observed with continuous creatine supplementation (Loike et al., 1988; Persky et al., 2003). While the 6 week supplementation regime in the current study is similar to that observed in human studies, it is fair to say that 6 weeks is a far greater proportion of a rat life than a human one. Thus, perhaps even in 6 weeks, a washout period, similar to that used in human studies, is required during the supplementation period to prevent down-regulation of the Cr transporters. As Cr levels were only measured at the completion of the supplementation, there is no way of knowing whether there were any changes in Cr levels throughout the 6 week regime. It is, however, interesting that the increases in SR protein content observed in the Obese animals were further increased following supplementation (which would be most likely attributable to the anabolic effects of creatine and whey/amino acid loading). Exactly what proteins are increased and whether they maintain their functionality would be an important avenue for future research.

5.5 Conclusion

It has been found that overall, the combination of ATP + PCr + ADP was the better energy substrate to utilise in determining Ca^{2+} transients in the SR compared to ATP alone, further providing evidence of the importance of linked CK in regulating the energy supply to- and functional activity of- the Ca^{2+} -ATPase pump. Furthermore, insulin resistant ZDF rats show

no significant alterations to skeletal muscle SR Ca^{2+} uptake rates (albeit a strong trend to slower rates in red muscles), slightly lower leak rates and significantly lower release rates compared to Lean littermates.

Creatine, whey protein and aspirin supplementation appear to have no beneficial effects on skeletal muscle SR function, perhaps due to the already high insulin resistance observed in these animals. However, elevated SR protein content in Obese animals, with further increases after supplementation, warrants further research, as does alternative methods of administration and use of these supplements.

CHAPTER 6

Effects of Creatine, Whey and Aspirin on Skeletal Muscle MAPR in Type 2 Diabetes.

6.1 Introduction

It has long been established that fatty acids contribute largely to insulin resistance resulting in reduced glucose uptake into muscle cells and hence less substrate available for metabolism. Given the importance of mitochondria in utilising fats and carbohydrates to provide fuel for muscle cells, and the difficulties in glucose transport across muscle cells in T2D due to insulin resistance, altered mitochondrial function is likely central to the diabetes condition. Indeed, healthy mitochondrial function is required for normal glucose metabolism, and any impairment would reduce the metabolic capacity and hinder muscle function.

Mitochondrial function in T2D remains controversial with a number of studies showing impaired function (Kelley et al., 2002; Schrauwen and Hesselink, 2004; Phielix and Mensink, 2008; Szendroedi et al., 2012), whilst others reported no changes (Boushel et al., 2007; De Feyter et al., 2008). Given that reduced oxidative capacity has been shown to be related to morphological abnormalities in mitochondria of Obese and T2D volunteers compared to healthy controls, and furthermore, the increase in FFA content associated with obesity and T2D can lead to several other mitochondrial defects, it is likely that a reduced oxidative enzyme capacity occurs in T2D (Simoneau and Kelley, 1997; Kelley and Mandarino, 2000; He et al., 2001). In addition to the higher FA content this may also be a result of 1) increased production of ROS such as superoxide and hydrogen peroxide, and reduced antioxidant defences available such as Manganese Superoxide Dismutase (MnSOD) and Glutathione (GSH) (Johansen et al., 2005); 2) increased inflammatory mediators leading to insulin resistance (Dandona et al., 2004) and apoptosis/necrosis (Shoelson et al., 2007); and 3) reduced membrane fluidity which inhibits complex V of the Electron Transport Chain (Lamson and Plaza, 2002). To date no studies have examined the potential fibre type differences in mitochondrial function in T2D, and given the differences in function of fast

versus slow twitch muscle fibres, it is important to ascertain if mitochondria are affected differently as this may clarify some of the discrepancies found in the mitochondrial function data to date.

Furthermore, chapter 1.4 outlined some potential compounds which may help improve mitochondrial function, in particular whey protein (see chapter 1.4.3). To summarise, whey protein has been found to act as an antioxidant precursor, in particular by providing the amino acids required for GSH synthesis (Bartfay et al., 2003), thereby reducing the oxidative stress to mitochondria. Whey has also been shown to increase satiety (Gentile et al., 2015), so animals may be less inclined to eat as much (although with leptin deficiency this may not occur). Whey increases Lean muscle mass (Burke et al., 2001) and reduces muscle fat content (Belobrajdic et al., 2003), and has been found to reduce insulin resistance (as has creatine (Op't Eijnde et al., 2006)) (Belobrajdic et al., 2004), which may improve mitochondrial function as there is more substrate available for metabolism. Of course the added benefit of aspirin to reduce the detrimental effects of excessive pro-inflammatory cytokines (to be discussed in Chapter 7), and reduce ROS such as superoxide, would also be beneficial to mitochondrial function.

Thus the aims of this study were to determine if ZDF rats had impaired skeletal muscle mitochondrial function (as measured by ATP production rate), and whether these impairments were specific to fast or slow twitch muscles. In addition it was aimed to determine if creatine, whey and aspirin treatment could improve mitochondrial ATP production rates in T2D.

6.2 Methods

The methods used to determine mitochondrial function are detailed in chapters 2.6, with details of animals and the supplementation regime employed is outlined in chapters 2.1 to 2.3. Mitochondrial protein methods are found in chapter 2.7.4, and overall histology is outlined in chapter 2.11 with specific staining methods utilised in this chapter detailed in the next section.

6.2.1 Oil Red O Staining Method

In order to determine the lipid content of skeletal muscle, the Oil Red O staining method was used as optimised by Koopman et al. (2001). Cryosections from EDL and Soleus muscles of 8 μ M were obtained at -20°C and air-dried for 15 minutes. (Note, due to sample shortage RG and WG muscles were not utilised as per MAPR). Samples were fixed in 3.7% Formaldehyde (Aldrich F15587) for 60 minutes and rinsed in deionised water 3 times for 30 seconds to remove excess formaldehyde. This followed an incubation period in Oil Red O Working Solution (Sigma, Catalogue # 01391-250mL) for 30 minutes. Oil Red O stock solution was combined with deionised water (in a 3:2 ratio), and filtered through Whatman paper number 42 (Whatman 1001-42, UK) to remove crystallised Oil Red O. Sections were then washed with three exchanges of deionised water 3 times for 30 seconds each. To visualise nuclei, sections were dipped in Mayer's Haematoxylin (Sigma 51275) for 60 seconds. Sections were rinsed under running tap water for 10 minutes, and covered with coverslips using 10% glycerol in Phosphate Buffered Saline (Sigma, Catalogue # P5368-10PAK, pH7.4) (Figure 6.0).

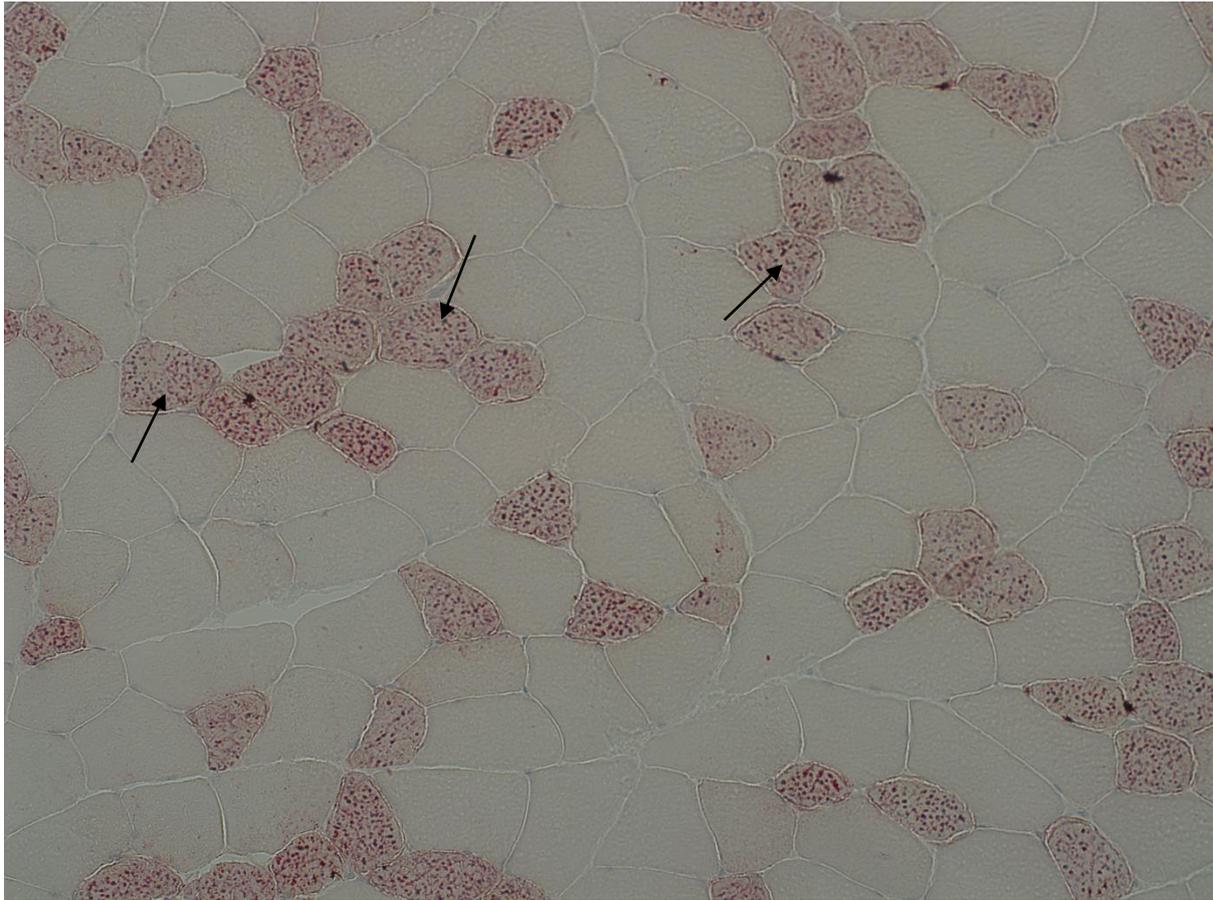


Figure 6.0 Example of Oil Red O Stain in EDL muscle of an Obese ZDF Rat x200 magnification. Areas of red pigmentation as indicated by black arrows show areas of intramyocellular lipid.

6.2.2 Statistical Analysis

A two-way ANOVA was used to detect differences between animal groups in MAPR and differences between muscle type. The post hoc Tukey test was done to determine where the differences were. Significance was set at $p < 0.05$. All results are expressed as means \pm SEM.

6.3 Results

6.3.1 Mitochondrial ATP Production Rates

In red gastrocnemius muscles Obese animals had lower MAPR compared to Lean littermates, whereby significant differences occurred with substrates P&M, α -KG, G&S and PPKM

($p < 0.05$) (See Figure 6.1). Supplementation significantly improved mitochondrial function across all substrate combinations ($p < 0.05$) in the obese animals, such that there were no differences compared to the Lean group. Conversely, supplementation of the Lean animal group did not alter MAPR across any of the substrate combinations in red gastrocnemius muscles. Overall, variability in the mitochondrial responses seems to be inherent to the diabetic condition as the same variability in MAPR was not observed in a rodent model of Duchenne Muscular Dystrophy (Rybalka et al., 2014).

White gastrocnemius muscles also demonstrated large variability in MAPR measurement, with few differences observed. Interestingly, the metabolic pathway for fat metabolism showed significantly higher MAPR in the Obese and ObSupp groups compared to the Lean and LSupp animals ($p < 0.05$), suggesting a heightened capacity for fat metabolism. Supplementation did not appear to have any effect on MAPR when measured with the individual substrates. However, the combination of metabolic pathways (PPKM) showed improved MAPR ($p < 0.05$) in the ObSupp group compared to the Obese animals.

6.3.2 Mitochondrial Proteins

The amount of mitochondrial proteins per mg of muscle was determined in white and red gastrocnemius muscles of all animal groups (Table 6.0). Unsurprisingly, the RG had significantly more mitochondrial protein than the WG. In the RG muscle, Obese and ObSupp groups had significantly higher mitochondrial protein content compared to Lean littermates ($p < 0.05$). WG mitochondrial protein content was significantly lower in the LSupp animals compared to all other animal groups ($p < 0.05$). Overall, the percentage of mitochondrial proteins in RG was 10% higher in the Obese compared to Lean groups ($p < 0.05$). Supplementation significantly improved the percentage of mitochondrial proteins in both

LSupp and ObSupp groups, with the ObSupp showing a 20% greater increase compare to the LSupp ($p < 0.05$). WG showed no differences between groups, with a trend to being higher in the ObSupp group compared to Obese littermates ($p = 0.06$).

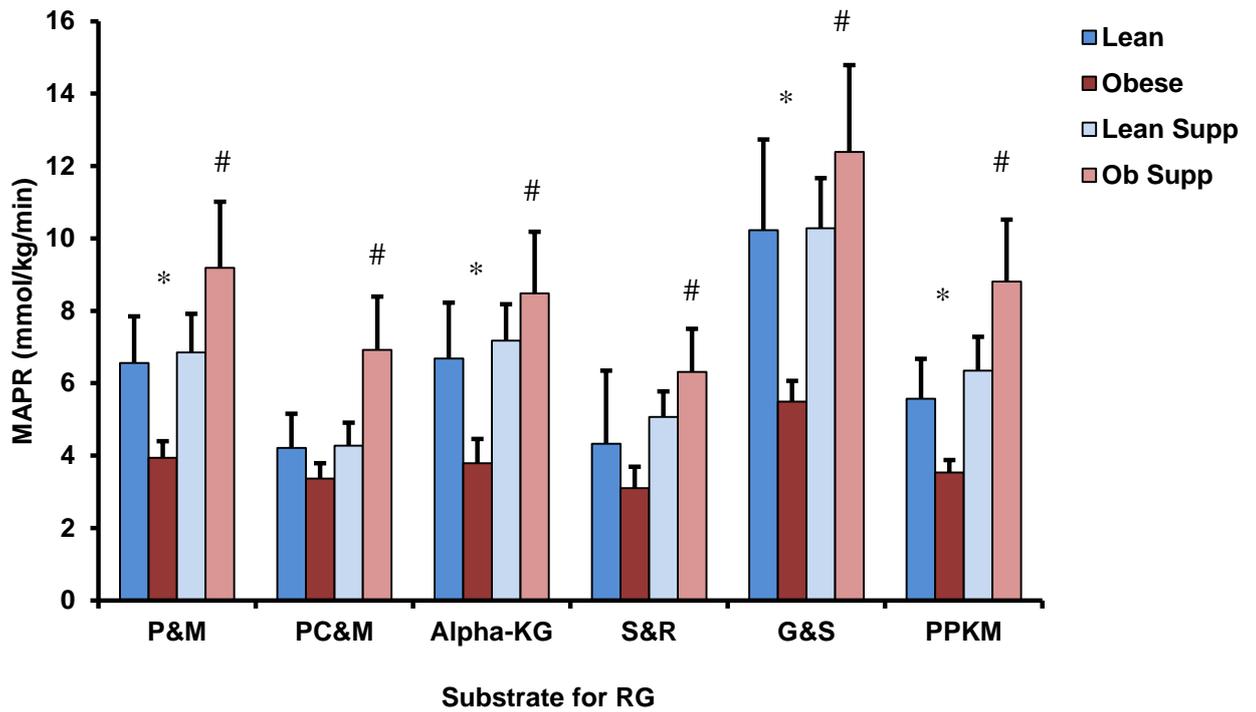
6.3.3 Histology – Oil Red O

Histological analysis of muscle fat content using Oil Red O indicated that in both Soleus and EDL (Figure 6.2), Obese and ObSupp animals had significantly higher fat content compared to their respective Lean littermates ($p < 0.05$). Whilst there appears to be more fat in the LSupp soleus muscles compared to the Lean group, this was not significant ($p = 0.2$). Finally, Obese and ObSupp animals in soleus had significantly less fat than the EDL from the equivalent groups ($p < 0.01$). A trend ($p < 0.08$) was observed between muscle groups of Lean animals and no differences found for LSupp animals.

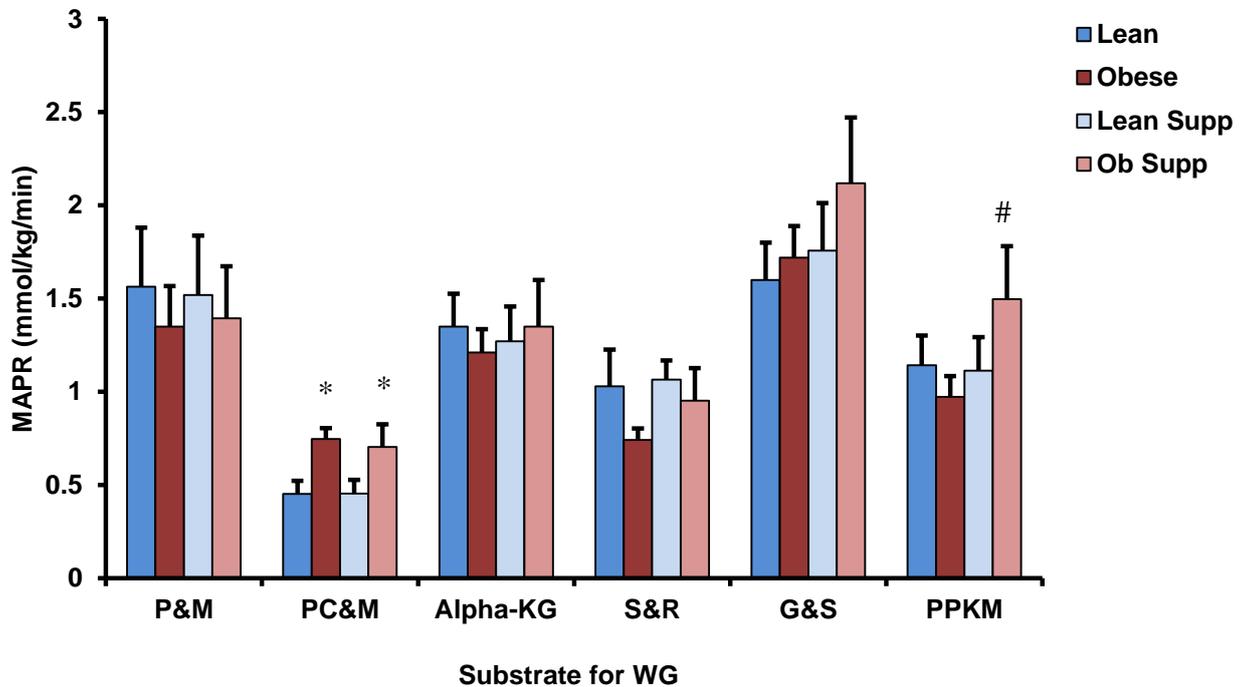
Table 6.0 Mitochondrial Proteins in RG and WG Muscles of ZDF rats.

	Lean	Obese	LSupp	ObSupp
RG Mitochondrial Protein (ug/mg muscle)	47.9 ± 3.7 (n=10)	67.8 ± 1.9* (n=7)	54.3 ± 3.8## (n=11)	64.7 ± 3.1* (n=11)
WG Mitochondrial Protein (ug/mg muscle)	20.8 ± 2.3 (n=10)	23.0 ± 2.6 (n=11)	12.9 ± 1.3 ψ (n=11)	22.2 ± 2.1 (n=12)
RG % Mitochondrial Protein	30 ± 0.4 (n=10)	40 ± 0.5* (n=7)	45 ± 0.4* (n=11)	66 ± 0.5*# (n=11)
WG % Mitochondrial Protein	13 ± 0.3 (n=10)	15 ± 0.4 (n=11)	14 ± 0.3 ψ (n=11)	22 ± 0.3## (n=12)

Results are expressed as means ± SEM. *Significant difference compared to Lean ($p < 0.05$). ψ Significant difference compared to Lean, Obese, and ObSupp ($p < 0.01$). # Significant difference compared to Obese ($p < 0.05$). ## Trend compared to Obese ($p = 0.06$).

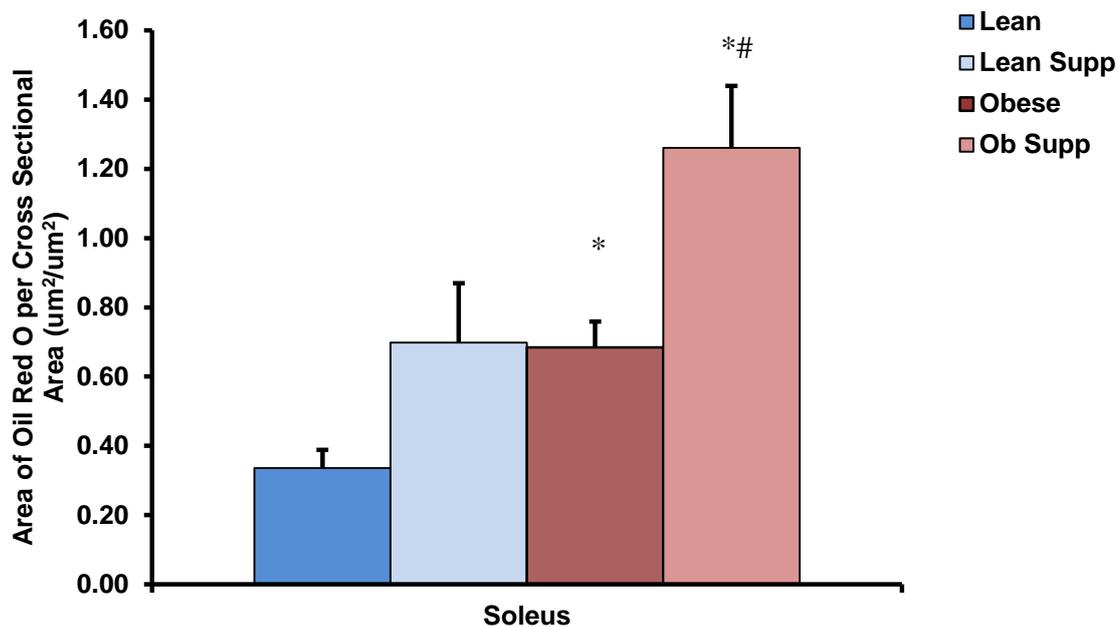


A.

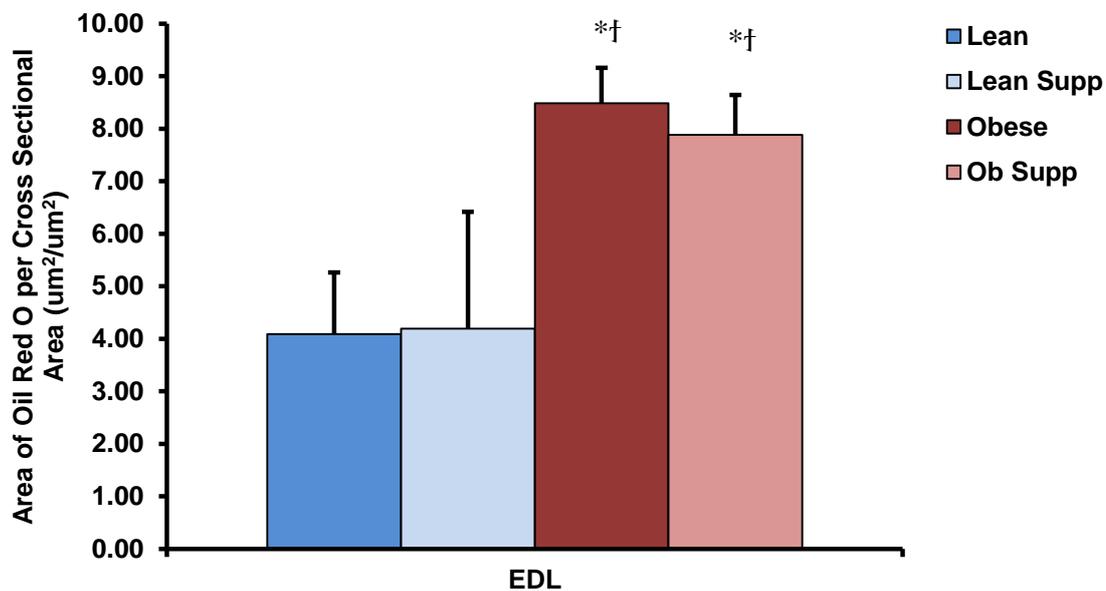


B.

Figure 6.1 MAPR in (A) Red Gastrocnemius (slow twitch), and (B) White Gastrocnemius (fast twitch) muscle of Lean (n=9), Obese (n=10), LSupp (n=11) and ObSupp (n=12) ZDF rats. Results are expressed as means \pm SEM. * Significant difference compared to Lean and LSupp animals ($p < 0.05$). # Significant difference compared to Obese animals ($p < 0.05$).



A.



B.

Figure 6.2 Oil Red O in (A) Slow Twitch and (B) Fast twitch muscles of Lean (n=3), Obese (n=4), LSupp (n=3) and ObSupp (n=4) ZDF rats. Results are expressed as means \pm SEM. * Significant difference compared to Lean animals ($p < 0.05$). # Significant difference compared to Obese animals ($p < 0.01$). † Significant difference compared to Obese and ObSupp respectively in Soleus muscles ($p < 0.01$).

6.4 Discussion

The overall findings of this study were that there was a fibre type-specific and pathway-specific reduction in MAPR of Obese ZDF rats, particularly for carbohydrate and protein metabolism in the RG. In contrast, MAPR via the fat pathway appeared to be enhanced in the WG. Supplementation significantly improved total MAPR when the mitochondria were given substrates from all metabolic pathways (carbohydrate, fat and protein) in both red and white muscles of the Obese animals only.

In the RG muscle, not only did the ZDF animals have significantly lower MAPR, but they also had significantly more mitochondrial protein compared to Lean, LSupp and ObSupp animals. This indicates a severe mitochondrial impairment, as despite an adaptive mechanism to increase mitochondrial protein content in an attempt to maintain mitochondrial function, it remains significantly lower. Having said that, stimulating the fat pathway (PC&M) produced no significant differences in MAPR between ZDF and Lean animals for RG, but in fact, increased MAPR in WG (albeit still well below the RG). This may indicate a shift in preference for fat metabolism in the Obese ZDF rats. This is perhaps not surprising due to their condition and is supported by the fact that Obese animals showed a significantly higher fat content (indicated by Oil Red O stains) and thus more fuel availability. It has been shown that increased fat oxidation occurs after acute exposure to lipids (Cameron-Smith et al., 2003), where fatty acid genes such as fatty acid translocase (FAT), and fatty acid oxidation enzymes such as malonyl CoA decarboxylase and β -hydroxyacyl CoA dehydrogenase are upregulated. This may have been the case in the WG, where a higher MAPR in Obese animals was observed despite no change in mitochondrial protein content. Further, Koves et al. (2008) show that excessive β -oxidation overloads muscle mitochondria with intermediate metabolites which results in a reduced capacity for glucose oxidation, as is supported by the

current results, at least in the RG. However, other studies have shown impaired lipid oxidation (Kelley and Simoneau, 1994; He et al., 2001) despite a higher lipid content, and this occurs via a down regulation of a subset of nuclear genes encoding enzymes of complexes I to IV in the mitochondrial respiratory chain (Sparks et al., 2005). The finding of comparable MAPR with PC&M despite higher mitochondrial protein in the RG in the current study suggests that the increased protein is not related to the electron chain complexes listed above. A limitation to this study is that the specific genes related to fat oxidation were not measured, and so the mechanism behind the mitochondrial impairments can only be speculated upon.

MAPR in fast twitch muscles generally showed no significant difference between all the animals groups across the different metabolic pathways except for the fat pathway, where it was significantly higher in Obese compared to Lean animals, indicating no mitochondrial defect. However, there were higher levels of intramyocellular lipids (IMCL) in EDL compared to soleus and this may explain the significantly higher fat metabolism that occurred in this muscle group. In addition, given we looked at two different muscles when measuring MAPR and IMCL content due to a lack of muscle availability, comparisons between these variables must be made with caution as every muscle tissue functions in its own right and has been the cause of much debate amongst the various findings reported in this disease. It should also be noted that although there was higher IMCL content in EDL, corresponding with higher fat metabolism, MAPR was still well below of the RG. Whilst it is expected that RG normally have higher MAPRs, the difference between the two fibre types might not be expected to be so vast given the high IMCL content. However this would require further investigation.

In the present study, there was almost ten times the amount of IMCL observed in fast-twitch compared to slow-twitch muscle. This is the opposite of what has been shown by others (De Feyter et al., 2006; De Feyter et al., 2008), where type I fibres were reported to have two-fold higher IMCL compared to type II fibres. This could be due to the muscles chosen, or be specific to this particular strain of animal. Despite this, it is interesting that the higher levels of fat found in the fast twitch EDL muscle corresponded with higher MAPR in the WG muscle, which has a predominance of fast-twitch fibres. It might be expected that a large degree of mitochondrial impairment would be seen (Kelley and Simoneau, 1994; He et al., 2001), however our results show that mitochondria in white muscles are not affected despite the excess IMCL content. It may be that whilst plasma FFA concentrations and extramyocellular lipid (EMCL) content might affect mitochondrial function, IMCL content does not (Daniele et al., 2014). Other studies have reported that IMCL content may not necessarily impact mitochondrial function. For example, Turner et al. (2007) looked at rodent models containing an oversupply of lipids and the influence on downstream markers of mitochondrial fatty acid oxidative capacity. They found that excess IMCL increased mitochondrial fatty acid oxidation, which indeed occurred in ZDF rat fast-twitch fibres in this study. Further, Meex et al. (2010) found that mitochondrial function was restored by improving insulin mediated glucose disposal through physical exercise in the presence of increased IMCL storage. Toledo et al. (2008) showed that diet-induced weight loss resulted in lower IMCL content but no improvement in mitochondrial function, whereas the combination of exercise and diet improved insulin sensitivity without changes to IMCL, which improved mitochondrial oxidative capacity. Hence it would appear that IMCL content is not the likely mechanism interfering with mitochondrial function in T2D.

The combination of creatine, whey and aspirin supplementation significantly improved MAPR in Obese ZDF rats across all metabolic pathways in the RG, however had no effect on the Lean animals. This indicates that supplementation alone may be beneficial for improving mitochondrial function of slow twitch fibres in T2D. Interestingly, in fast twitch fibres, supplementation had no effect on MAPR under any of the pathways stimulated or in any of the supplemented groups, except for PPKM (a more representative combination of *in vivo* processes), where it was significantly improved in the WG of the Obese ZDF animals only. Overall, this highlights that when no impairments are observed (as in the Lean animals), no supplementation-instigated improvements in mitochondrial function occur. However when there are substantial mitochondrial impairments, as were observed in the RG muscle, significant improvements were observed following the supplementation regime employed in this study.

Several mechanisms that are thought to be involved in improving mitochondrial function with the supplement regime used are improvements to insulin resistance and glucose tolerance, increased lean muscle mass, fat loss and weight reduction through satiety control, and enhanced antioxidant defences. Indeed these are whole body mechanisms, whereas experiments herein were conducted on isolated mitochondria, suggesting these mechanisms are causing long-lasting changes to mitochondrial structure and/or function. All three supplements have been shown to improve insulin sensitivity (Nishikawa et al., 2000; Hundal et al., 2002) and improve glucose tolerance (Hopper et al., 1985; Mortensen et al., 2009) particularly of muscle cells (Ferrante et al., 2000; Eijnde et al., 2001; Belobrajdic et al., 2003; Belobrajdic et al., 2004). In particular, insulin sensitivity can be improved by reducing fat mass. Whey has been shown to promote weight loss (Pilvi et al., 2009; Baer et al., 2011), reduce fat mass (Frestedt et al., 2008; Mortensen et al., 2009; Pal et al., 2010) and increase

lean muscle mass (Frestedt et al., 2008), through satiety control (Bowen et al., 2006; Pichon et al., 2008) via increasing leptin sensitivity (Pilvi et al., 2008). Improving insulin resistance is important given that ATP production is dependent on the availability of energy substrates such as carbohydrate, fat and protein to the mitochondria. Whilst systemic insulin resistance was not seen to be improved with supplementation in this study, it is still possible that local improvements at the myocellular level may have taken effect given the significant improvements to MAPR for carbohydrate metabolism observed in RG fibres of ZDF rats. However local myocellular insulin resistance was not measured, thus would require further investigation to confirm such an effect. In addition, IMCL content was not reduced but increased in ObSupp animals. This may be because ZDF rats are leptin deficient, therefore the action of whey to reduce fat mass by increasing leptin sensitivity and altering satiety levels could not occur – in fact ObSupp rats ate significantly more, and had the highest weight gain than all other animal groups, as shown in Chapter 4.3.2. Alternatively, whey protein has been shown to enhance muscle protein synthesis (Ha and Zemel, 2003), and Pilvi et al. (2007) showed that in high fat-fed mice, whey-induced increases in plasma leucine levels modulated insulin signalling by PI3K, and may be an alternative pathway for any potential improvements to local insulin resistance and hence MAPR. However, any protein synthesis changes due to supplementation did not appear to influence the mitochondrial protein content, as the supplemented groups had comparable levels to unsupplemented controls – in the WG, there was a surprising decrease from the Lean group.

Bonnard et al. (2008) has shown that skeletal muscle mitochondrial impairments are a result of oxidative stress in diet-induced insulin resistant mice. Intracellular glutathione levels are known to be increased with whey supplementation (Ebaid et al., 2011; Hamad et al., 2011), leading to a decrease in oxidant-induced muscle dysfunction (Lands et al., 1999; Xu et al.,

2011), specifically by acting on H₂O₂. Thus, it's possible that there was less damage to the mitochondria from production of oxygen free radicals during metabolism as a result of higher levels of glutathione, and thus a better maintenance of mitochondrial function, despite the higher fat content observed with supplementation. However ROS was not measured in these mitochondrial isolates as further methodological considerations would be required if the methods utilised in chapter 3.2.5 were to be employed for comparative purposes. Of course, given the actions of aspirin on superoxide dismutase, it is also likely that this could be a mechanism of improving ROS and thus MAPR. These results could also indicate that fat content is not necessarily the cause of the mitochondrial impairments seen in T2D, but rather impaired anti-oxidant defences available to cells. Glutathione, superoxide and ROS were not measured in this study - nevertheless the addition of the supplement to the diet of the ZDF rats was able to reverse the detrimental effects on MAPR seen in the non-supplemented Obese animals. This is important as Obese and diabetic patients who suffer from poor metabolic control often complain of fatigue and weakness, leading to exercise intolerance (Demir et al., 2001). Therefore creatine, whey and aspirin supplementation may potentially help reduce exercise intolerance in these patients, by improving the ability of mitochondria to produce energy.

6.5 Conclusion

In conclusion, MAPR was found to be impaired in RG muscle fibres of ZDF rats, which was attenuated with creatine, whey and aspirin supplementation. This improvement occurred independently of increasing/improving the mitochondrial protein content, IMCL content and whole body insulin resistance. However it is possible that local effects have taken place by reducing oxidative stress (through increases to glutathione levels and reductions in H₂O₂ and

superoxide anions) and myocellular insulin resistance, however further studies would be required to ascertain this.

CHAPTER 7

Effects of Creatine, Whey and Aspirin on Plasma and Skeletal Muscle Cytokines In Type 2 Diabetes.

7.1 Introduction

As discussed in Chapter 1, it was postulated that inflammation (amongst other factors) causes insulin resistance which could reduce ATP production as a result of reduced substrate availability and/or mitochondrial dysfunction. This in turn was postulated to impair SR function causing Ca^{2+} overload leading to further insulin resistance, as well as reduced protein synthesis rates, thus contributing to the muscle atrophy and dysfunction seen in T2D. Cytokines have been linked to muscle atrophy however it's not known if the same occurs in diabetes-associated atrophy. In addition, whilst Obese and diabetic patients are encouraged to exercise to help improve insulin sensitivity and hyperglycaemia, this exercise can potentially have detrimental effects by inducing muscle damage, which in turn has been shown to activate inflammation and compound the atrophy and muscle dysfunction, leading to a vicious cycle.

The aim of this study was to determine if ZDF rats had increases in the inflammatory cytokines Il-6 and TNF- α . Il-6 and TNF- α were chosen as high levels have been shown to contribute to insulin resistance and the development of T2D (Mohamed et al., 1999; Dandona et al., 2004). Further, whether the combination of Creatine, Whey and Aspirin treatment could positively modulate changes in cytokine content in order to improve insulin resistance was also examined.

7.2 Methods

The methods used to determine muscle and plasma cytokines in this section are detailed in chapter 2.9. Animals, tissues acquisition and supplementation regime were performed as per chapters 2.1 to 2.3, respectively. Fasting plasma insulin and glucose were also measured and

an index of insulin resistance was determined using the QUICKI method (Refer to chapter 2.10). These measures were also determined in the supplemented animal groups.

Skeletal muscle atrophy was also determined in this group of animals, by calculating the cross-sectional area as explained in section 2.4.2

7.2.1 Statistical Analysis

For muscle and plasma samples, a two-way ANOVA was used to detect differences between treatment and animal strain. A two-way ANOVA was used to detect differences between muscle type and cytokine type. Significance was set at $p < 0.05$. Refer to Chapter 3.2.7 for methods and statistical analyses pertaining to insulin resistance. All results are expressed as means \pm SEM.

7.3 Results

7.3.1 Skeletal Muscle Cytokines

Obese animals were found to have 56% more TNF- α and 65% more Il-6 in EDL muscle compared to their Lean littermates ($p < 0.05$) (Figure 7.0A). Supplementation with Creatine, Whey and Aspirin significantly reduced both cytokines by 59% and 63% respectively, in the ObSupp group compared to the Obese group ($p < 0.05$) with levels comparable to Lean animals; indeed, there were no differences between the Lean and the ObSupp group. No differences were found between Lean and LSupp groups indicating the supplementation had a specific effect on Obese animals.

Cytokine concentrations in soleus muscle showed no differences between animal groups (Figure 7.0B). Interestingly, both cytokines in soleus muscle were approximately double that in EDL of the same animal group ($p < 0.05$).

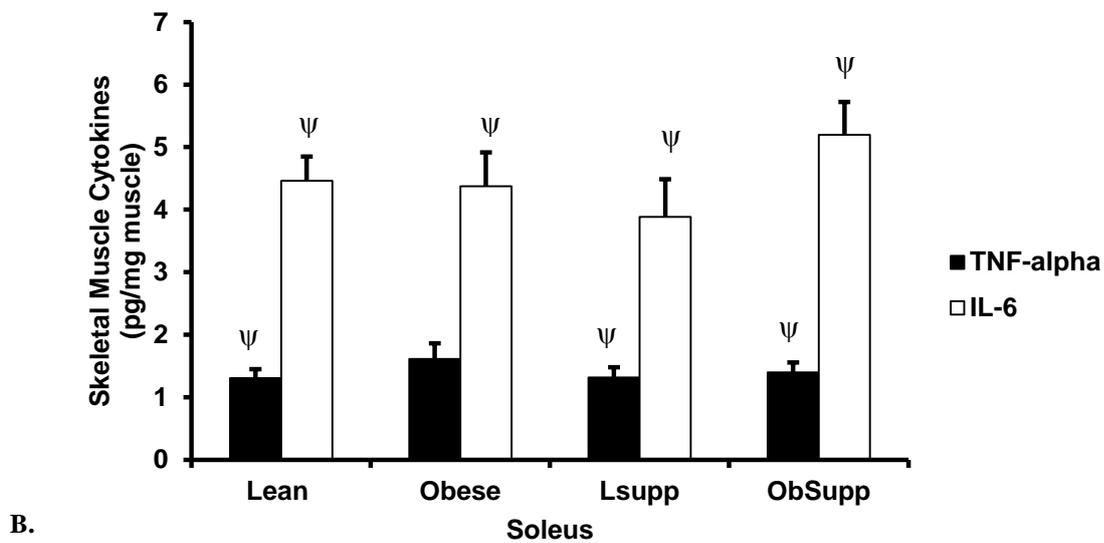
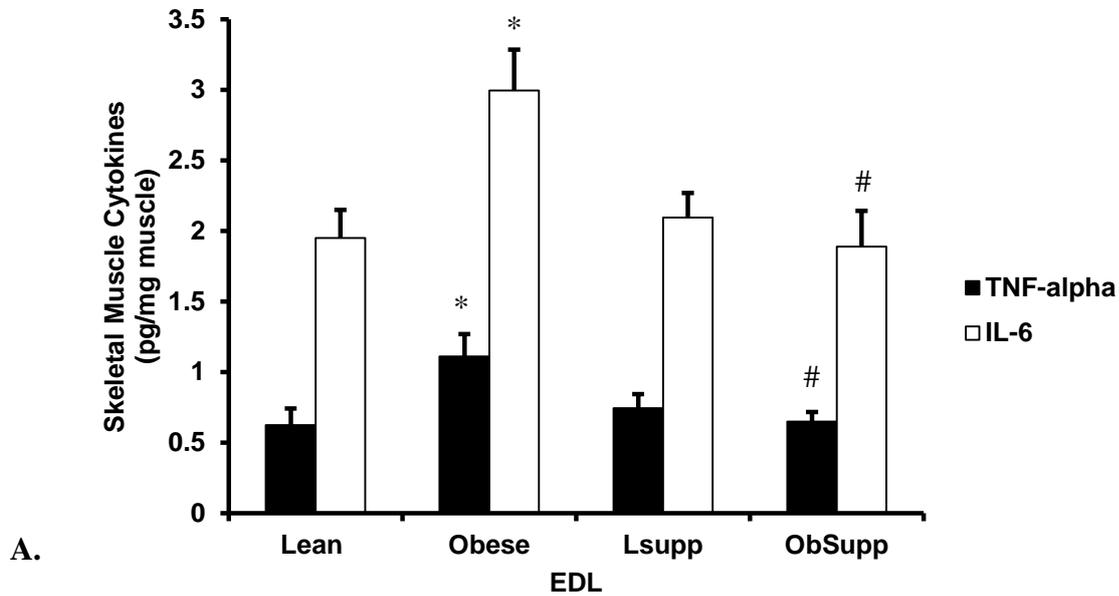


Figure 7.0 A) EDL Muscle Cytokines (TNF- α & IL-6) in Lean (n=6 & 9 respectively), Obese (n=10 & 10 respectively), LSupp (n=9 & 11 respectively) and ObSupp (n=9 & 10 respectively) ZDF rats. B) Soleus Muscle Cytokines (TNF- α & IL-6) in Lean (n=8 & 9 respectively), Obese (n=9 & 9 respectively), LSupp (n=10 & 9 respectively) and ObSupp (n=13 & 13 respectively) ZDF rats. * Significant difference between Lean and Obese animals ($p < 0.05$). # Significant difference between Obese and ObSupp animals ($p < 0.05$). ψ Significant difference between Soleus and EDL for respective animal groups ($p < 0.05$).

7.3.2 Plasma Cytokines

Both TNF- α and Il-6 were measured in plasma samples of all animal groups, however TNF- α was undetectable in these samples and so have not been reported herein. There was a trend towards a higher concentration of plasma Il-6 in the Obese group compared to the Lean group ($p=0.099$) (Figure 7.1), and of Il-6 (33% reduction) in the ObSupp group compared to the Obese group ($p=0.067$). A significantly lower concentration of Il-6 in LSupp compared to Obese group ($p<0.01$) was observed.

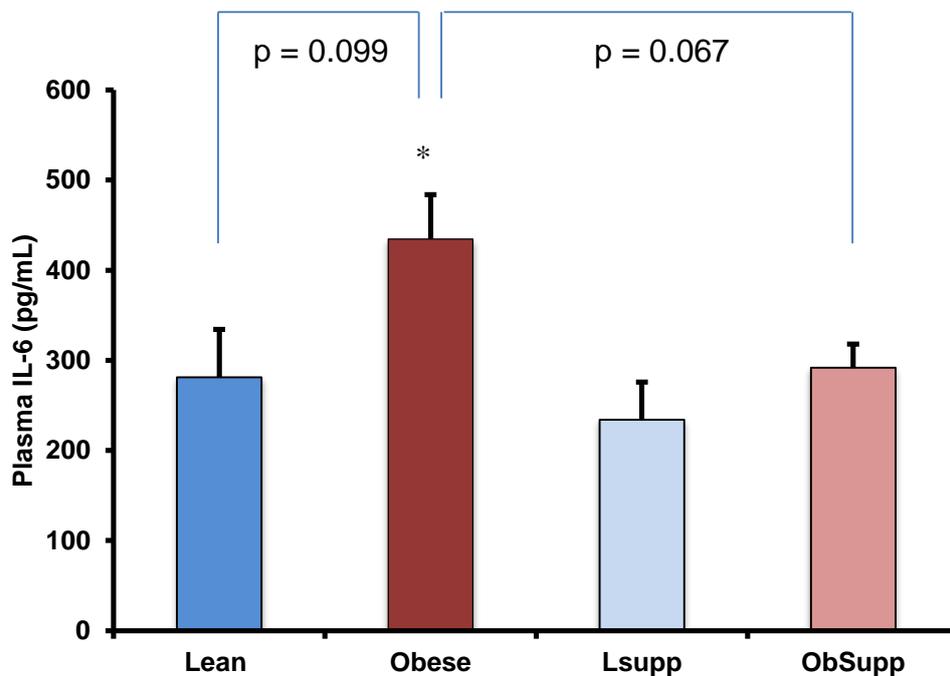


Figure 7.1 Plasma Il-6 in Lean (n=5), Obese (n=7), LSupp (n=7) and ObSupp (n=9) ZDF rats. *Significant difference compared to LSupp animals ($p<0.01$).

7.3.3 Insulin Resistance

As discussed in chapter 3.3.1, the ZDF animals were confirmed to have hyperglycaemia and hyperinsulinaemia, as well as insulin resistance. Unfortunately, the supplementation regime did not improve any of these indices (Table 7.0).

Table 7.0 Effects of supplementation on Insulin, Glucose and Index of Insulin Resistance (QUICKI) in ZDF rats.

	Lean (n=7)	Obese (n=8)	LSupp (n=10)	ObSupp (n=11)
Insulin (microunits/mL)	3.82 ± 0.69	11.78 ± 0.03*	4.72 ± 1.05	11.35 ± 0.48*
Glucose (mg/dL)	110.44 ± 18.21	271.24 ± 36.23*	141.95 ± 15.58	350.99 ± 39.41*
Insulin Resistance (QUICKI)	0.40 ± 0.08	0.29 ± 0.01*	0.37 ± 0.02	0.28 ± 0.01*

Results are expressed as means ± SEM. *Significant difference compared to Lean and LSupp animals (p<0.01).

7.4 Discussion

Data from the present study demonstrated that compared to Lean ZDF rats, Obese ZDF rats had significantly higher levels of TNF- α and Il-6 in EDL but not soleus muscle indicating a fibre type specific activation of these cytokines. Contrastingly, healthy humans have been shown to express TNF α solely in Type II fibres, whereas Il-6 is expressed in Type I fibres (Plomgaard et al., 2005). Furthermore, skeletal muscle transient production and short term action of Il-6 and TNF α have positive effects on muscle growth and energy regulation (for review see Munoz-Canoves et al. (2013)), however if these increases are long-lasting and elevated, they may induce muscle atrophy. This has been postulated to occur via the inhibition of Insulin Growth Factor-1 (IGF1)-dependent signalling (De Benedetti et al., 1997). Indeed we have shown that both Il-6 and TNF α are expressed in type I and II fibres in the ZDF rat strain, which may indicate a direct relationship between high levels of muscle cytokines and the atrophy seen in these muscles, especially given that both cytokines are specifically elevated in EDL of Obese ZDF animals, and EDL fibre areas were shown to be smaller compared to soleus (refer to chapters 3 and 4). Febbraio et al. (2003) suggested that Type 2 diabetic skeletal muscle does not release TNF- α or Il-6 into the localised blood stream at rest. This may be true for TNF- α , which was undetectable in the plasma, but not Il-6, as this study showed that intramyocellular cytokines are in fact higher in diabetic muscle at rest compared to Lean, at least in EDL but not soleus, and plasma IL-6 showed a trend in the

Obese animals compared to Lean ($p=0.099$). Of course, the IL-6 could also be coming from the soleus muscle, which has a high type I component, particularly as IL-6 concentrations were two-fold higher in the soleus compared to the EDL, or indeed from other sources. Whilst plasma samples tested for TNF- α lied below the range of the standard curve, indicating that it wasn't released by skeletal muscle as suggested by Febbraio et al. (2003), it is also possible that samples were over-diluted, and hence repetition of the assay with a more concentrated sample would confirm this. However limited sample availability precluded this from being re-tested.

The combination of creatine, whey and aspirin supplementation significantly attenuated these two cytokines in Obese ZDF animals, at least in the EDL muscle, yet had no effect on the supplemented Lean littermates, indicating a potential beneficial role in ameliorating pro-inflammatory cytokines in T2D. These results indicate that the supplements may only take effect when there are chronic levels of cytokines in muscles, and that perhaps EDL is more so affected than soleus, despite soleus having higher levels of cytokines compared to EDL (which for IL-6 at least, has been shown to be the case in healthy humans as mentioned in the previous paragraph). Indeed Type II fibres normally do not express IL-6, and so given that it is expressed in these rats, it confirms that EDL is more so affected than soleus. Given the likely bursts of force required to carry the heavier mass of Obese animals, which is characteristic of the types of contractions often performed by fast twitch EDL muscles, EDL may be utilised more in this disease compared to soleus and hence bearing the effects of higher ROS production and poor antioxidant defences compared to soleus. Nonaka et al. (2014) recently measured the major antioxidant enzymes and lipid peroxidation in streptozotocin diabetic rats of these two muscle groups. They found that adaptive responses to oxidative stress were adequate in the soleus muscle, but not in the EDL. Thus fast twitch

muscle fibres may be more susceptible to oxidative stress than slow twitch muscle fibres and this may contribute to muscle atrophy under diabetic conditions.

Given the anti-inflammatory properties of aspirin (Hundal et al., 2002), it is likely to be a significant contributor to the reduction of Il-6 and TNF- α found in EDL in this study. For example, as highlighted in chapter 1, high plasma FFA content results in an increase in ROS production (namely superoxide) which stimulates NF-k β to produce pro-inflammatory changes in Il-6 and TNF- α , resulting in insulin resistance and T2D. Aspirin has been shown to specifically target superoxide anions (Midaoui and de Champlain, 2002) and thus inhibit activation of NF-k β , reducing the pro-inflammatory changes occurring. Logically, one might assume that insulin resistance would be improved, however our results have shown that the supplements utilised herein have not improved systemic insulin resistance in the Obese ZDF animals as was hypothesised and has been reported previously (Hundal et al., 2002; Carvalho-Filho et al., 2009). One limitation to this study was that local insulin resistance in each muscle group was not measured, and so it cannot be concluded with certainty that it had no local effect despite the changes not being substantial enough to improve whole body insulin resistance. In fact, our analysis of plasma Il-6 indicated that the Obese ZDF rats had elevated levels, with the supplementation showing a strong trend to reduce this back to values similar to Lean animals. TNF- α could not be detected in these plasma samples, despite it following the same pattern intramyocellularly as Il-6, albeit at a lower concentration. Thus it would have been interesting to observe if similar alterations to TNF- α occurred. However, due to a limited amount of sample remaining, and the number of freeze-thaw cycles that had thus far taken place, further testing was not possible.

Another limitation to this study is that levels of superoxide anions were not measured given their transient lifetimes and need for substantial samples for the concomitant use of ≥ 2 assays to ensure accuracy and specificity, for which we were limited to. Thus, it could not be determined if Aspirin had any effect on this hypothesised pathway, despite others having shown this to be improved following only 3 weeks of aspirin supplementation (Midaoui and de Champlain, 2002). It is also possible that 6 weeks of aspirin supplementation at 100mg/kg/day was not sufficient to overcome the adverse changes in muscle properties seen in ZDF rats. Recently, Raghavan et al. (2014) looked at the effect of different doses of aspirin on inflammation, oxidative stress, and insulin resistance on endothelial function. Even at 300mg/day no significant changes to these parameters were evident, although it was only over 2 weeks of supplementation. Carvalho-Filho et al. (2009) however showed that whilst aspirin treatment reversed the increases in IKK β , JNK phosphorylation, and IRS-1 serine phosphorylation in diet-induced Obese rats, only a mild reduction in insulin sensitivity was seen. However they were only supplemented for 2 days by oral gavage at 120mg/kg/day. Interestingly, a concept known as Aspirin Resistance has been reported in Type 2 Diabetic patients taking 100mg Aspirin/day, which is correlated to body mass index and glycaemic control (Ertugrul et al., 2010). Grimaldi et al. (2014) showed that an initial booster dose of lysine acetylsalicylate was required to reverse aspirin resistance following treatment of 100mg/day. Thereby it can be speculated upon that perhaps a form of aspirin resistance was occurring in the Obese ZDF skeletal muscle, thus not elucidating the desired effects of improving insulin resistance, especially given no improvements to blood glucose or body mass were observed in the supplemented animals. Further this may occur in a fibre-type specific manner given that slow-twitch muscles showed no alterations in cytokine concentrations with supplementation, despite fast-twitch muscles demonstrating a significant reduction.

Whey protein has recently been shown to enhance the normal inflammatory responses during cutaneous wound healing (Ebaid et al., 2011) by specifically increasing glutathione, thereby reducing ROS in skin tissue, resulting in lower levels of Il-6 and TNF- α . Hence there is evidence that whey protein may have acted in a similar manner in skeletal muscle tissue, not only by improving anti-oxidant properties, but also by specifically reducing these pro-inflammatory cytokines. Indeed further investigations would be required in order to determine the specific actions of each supplement on these parameters to provide more conclusive results as to which supplement is providing the main effect, as well as to elucidate how the combination of these supplements influence one another when administered simultaneously. Although, it was deemed likely that given the different pathways each supplement works on, their effects would have been additive, thus maximising its beneficial potential, we cannot rule out that in combination they may have negative effects, or at least inhibit some of the benefits of the other. Indeed, if “aspirin resistance” did occur in the Obese ZDF animals then the aspirin may not have been having any effect, but rather the whey protein was inducing the over-riding anti-inflammatory effect. Given the usual targets of whey protein, this may have been why the responses were more pronounced in type II fibres. Despite this, and given the complexities and heterogeneity of this disease, there is an advantage of having multiple treatments that target multiple comorbidities which should be a common goal for any new treatment regime, given that patient adherence to taking pills is inversely proportional to the number of pills prescribed (Donath, 2014).

It has long been determined that high levels of TNF- α and Il-6, as seen in T2D (Sell et al., 2006; Wei et al., 2008), have catabolic effects on skeletal muscle (Goodman, 1994) resulting in muscle atrophy (Fujita et al., 1996; Tsujinaka et al., 1996; Pedersen et al., 2003; Janssen et

al., 2005; Wei et al., 2008). This has been shown to occur via a reduction in growth factor-mediated intracellular signalling (Haddad et al., 2003) and apoptosis (Wei et al., 2008). Therefore it was hypothesised in this study that by improving pro-inflammatory cytokines there would be improvements to insulin resistance and therefore muscle atrophy. In chapter 4 it was reported that ZDF animals had significantly smaller muscles in terms of muscle weight, fibre area and cross-sectional area compared to Lean littermates in EDL but not in soleus. Correspondingly, measurements of skeletal muscle pro-inflammatory cytokines showed significantly higher levels of TNF- α and Il-6 in EDL but not in soleus suggesting involvement of these cytokines in some of the muscle catabolic processes mentioned above, and that EDL is predominantly affected. However, whilst the supplements utilised in this study significantly reduced both Il-6 and TNF- α in EDL, systemic insulin resistance and thus skeletal muscle atrophy were not altered in this muscle group at rest. This could either indicate that muscle anabolic and catabolic activity occurs independently of cytokine activity in contrast to previous reports (Sishi and Engelbrecht, 2011; Munoz-Canoves et al., 2013), and that the degenerative pathway has not been inhibited, given the role of insulin resistance in causing higher protein breakdown rates (Price et al., 1996; Wang et al., 2006). Alternatively, insulin has been shown to stimulate protein synthesis and anti-apoptotic effects in cardiac myocytes (Flaim et al., 1983; Aikawa et al., 2000), so it is possible that insulin has not been able to promote the anabolic pathway due to the persistent level of insulin resistance still apparent in these animals.

Unfortunately, ROS such as superoxide anions and antioxidants such as glutathione and manganese superoxide dismutase were not measured in this study, and so would be beneficial in determining which supplements had the greater effects (in addition to testing singular supplement effects), not only in obesity and insulin resistant states, but in type I versus type II

muscle fibres. This would elucidate which muscles may be more affected and hence allow more precise targets for future drug treatment development. In addition, it would be useful to determine if insulin resistance is affected locally in the muscle by potentially measuring IRS-1 serine phosphorylation. Furthermore if insulin resistant states cause resistance to any supplement/drug therapies, investigating ways to overcome them in order to exert their therapeutic effects would be paramount. Finally, determining the atrophy pathways, such as components of protein synthesis and breakdown rates and other protein markers such as JNK, IGF1, MURF, akt/mTOR, NF- κ B/ I κ B and how pro-inflammatory cytokines affect these would allow us to understand the mechanisms behind some of the skeletal muscle changes occurring in T2D.

7.5 Conclusion

In conclusion, this study has shown elevated intramuscular levels of Il-6 and TNF- α in ZDF rats compared to Lean littermates, and that this elevation is specific to fast-twitch muscle fibres. Systemic insulin resistance was also apparent in ZDF animals. Although supplementation with aspirin, creatine and whey protein reduced Il-6 and TNF- α in EDL, and plasma Il-6, this did not result in improvements to insulin resistance or muscle atrophy of diabetic animals. The mechanisms by which these supplements interact together and separately requires further investigation.

CHAPTER 8

Conclusions and Future Directions

8.1 Conclusions

Finding a cure for T2D is no easy feat, and in order to do so, determining the precise cause would be paramount, as it would be in any disease. However, T2D is complex given its heterogeneous nature and the multitude of interactions occurring between organs and organ systems. As such, it is important to look at these systems and how each of the organs function within them to provide more insight into how they impact upon the disease state. Skeletal muscle is the main site for glucose disposal, with insulin-stimulated glucose uptake highly impaired in insulin resistant states like T2D (De Fronzo, 2004; De Fronzo, 2009). Overall, this thesis aimed to look at some of the changes to skeletal muscle morphology and function that potentially occur in T2D and give further insight to some of the mechanisms behind these changes. The efficacy of utilising supplements such as creatine monohydrate, whey protein isolate and aspirin to ameliorate any of the changes seen to skeletal muscle was also examined.

Skeletal muscle atrophy has been shown to be one of the morphological changes that occur in T2D (Cotter et al., 1993), and is certainly a change confirmed in chapter 3 of this thesis (Pompeani et al., 2014). In this chapter, whilst the mechanisms leading to this change were not elucidated, it was determined to be independent of membrane damage induced by Ca^{2+} dysregulation or oxidative stress as indicated by calpain autolysis and lipid peroxidation, respectively. The lack of changes in SR Ca^{2+} leak found in chapter 5 further suggests that Ca^{2+} dysregulation is not a mechanism involved in any changes to muscle in this condition. Nevertheless, it was found that there was excessive extracellular albumin accumulation (sarcolemmal and interstitial) in relation to the hyperglycaemic environment, which as discussed in chapter 3.4, can alter membrane fluidity potentially impairing nutrient availability for ATP production and impairing muscle contractile function.

Indeed, analysis of skeletal muscle contractile function in chapter 4 indicated that peak twitch and tetanic force production, $\frac{1}{2}$ RT and TTP were not altered in insulin resistant rats, however fatigue and recovery to fatigue were, particularly in soleus, which subsequently had a higher percentage of EBD/albumin accumulation compared to EDL. In addition, MAPR was found to be impaired in Obese compared to Lean red muscle, as shown in chapter 6. Taken together, the results of this thesis show that slow twitch muscles are more susceptible to the effects of insulin resistance and hyperglycaemia, which result in albumin accumulation either on the extramyocellular surface or in interstitium. The downstream effects of this are interference with mitochondrial function, resulting in a higher fatigability index in sedentary Obese insulin resistant rats. This is an important finding as the majority of skeletal muscle work on animal tissue investigating glucose uptake and insulin-related pathways are performed on fast-twitch (white) muscles such as the EDL (Hong et al., 2015). Hence future work needs to include both white and red muscles.

Another mechanism thought to influence muscle function was SR activity (chapter 5). Given that the release and uptake of Ca^{2+} from the SR is responsible for the contraction and relaxation of skeletal muscle, respectively, it was hypothesised that any impairment in contractile force would be directly linked to impairments in SR function. As mentioned above, no changes to force production, $\frac{1}{2}$ RT or TTP were observed in Obese rats. Obese animals likewise showed no significant differences in Ca^{2+} uptake rates, which would explain why no changes in $\frac{1}{2}$ RT time were observed. However in red gastrocnemius muscles, there was a strong trend toward a reduction in uptake rates, indicating a potential for developing impairments with disease progression. Indeed this may also impact upon the fatigue index shown in this muscle group with subsequent stimulation. On the other hand, Ca^{2+} release rates of both RG and WG were shown to be lower in Obese animals and hence does not

explain why no differences were observed in TTP measurements in EDL and soleus of this group of animals. A limitation to this analysis may be the use of different muscles for the contractile experiments (EDL; almost exclusively fast-twitch and soleus; almost exclusively slow-twitch) and the use of gastrocnemius muscles for the SR and mitochondrial function assays, which comprises of mixed muscle fibres. Whilst every attempt was made to separate out red and white fibres to delineate fast and slow properties, the percentage of each fibre type was not determined in each sample. Indeed it would be beneficial to utilise one set of muscles to determine each of the characteristics measured above, especially given the variability of results obtained across different muscles already found in this disease (Lennmarken et al., 1986; Smith et al., 1989; Stephenson et al., 1994; He et al., 1995; Warmington et al., 2000; He et al., 2001; Adachi et al., 2007; Ouwens et al., 2007; Barzilay et al., 2009; Katta et al., 2009; Shortreed et al., 2009; O'Neill et al., 2010). Nevertheless, it appears unlikely that SR function is pivotal in the initial changes to skeletal muscle observed in T2D.

Another factor thought to influence insulin resistance and hence muscle function are the pro-inflammatory cytokines IL-6 and TNF- α (Mohamed et al., 1999; Dandona et al., 2004). Whilst these two cytokines were shown to be higher in EDL of Obese animals, the same observation was not shown in soleus. Hence, any effects these cytokines may have on muscle function do not appear to impair contractile or SR function or MAPR given all of the impairments found were in soleus. As such the influence of these cytokines on skeletal muscle requires further investigation.

Supplementation with creatine, whey and aspirin was shown to have some beneficial effects on muscle function. In particular, the fatigue index and recovery to fatigue was significantly

improved in soleus muscle of ObSupp animals, such that they became comparable to their Lean littermates. This occurred independently of muscle mass, given no increases in muscle mass were observed following supplementation. This may be related to the significant improvements to MAPR in red muscle (RG) indicating that mitochondrial impairment played a vital role in the fatigue/recovery process. Whether this is due to improved substrate uptake and utilisation cannot be determined as local insulin resistance was not measured. Whilst it was speculated above that the increased extracellular albumin content may interfere with substrate uptake, we did not determine if albumin content had been altered with supplementation. Certainly, the fat content had not been reduced with supplementation, although it is possible that supplementation improved/increased the use of this intracellular fat for energy production, as supported by increased ATP production with PC&M, at least in RG. Furthermore, Ca^{2+} uptake and release rates were not altered with supplementation. It is possible that given minimal changes were observed in SR and twitch and tetanic force production, that the muscle utilised the supplements in other areas where it was needed more, such as the mitochondria. This concept has been reported in other disease states, such as muscular dystrophy (Rybalka et al., 2015) where despite being characterised by muscle wasting and weakness, whey protein and creatine supplementation preferentially increased proteins associated with mitochondrial function. The results of the current study suggest metabolism (and hence mitochondria) is the main target for the supplements used.

Previous studies have shown a role for inflammation in developing insulin resistance (Perseghin et al., 2003; Dandona et al., 2004; Shoelson et al., 2006; Shoelson et al., 2007). This thesis has shown that although the combination of creatine, whey and aspirin reduced IL-6 and TNF- α in EDL, no effects occurred in Soleus, which was the muscle affected by heightened fatigue, slower recovery and reduced MAPR. Furthermore, despite reductions in

cytokines of EDL, no improvements to systemic insulin resistance occurred. As discussed in chapter 7.4, the effects of these supplements to local muscular insulin resistance were not determined, as such it remains unknown as to whether any effect had taken place locally.

8.2 Future Directions

This thesis has confirmed a degree of muscle atrophy in the sedentary ZDF rat model of T2D, however the mechanisms behind this remain to be elucidated. Furthermore, while organelle function was assessed in this thesis, molecular regulation of atrophy pathways need also be considered. The effects of a more sedentary lifestyle appear to impact on muscle and act as a “stressor” causing phosphorylation and activation of the p38 and c-Jun N-terminal kinase (JNK) pathways leading to p53 activation (Machida and Booth, 2005) and reductions in muscle mass, irrespective of whether it is primarily muscle wasting or growth retardation. Downstream of this pathway, a family of growth arrest and DNA damage-inducible (GADD45) proteins exist. These proteins function to protect cells and induce cell growth, DNA repair and apoptosis (Sytnikova et al., 2011). However, over-expression of GADD45 in particular during muscle disuse has been shown to lead to growth inhibition and is involved in the atrophy process in skeletal muscle (Ebert et al., 2012). These authors have shown that GADD45a induces myonuclear remodelling and a comprehensive program for muscle atrophy, by repressing genes involved in anabolic signalling and energy production, and it induces pro-atrophy genes. Interestingly these authors also showed a reduction in PGC1 α (a regulator of mitochondrial content and oxidative metabolism, critical in maintaining glucose, lipid and energy homeostasis in muscle (Lin et al., 2002)) and mitochondrial DNA. Given the atrophy and mitochondrial impairments in ZDF rats found in this thesis, future research in determining the involvement of GADD45a as a potential mechanism in causing these changes would be beneficial, as to my knowledge, no studies have assessed GADD45a in

T2D and so would be useful to ascertain its role in the disease and become a potential target for therapy.

Another stressor found to activate GADD45a and lead to muscle wasting is muscle denervation (Ebert et al., 2012). Interestingly, one of the complications found in T2D is peripheral diabetic neuropathy as reviewed by Said (2007). Diabetic neuropathy is a form of nerve damage that can occur if you have diabetes as a result of hyperglycaemia, which most often damages nerves in your legs and feet. Indeed the ZDF rat has been shown to develop diabetic neuropathy with the onset of hyperglycaemia (Coppey et al., 2002; Oltman et al., 2005), and furthermore it has a complex aetiology which is difficult to reverse (Oltman et al., 2008). Nevertheless, it is postulated that the development of diabetic neuropathy may activate GADD45a, which further adds to the muscle wasting condition seen in this disease. As such finding ways to prevent or delay this complication would be of utmost importance given its irreversible nature.

The GADD45a proteins have also been shown to be indirectly activated by oxidative stress (which activates JNK, p38 phosphorylation, p53 and subsequently GADD45a), and directly by increases in oxygen radicals, interleukins, and non-steroidal anti-inflammatory drugs (Rosemary Siafakas and Richardson, 2009). Not only are all these components implicated in T2D, of interest are the effects of non-steroidal anti-inflammatory drugs on the system. One such drug is aspirin, which whilst may have its benefits as an anti-inflammatory, may also be counteractive by activating GADD45a to amplify the catabolic effects on muscle. It may be that activation of GADD45a by aspirin, and hence the atrophy pathway, impeded the anabolic effects of the whey and creatine used in this series of experiments, and a possible mechanism as to why improvements to atrophy were not seen utilising this supplementation regime.

Hence it would be essential to not only study the effects of aspirin on GADD45a in T2D, but also to more closely study the mode of action of each individual supplement to ascertain how each one influences each system, and give further insight as to how they interact in combination. Indeed the dosage rate and regime may also be reviewed as it may be of value to administer each supplement in succession of one another as opposed to in combination to prevent counteractive mechanisms taking place. For example, administering aspirin first to help reduce inflammatory effects on muscle and improve insulin resistance, then supplement with whey protein to further improve insulin resistance and build muscle mass and then administering creatine, in order to achieve better insulin-mediated creatine loading. The addition of carbohydrate to creatine may also be tested in such animal models of T2D, provided some improved insulin sensitivity as already been achieved.

While further work is clearly needed with optimising the type and mode of implementation of potentially beneficial supplements, given the complexity of the diabetic condition, a whole body approach, such as seen with exercise, is desirable. The use of whey and creatine in combination have been shown to increase Lean muscle mass to a greater degree than either supplement alone, and these improvements are even greater when combined with exercise in healthy males (Burke et al., 2001). More specifically, the timing of supplement administration with respect to exercise training in healthy males has also been shown to influence the changes in muscle cross-sectional area observed (Cribb and Hayes, 2006a). As such, timing of administration is another variable to consider when looking at supplementing with creatine and whey in T2D. The type of exercise that would be most beneficial would also need to be considered given the different stages of disease progression and what would be most beneficial at each stage as discussed below.

One of the main problems with reduced insulin sensitivity and impaired glucose transport into the cell is that it reduces the potential for ATP production by the mitochondria, as has been shown in this thesis, hence reducing the amount of energy substrate available to the muscle for efficient contraction-relaxation cycling. This may account for the exercise intolerance seen in people with T2D (Demir et al., 2001). This is further exacerbated by the decreased ability of diabetic individuals to oxidize fatty acids (Kelley and Goodpaster, 2001), with the subsequent accumulation of fatty acid intermediates driving the increase of inflammatory factors such as JNK and NF- κ B, which further decreases insulin sensitivity. Therefore therapies aimed at improving insulin resistance may potentially reverse this effect, with the benefits of exercise to improve insulin sensitivity being well known (Borghouts and Keizer, 2000), as well as there being many other positive effects of exercise (Horowitz, 2007; Praet and van Loon, 2007; Wang et al., 2009).

Exercise has long been known to improve insulin sensitivity, with even a single bout of endurance exercise eliciting improvements (Devlin et al., 1987; Cartee et al., 1989), and exercise training, along with other lifestyle modifications, decreasing the severity of symptoms and/or likelihood of developing T2D (Dunstan et al., 1997; Tuomilehto et al., 2001; Knowler et al., 2002; Lindstrom et al., 2006). Interestingly however, the “optimal” type, intensity and duration has not been confirmed (Horowitz, 2007), particularly due to age of onset and relative progression of the disease (Praet and van Loon, 2007). While endurance exercise may be of benefit for improving fitness, maximal oxygen uptake and, with the right circumstances, weight loss, it does not have any major effect on increasing muscle fibre size (Kim et al., 2004) or reversing the muscle weakness observed in diabetic patients (Andersen et al., 2004; Sayer et al., 2005). Thus, exercises promoting increases in Lean mass, i.e. resistance training, should be included as part of any exercise therapy. Skeletal muscles have

been shown to be able to adapt to resistance training even in very old age (Seynnes et al., 2004) and thus muscles do not lose their capacity to adapt. Importantly for diabetic sufferers, the improvements observed from endurance exercise in diabetes are also observed following resistance exercise training, such as improved insulin sensitivity (Fenicchia et al., 2004; Brooks et al., 2007) and whole body glucose tolerance. More importantly, resistance exercise training has been shown to increase the area of both type I and type II fibres in diabetic individuals (Holten et al., 2004; Gordon et al., 2006; Brooks et al., 2007), whereas this effect is not observed after endurance training (Kim et al., 2004). Further, increasing contractile activity as seen with resistance training is able to stimulate regulatory pathways, such as p70^{S6K}, a controller of muscle protein synthesis downstream of mTOR, independently of Akt and insulin action (Baar and Esser, 1999; Hornberger and Esser, 2004). In addition, there is likely to be endurance-like benefits, such as improved maximal oxygen uptake, derived from the resistance training, especially if a circuit training approach is used. Indeed, such improvements in both strength and muscle oxidative function have been observed in CHF patients following a structured resistance training program (Williams et al., 2007), and where resistance training is combined with endurance training (Beckers et al., 2008).

While resistance training improves mass and strength of skeletal muscle, obesity is commonplace for many diseases, and is particularly pertinent as a forerunner of diabetes, and thus exercises that promote fat loss also need to be considered. Exercise for weight loss (or more specifically fat loss) has traditionally been prescribed as continuous, low- to moderate-intensity exercise. This is due to the fact that the relative contribution of fat oxidation to exercise is higher at lower exercise intensities compared to higher intensities, which see a rise in the respiratory exchange ratio (RER) as muscle metabolism shifts to CHO use (Romijn et al., 1993; Venables and Jeukendrup, 2008). However, Obese people exhibit a higher resting

RER, indicative of a higher CHO contribution (or more importantly a decreased fat oxidation, despite the higher availability of FFA), and then a decreased elevation in RER during exercise (Phielix and Mensink, 2008). Presumably this occurs due to the difficulties in glucose uptake into exercising muscles, meaning an earlier introduction of anaerobic metabolism and the subsequent limitation on the time and/or benefits of exercise, particularly for fat loss. Indeed, during submaximal exercise, a lower total body glucose uptake and oxygen consumption has been observed in Type 2 Diabetic patients (Bauer et al., 2007), with this effect not due to impaired capillary recruitment unless there is also microvascular complications (Womack et al., 2009). Thus, in a “time-poor” society and a likely limit to exercise time in obese and diabetic individuals, it can be suggested that long duration sub-maximal exercise will not be either (a) sustainable, or (b) useful in producing significant fat loss. Indeed, moderate intensity exercise of less than 1 hour consistently fails to elevate 24-hour fat oxidation (Melanson et al., 2009), whereas vigorous sporting activities reduce the incidence of T2D (Helmrich et al., 1991).

Individuals participating in regular vigorous activities are leaner than those that do not (Tremblay et al., 1990), and high-intensity intermittent exercise (HIIE) training elicits a greater decrease in adiposity than steady state moderate-intensity exercise training, despite over twice as much estimated total energy cost of steady state exercise compared with HIIE (Tremblay et al., 1994), or a similar energy expenditure, yet achieved with only half the exercise time (Trapp et al., 2008). Thus, high-intensity exercise is seen as beneficial for diabetic individuals, especially with accompanying obesity (Praet and van Loon, 2007) and using this intensity “intermittently” may produce significant benefits.

Indeed, by its very nature, resistance training is a high-intensity intermittent type of exercise, and progressive resistance training has been shown to reduce adiposity in overweight children (Benson et al., 2008), and in addition to its already listed desirable effects for diabetes, also has additional benefits. Firstly, maintenance of a good lean muscle mass is a key contributor to resting metabolic rate (and hence weight maintenance), and a higher lean muscle mass improves whole body glucose disposal. Secondly, a higher lean mass, and the accompanying strength gains, will offset some of the decreased activity commonly observed with diabetes. Only when sufficient muscle mass and strength has been achieved can exercise regimes revert to a more traditional endurance (preferably of a high- to moderate-intensity approach (Holten et al., 2004; Praet and van Loon, 2007). However some lean muscle mass retaining resistance exercises should still be incorporated, and strategies to aid this, such as protein supplementation discussed earlier in this thesis should be further tested.

Trying to reduce obesity and hence the risks of diabetes may be hugely beneficial, however, what makes T2D more complex, is that up to 15% of those suffering from the disease are in fact lean (Zimmet et al., 2001). As such it would be useful to determine if the same skeletal muscle aberrations occur as per the obese condition, or if there are different mechanisms causing the changes seen. Indeed a recent study in the Goto-Kakizaki rat, a lean model of T2D, assessed mitochondrial function in gastrocnemius muscle by using magnetic resonance (MR) imaging and 31-phosphorus MR spectroscopy (³¹P-MRS) (Macia et al., 2015). They found that basal oxidative ATP synthesis rates were not altered in the diabetic rats despite having the same IMCL content and furthermore, after a fatiguing protocol, whilst mechanical performance and glycolytic ATP production were reduced, oxidative ATP production rates were unchanged in diabetic animals. In addition, no differences in fatty acid content were detected between diabetic and non-diabetic animals. Clearly there were different

methodological considerations between measurements made in this thesis and that by Macia et al., (2015), and hence further investigation into both contractile and mitochondrial function in lean models of T2D is required.

One of the main difficulties of studying T2D involves the changes that occur throughout disease progression. This makes comparisons between studies difficult as different animal models, different muscle groups, and different stages of the disease are measured yielding different results. Indeed this may also be a limitation for some of the results found in this thesis. As such it would be beneficial to characterise the time course of the progression of skeletal muscle morphological, contractile, mitochondrial, and SR changes, at each stage of the disease. This may shed some light as to what aspect of muscle function begins to deteriorate first and as such bring us closer to determining causal links or at least an avenue as to which part of muscle function to commence treatment on. For example, given that the results of this thesis have shown mitochondrial impairments and muscle fatigability to be the first variables affected, perhaps supplementation with whey protein initially should commence first. However this argues against the previous suggestion of aspirin supplemented first to limit and/or decrease insulin resistance, again highlighting the difficulty in demonstrating an effective supplementation regime in this disease.

A further limitation to this series of experiments is that all parameters were measured shortly after the commencement of the diabetic stage, despite supplementation commencing in the pre-diabetic stage (for profiling of ZDF rat in their progression to overt diabetes see Etgen and Oldham (2000)). As such, supplementing animals within each phase alone without any overlap between phases, and utilising the same one set of fast/slow muscles such as EDL/Soleus respectively for each parameter, would allow for more controlled and conclusive

results without interference from the potential diabetic complications that develop with disease progression, as well as minimising different effects of different muscle groups.

One of the main characteristics of T2D is the presence of insulin resistance. One of the limitations of this thesis in studying insulin resistance is that it was only measured at the systemic level, hence any potential local changes were not determined. Changes to individual organs, skeletal muscles in particular, are likely given the muscle specificity of the supplements used. Hence any future research in this area would benefit from such local determinants to give greater insights into the influence of intramuscular processes on insulin resistance. Nevertheless, given that systemic insulin resistance was not altered with this particular supplementation regime, this may in itself provide more information about the disease process.

It is a common view that insulin resistance brings about many of the skeletal muscle metabolic perturbations as a result of nutrient oversupply thus restricting substrate uptake and hence utilisation. However an emerging view is that perhaps insulin resistance acts more as a protective mechanism (Connor et al., 2015). For example, mitochondria are a source of ROS production which is increased in response to increased lipid availability (Anderson et al., 2009). It reaches a point where the anti-oxidative capacity of muscle cells is compromised as discussed in chapter 1, and as such, to prevent further deleterious effects from excess substrate utilisation, insulin resistance results as a preventive measure. Indeed this would help explain why no improvements were seen in this thesis regarding insulin resistance, given no changes to IMLC were observed. It can further be speculated that any improvements to mitochondrial function may have occurred due to potential reductions in ROS, particularly superoxide anions, allowing a greater capacity for mitochondrial function. However, ROS

and anti-oxidant defences were not measured herein and would require further research. Another potential protective mechanism that may have occurred in this thesis results from the excessive albumin found on the extracellular surface of muscle cells which binds extracellular Ca^{2+} hence preventing Ca^{2+} -mediated cell damage, as discussed in chapter 3.4. Furthermore the significant reductions in Ca^{2+} leak from the SR of Obese animals discussed in chapter 6 may be an additional source of cellular preservation, however the underlying mechanisms such as increased calsequestrin or other SR regulatory proteins such as sarcolipin requires further investigation and its role in preventing intracellular Ca^{2+} accumulation.

In conclusion, this thesis has shown that skeletal muscle of sedentary Obese ZDF rats are significantly smaller, more fatigueable, with impaired recovery of mainly slow twitch muscle fibres. The underlying cause of the fatigue and recovery processes may be a result of the impaired MAPR and thus reduced energy available to the contractile apparatus of Obese ZDF animals, thus restricting prolonged contractile function. Supplementation with creatine, whey and aspirin was beneficial in improving some parameters of muscle function but not all. MAPR was improved with supplementation as was the fatigue and recovery processes such that they returned to control conditions. This thesis gives further evidence that mitochondria may play a significant role in disease progression, given that inflammation and SR function had little influence on the outcomes observed herein, and that supplementation with creatine, whey and aspirin may show promise as an aid to improving muscle endurance. However the supplementation regime as well as mechanisms behind them and the altered mitochondrial function require further investigation.

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APPENDICES

APPENDIX A

Calculation of K_d for Indo-1:

$$\text{Rel. Fluor.} = \log_{10} \left[\frac{(\text{Fluorescence} - \text{Fluorescence}_{\min})}{(\text{Fluorescence}_{\max} - \text{Fluorescence})} \right]$$

$$\text{Relative fluorescence @ 405nm: } y = -1.9674x + 12.705$$

$$\text{Relative fluorescence @ 485nm: } y = 1.1524x - 7.5338$$

When $y = 0$ (i.e. the point at which the dye is 50% bound such that $\text{Rel. Fluor.} = \log_{10} 1$)

$$\rightarrow -1.9674x + 12.705 = 1.1524x - 7.5338$$

$$\rightarrow (1.152 + 1.9674)x = 12.705 + 7.5338$$

$$\rightarrow x = 20.2388 / 3.1194$$

$$\rightarrow x = 6.488$$

Therefore, the $pCa @ K_d = 6.488$

$$\rightarrow -\log[Ca^{2+}] = 6.488$$

$$\rightarrow [Ca^{2+}] = 10^{-6.488}$$

$$\rightarrow K_d = 356.45\text{nm}$$