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Mitochondrial Capacity in Cardiac Muscle of Diet-Induced, Insulin Resistant Mice

by

Zahra Ezzat Zadeh

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Mitochondrial capacity in cardiac muscle of diet-induced, insulin resistant mice " submitted by Zahra Ezzat Zadeh in partial fulfilment of the requirements of the degree of Master of Science.

Supervisor, Dr. Jane Shearer, Faculty of Kinesiology

Dr. David L. Severson, Faculty of Kinesiology

Dr. Dustin Hittel. Faculty of Kinesiology

Dr. Darrell Belke. Faculty of Kinesiology

Dr. Andrew G. Howarth. Faculty of Medicine

May 18, 2010

Date

Abstract

Obesity is highly correlated to insulin resistance and cardiovascular complications. Cardiac dysfunction can result from impaired energy metabolism and has been found in association with abnormal mitochondrial structure and function. The purpose of this study was to examine the effects of a high fat diet (HF) on mitochondrial respiratory capacity and its contribution to left ventricular dysfunction. Male C57BL/6J mice were fed either chow (CH) or HF for 20 weeks (n=10/treatment). Physiological and biochemical parameters, cardiac mitochondrial structure, electron transport chain (ETC), enzymatic activities as well as left ventricular function were examined. HF was associated with mitochondrial adaptation including increased mitochondrial content and ETC activity. However, this increase was not accompanied by enhanced cardiac oxidative metabolism. As a result, the insulin resistant heart has impaired mitochondrial structure and function that is associated with reductions in myocardial efficiency.

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Dedication

To my mother who have been there for me during my ups and downs throughout this project, giving me unconditional love and support. And in the memory of my father, whose presence and support is sorely missed.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
ATP	Adenosine triphosphate
BMI	Body mass index
CD36	Cluster of Differentiation 36
CH	Chow
CHO	Carbohydrate
CoA	Coenzyme A
COX	Cytochrome *c* oxidase
CS	Citrate synthase
Cu/Zn-SOD	Cytosolic superoxide dismutase
DNA	Deoxyribonucleic acid
DTT	DL-Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EF	Ejection fraction
EM	Electron microscopy
ETC	Electron transport chain
FA	Fatty acids
FADH	Flavin adenine dinucleotide
FAT	Fatty acid translocase
FCCP	carbonyl cyanide *p*-(trifluoromethoxy) phenyl-hydrazone
FS	Fractional shortening
HF	High fat
IFM	Interfibrillar mitochondria
IVSs	Interventricular septal thickness in systole
IVSd	Interventricular septal thickness in diastole
GLUT1	Glucose transporter 1
GLUT4	Glucose transporter 4
KCN	Potassium cyanide
LV	Left ventricular
LVIDs	Left ventricular end-systolic dimension
LVIDd	Left ventricular end-diastolic dimension
LVPWs	Left ventricular posterior wall thickness in systole
LVPWd	Left ventricular posterior wall thickness in diastole
MN-SOD	Mitochondrial superoxide dismutase
mRNA	Messenger ribonucleic acid
Mt DNA	Mitochondrial DNA
NADH	Nicotinamide adenine dinucleotide
NEFA	Non-esterified fatty acids
O_2	Oxygen

OXPHOS	Oxidative phosphorylation
PDH	Pyruvate dehydrogenase
PPAR-α	Peroxisome proliferator-activated receptor-α
PRO	Protein
^{31}P NMR	Phosphorus-31 NMR (for nuclear magnetic resonance)
ROS	Reactive oxygen species
SEM	Standard error of the mean
SOD	Superoxide dismutase
SDH	Succinate dehydrogenase
SSM	Subsarcolemmal mitochondria
T2D	Type 2 diabetes mellitus
TCA	Tricarboxylic acid
TG	Triglyceride
UCPs	Uncoupling proteins

Chapter One: Introduction

1.1 Introduction

Type 2 diabetes mellitus (T2D) is a chronic metabolic disorder. It is characterized by insulin resistance, hyperglycemia, hyperinsulinemia and increased plasma free fatty acids. The incidence of T2D is rapidly increasing and is reaching epidemic proportions. It is estimated that currently 190 million people all over the world suffer from T2D and it is predicted that this number will increase to 300 million in 2025 (68, 155). One of the main causes of morbidity and mortality in insulin resistant, obese and T2D patients is cardiovascular complications (40, 86). Both T2D and obesity are independent risk factors for the development of cardiac dysfunction and heart failure (10, 11, 140). Alterations in substrate metabolism may be the key to the development of myocardial dysfunction in T2D (85, 109). The healthy adult heart mainly metabolizes free fatty acids, glucose, and lactate for ATP production in the mitochondria. However, under diabetic conditions, glucose and lactate oxidation in the heart are decreased (26, 131) and fatty acid oxidation is increased (134).

Cellular studies support the hypothesis that obesity and T2D are associated with impaired mitochondrial biogenesis and function (107, 109, 118). Mitochondria have a very important role in the energy supply for active metabolic tissues like the heart. The association between impaired mitochondrial function and T2D and insulin resistance has been reported in skeletal muscle (6, 74, 89) as well as other tissues, including liver, fat, heart, blood vessels, and pancreas (5, 18, 99, 150). Different *in vitro* methods have been used to obtain data for these studies, including measurements of mRNA and/or protein

expression of oxidative phosphorylation genes (56, 91, 92, 104), oxidative enzyme activities (51, 52, 66, 132), mitochondrial content, morphology and respiration (66, 89, 92). In cardiac muscle, several studies have shown an association between cardiac mitochondrial oxidative energy and alterations in cardiac mitochondrial morphology and function (64, 102, 123). Increased numbers of morphologically abnormal mitochondria have been demonstrated in myocardial tissues of insulin-resistant rodent models using transmission electron microscopy (33, 98, 135). These observations have been associated with left ventricular hypertrophy, displaying oxidative stress (135, 137, 149). Although increased numbers of morphologically abnormal mitochondria could be an early adaptive response to oxidative stress and an increased energy demand, at the later stages in the pathogenesis of cardiac dysfunction and hypertrophy it has been shown that mitochondria DNA content is reduced (38).

1.2 Objectives

The objective of this study is to assess the effect of high fat feeding on cardiac mitochondrial structure and function and their contribution to development of cardiac dysfunction. Overall, this study aims to provide insight into mitochondrial oxidative phosphorylation and cardiac function in parallel providing comprehensive insight into the alterations in mitochondrial morphology induced by high fat feeding.

1.3 Hypotheses

The overall hypothesis of this project is that oxidative phosphorylation and electron transport capacity are increased in high-fat feeding. This is, in part, explained by increased delivery of reducing equivalents to the electron transport chain and increased

mitochondrial content. Despite enhanced mitochondrial density, mitochondria will have a reduced functional capacity. As a result, insulin resistant hearts will have impaired mitochondrial structure and function leading to reductions in myocardial efficiency.

Chapter Two: Literature Review

2.1 Diabetes and Cardiovascular Disease

Diabetes increases the incidence of myocardial infarction two to four fold, and diabetic patients suffer more mortality from any acute cardiovascular event compared to non-diabetic patients (88). Several studies have shown that the increased risk of developing heart failure persists in diabetic patients after adjusting cases for age, weight, cholesterol, blood pressure, and coronary atherosclerosis (54, 63). This has led to the use of the term *"diabetic cardiomyopathy"*, a clinical condition that is diagnosed when ventricular dysfunction occurs in diabetic patients in the absence of hypertension and coronary atherosclerosis (9, 36, 116). Diastolic dysfunction with a high prevalence of 60% in well-controlled T2D patients is now included in the term (9). Thus, developing left ventricular dysfunction may be a very common outcome of diabetes in addition to the increased prevalence of coronary atherosclerosis (9, 112). Contractile dysfunction has also been confirmed in rodent models of obesity, insulin resistance and T2D. Db/db and ob/ob mice are both models of T2D. Db/db mice are characterized by deficient leptin receptor activity as a result of mutation in the leptin receptor gene. Ob/ob mice are mutant mice that can not produce leptin. As a result, both strains have excessive appetite and become obese (4). Both systolic and diastolic dysfunction were reported using echocardiography of db/db and ob/ob mice (125). In isolated hearts of the db/db mouse, increased left ventricular end-diastolic pressure and decreased cardiac output and cardiac power have been reported (1, 8).

2.2 Glucose and Fatty Acid Uptake and Metabolism in Heart

In the healthy, normal heart, cardiac myocytes oxidize predominantly fatty acids (FA) (60–70%), and to a lesser extent carbohydrates (glucose 20% and lactate 10%) (46, 143). There are multiple steps involved in the regulation of glucose metabolism, including uptake, glycolysis, and pyruvate decarboxylation. Cardiac glucose uptake is regulated by the transmembrane glucose gradient and the content of glucose transporters in the sarcolemma (GLUT1 and GLUT4) (72, 83, 106). Although both GLUT1 and GLUT4 are located at the sarcolemma and in the intracellular storage compartments, under basal conditions, the majority of GLUT4 which is most abundant in the adult heart is located in an intracellular pool (83). Translocation of GLUT4 from an intracellular compartment to the sarcolemmal membrane is stimulated by insulin (106). In addition to translocation of glucose transport proteins, insulin also influences GLUT4 through its regulation of gene expression (81, 100). Inside the cardiomyocyte, glucose is rapidly phosphorylated into glucose-6-phosphate by hexokinase, resulting in intracellular trapping. From there, glucose-6-phosphate can enter the glycolytic pathway or be stored as glycogen (29, 117). Pyruvate, being the end product of glycolysis, crosses the mitochondrial membrane. Inside the mitochondria pyruvate dehydrogenase (PDH) converts it into acetyl-CoA (106). Acetyl-CoA is used for the synthesis of ATP by oxidative phosphorylation.

Two sources supply FA to the heart: albumin-bound FA and triglyceride (TG)-rich lipoproteins (139). Fatty acids can translocate into the cardiomyocyte by passive diffusion or by protein-mediated transport across the plasma membrane. Several FA

transport proteins have been identified and are thought to act together to facilitate cardiac FA uptake. The main putative FA transport protein is 88kDa fatty acid translocase (FAT) which is a rat mouse homologue of human CD36 (16). FAT/CD36, like GLUT4, is located at the sarcolemma as well as the intracellular storage compartments (47). Investigating the role of insulin in translocation of FAT/CD36, it has been demonstrated that insulin stimulates translocation of FAT/CD36 from intracellular sites to the sarcolemma in adult rat cardiomyocytes. In addition, there is a correlation between the amount of FAT/CD36 present at the sarcolemmal membrane and myocardial FA uptake (84). In the healthy normal heart, 70 to 90% of the fatty acids entering the cell are transported into the mitochondria for oxidation, and 10 to 30% are used for intracellular triglyceride synthesis or transformed to structural lipids (4). Carnitine-dependent transport system transfers acyl CoA molecules from the cytosol into the mitochondrial matrix. Once taken up by the mitochondria, fatty acids go through β oxidation and reduce nicotinamide adenine dinucleotide (NAD+) to NADH and flavin adenine dinucleotide (FAD) to $FADH_2$ which are the direct source of electrons for the electron transport chain (142).

2.3 Alteration of Substrate Metabolism in Heart during Diabetes

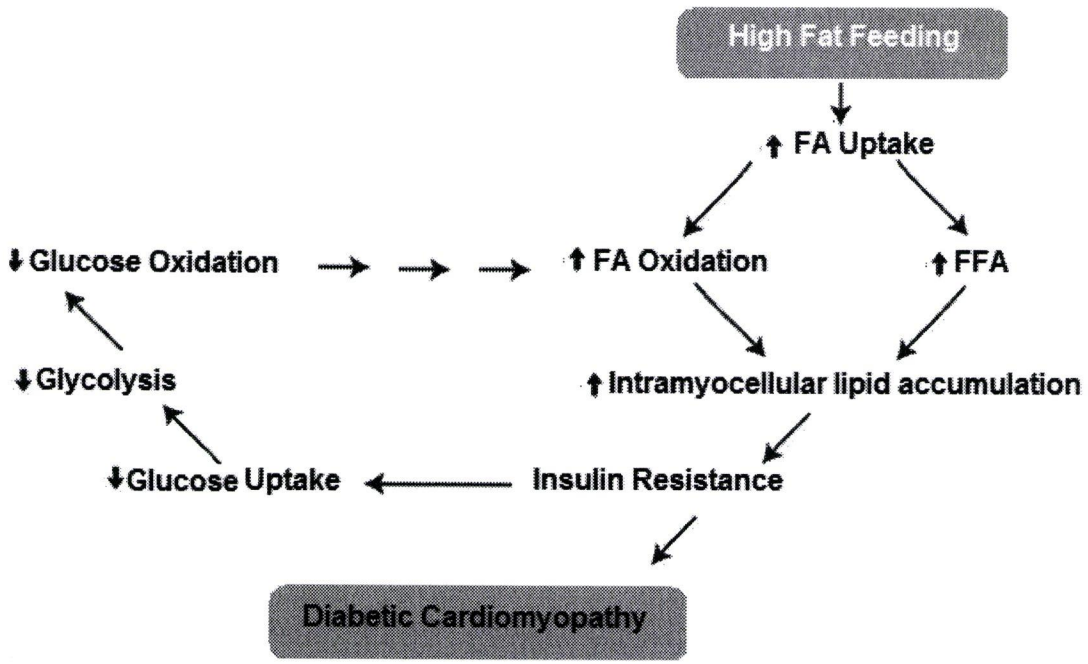
In T2D animals, cardiac glucose uptake is decreased as a result of reduced GLUT4 protein and impaired insulin signalling (22, 148). A reduced cardiac glucose oxidation associated with increased FA oxidation was observed at 4 weeks of age in ob/ob and db/db mice (20). Since this lower glucose oxidation was observed earlier than the onset of impaired insulin signalling in the heart and development of hyperglycaemia,

it has been concluded that reduced glucose utilization is more due to inhibition by high FA oxidation than impaired cardiac-specific insulin signalling. This has been confirmed with another study that used db/db mice at different ages and showed that increased cardiac FA oxidation preceded the reduction in glucose oxidation (2). An impaired myocardial glucose uptake associated with hypertriglyceridemia and increased plasma FA levels was observed in T2D patients (90). These findings suggest that in T2D, elevated FA levels in the plasma may result in impaired expression of GLUT4 (Figure 1).

Conversely, the utilization of FA by cardiac tissue increases in T2D. This change occurs as a result of a higher FA supply and an intrinsic adaptation/maladaptation to elevated FA (138, 154). The increased fatty acid metabolism may be the consequence of Randle's glucose fatty acid cycle hypothesis (114). According to the Randle hypothesis, an increased FA oxidation as a consequence of increased availability of intracellular FAs, contributes to the development of decreased cardiac glucose utilization (115). Increased FA oxidation leads to increased concentration of intracellular acetyl-CoA. Increased acetyl-CoA causes accumulation of citrate in the cytosol most likely due to increased synthesis of citrate through more available acetyl-CoA. High intracellular citrate leads to inhibition of phosphofructokinase-1 (PFK1) activity that inhibits the glycolytic pathway. Increased accumulation of intracellular acetyl-CoA and citrate also lead to increased glucose-6-phosphate concentrations and decreased hexokinase activity that inhibit the glycolytic pathway. Subsequently cardiac glucose uptake and oxidation are reduced (115).

While the enhanced FA uptake and oxidation by diabetic heart can be partially explained by Randle's glucose fatty acid cycle, altered gene expression in response to increased FA delivery to myocardium is known to have an equally important role (21). Activation of the nuclear receptor transcription factor, the peroxisome proliferator-activated receptor (PPAR-α) in the heart has been observed in several studies using obese or diabetic animal models including ob/ob and db/db mice and ZDF rats (39, 127). Activated PPAR-α promotes the expression of many genes encoding enzymes involved in fatty acid oxidation in the heart (35, 70).

Figure 1. Consequences of High Fat Feeding in Cardiac Metabolism.



High fat feeding or obesity increases fatty acid (FA) delivery and uptake. This will cause an accumulation of lipid inside cardiomyocytes and insulin resistance which together with a reduction in glycolysis and glucose oxidation contributes to the development of the diabetic cardiomyopathy.

Lastly, although lactate makes an important contribution in cardiac energy generation, there have been very few investigations regarding myocardial lactate metabolism in T2D. A greater decrease in lactate oxidation relative to glucose oxidation was observed in hearts from diabetic animals (25). A lower carbohydrate oxidation which was almost entirely due to reduced lactate utilization rather than glucose oxidation was observed in hearts from 12 week old ZDF rats (26). The mechanisms involved in inhibition of cardiac lactate oxidation in diabetes are unclear but are likely independent of lactate dehyrogenase or lactate transporter (25).

2.4 Role of Mitochondria

To maintain contractile function, the heart relies on continuous oxidative metabolism for ATP production. Energy production in the form of ATP is controlled mainly by mitochondria that account for approximately 40% of cardiomyocyte volume (67). Thus, examining the cause of metabolic disturbance and impaired mitochondrial function in diabetes is of importance. In addition, efficient substrate metabolism and fuel switching require proper mitochondrial function (67).

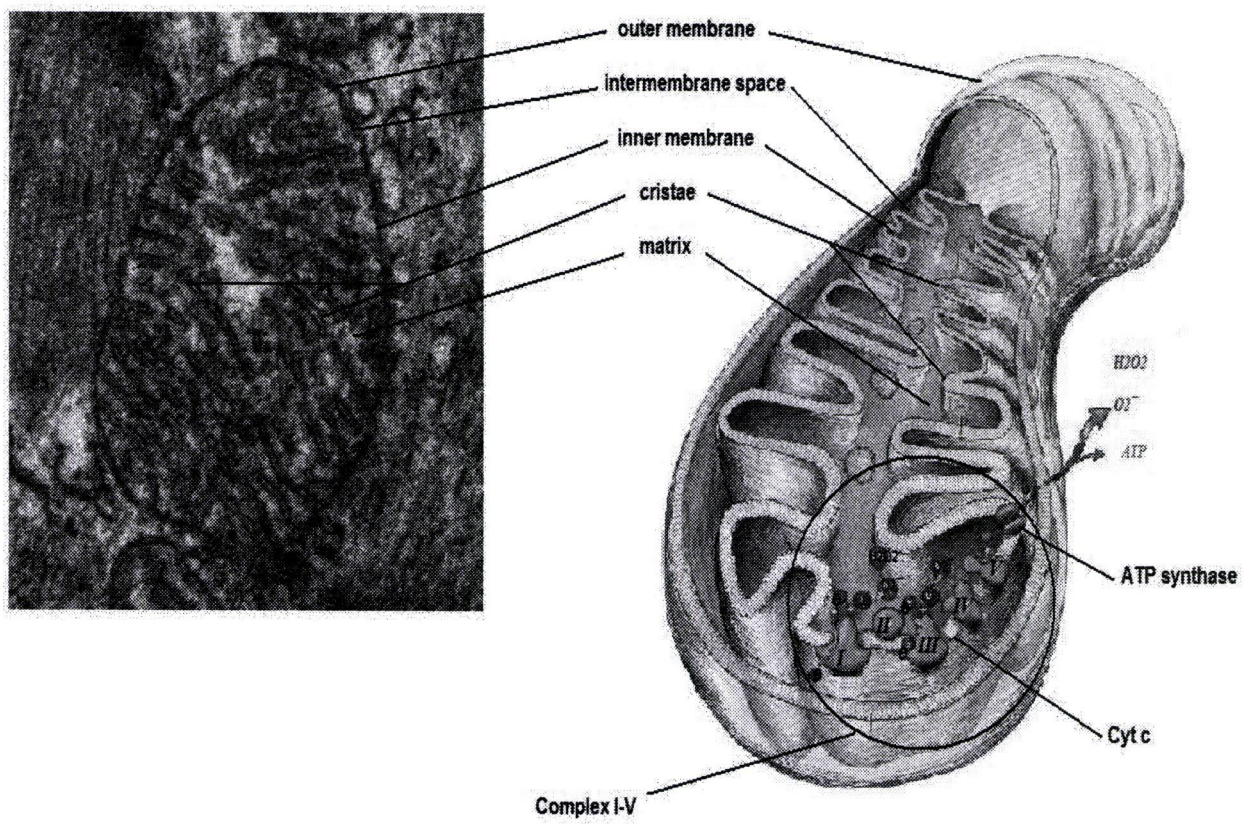
2.5 Mitochondria Structure and Function

Structurally, mitochondria are compartmentalized by: the outer membrane, the inner membrane, the intermembrane space, cristae and the matrix (the region inside the inner membrane) (Figure 2A). More than 90% of our cellular energy is produced by mitochondria that link oxidative phosphorylation (OXPHOS) with the metabolism of

substrates (31). Energy production is the result of two metabolic processes—the tricarboxylic acid (TCA) (69) cycle, also known as the Kreb's or citric acid cycle, and the electron transport chain (69). The TCA cycle produces ATP by converting carbohydrates and fats into carbon dioxide and water, but its main function is reducing the coenzymes nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH) to enter into the ETC (82). NADH and FADH carry electrons to the ETC, which is located in the inner mitochondrial membrane and consists of five enzyme complexes, known as I–V. Complex I is NADH dehydrogenase, or NADH:ubiquinone oxidoreductase; complex II is succinate dehydrogenase (SDH), or succinate:ubiquinone oxidoreductase; complex III is the bcl complex, or ubiquinone:cytochrome *c* oxidoreductase (UQCCR); complex IV is cytochrome *c* oxidase (COX), or reduced cytochrome *c*:oxygen oxidoreductase; and complex V is ATP synthase or proton-translocating ATP synthase (146). Electrons flow through the ETC complexes and pass down an electrochemical gradient to diatomic oxygen (O_2) through a chain of respiratory proton (H^+) pumps (82). Ubiquinone shuttles electrons from complexes I and II to complex III and Cytochrome *c*, transfers electrons from complex III to IV. Through complexes, protons are pumped from the inner mitochondrial membrane to the intermembrane space to establish a proton motive force, which is used by complex V to phosphorylate adenosine diphosphate (ADP) (61) by ATP synthase, and create ATP (Figure 2B) (146).

Figure 2. Mitochondrial Structure

A *B*



A. Normal cardiac mitochondrial morphology in male C57BL/6J mice. (Magnification, × 15 000, University of Calgary Microscopic Imaging Facility, Zahra Ezzat Zadeh). Structurally, mitochondria contain five different compartments: the outer membrane, the inner membrane, the intermembrane space, cristae and the matrix.

B. Electron transport chain (ETC) is located in the inner mitochondrial membrane and consists of five enzyme complexes, known as I–V. Electrons flow through the ETC complexes and pass down an electrochemical gradient to diatomic oxygen (O_2) through a chain of respiratory proton (H^+) pumps. Through complexes, protons are pumped from the inner mitochondrial membrane to the intermembrane space to establish a proton motive force, which is used by complex V to phosphorylate adenosine diphosphate by ATP synthase, and create ATP. Modified from Beil L,. *Mitochondria gone bad.* Science News. 175: 20, 2009 (7).

2.6 Alterations of Mitochondrial Functions in Diabetes

Mitochondria are the main consumers of oxygen in the body. An association between low expression of key proteins required for mitochondrial function with the occurrence of various cardiovascular risk factors including insulin resistance, hyperlipidemia and hypertension was found in skeletal muscle of rats with low oxidative capacity (151). A co-ordinated down-regulation of genes regulated by peroxisome-proliferator-activated receptor gamma co-activator 1 (PGC-1α), the main regulator of mitochondrial biogenesis and mitochondrial gene expression was found in skeletal muscle of T2D patients (91, 104). Studies of mitochondrial function and morphology in different tissues in prediabetic and diabetic states have provided more support for an association between altered mitochondrial function and diabetes. ATP synthesis in severely insulin-resistant offspring of T2D was 30% decreased. This observation was most likely attributable to a 38% reduction in skeletal muscle mitochondrial content reported in this study (92, 107). Impaired mitochondrial NADH: O_2 oxidoreductase activity and reduced mitochondrial citrate synthase activity in skeletal muscle of T2D patients were reported (66). Abnormal mitochondrial morphology, reduced mitochondrial content, impaired mitochondrial respiration, and reduced OXPHOS gene expression were found in adipocytes of diabetic db/db mice (27).

2.7 Alterations of Myocardial Mitochondrial Function

There are very few studies that have investigated mitochondrial function in the human heart in obesity and T2D. No direct measurement of mitochondrial respiratory

function has been published to date, likely due to the intrinsic difficulties in obtaining appropriate human heart samples. However, there are a few indirect studies indicating altered myocardial mitochondrial function in obesity and T2D. An increase in BMI (>30 kg/m^2) is associated with elevated myocardial oxygen consumption (MVO_2), reduced cardiac efficiency and impaired glucose tolerance in obese and insulin-resistant women (108). The impaired glucose tolerance is correlated with increased fatty acid oxidation. It is well known that mitochondria are the site of oxygen consumption and fatty acid oxidation, suggesting impaired mitochondrial energy metabolism may contribute to the impaired cardiac function observed in obese subjects (108). Studies using nuclear magnetic resonance (^{31}P NMR) spectroscopy have provided more direct support for cardiac altered mitochondrial function in T2D. It is reported that T2D patients have reduced cardiac phosphocreatine/ATP ratios. This indicates that metabolism of the high-energy phosphate is impaired, suggesting a cardiac energy deficit in these patients (30, 124). While electron transport chain dysfunction has been seen in failing hearts of other aetiologies, decreased phosphocreatine/ATP ratios has also been reported for these hearts, highlighting the possibility that alterations in mitochondrial function may occur in the human heart (23, 62, 94, 95).

In contrast with human studies, direct measurement of mitochondrial function has been accomplished in several animal studies. Reduced ADP-stimulated state 3 respiration of mitochondria isolated from db/db mouse hearts was observed using both pyruvate and palmitoyl carnitine as a substrate (75, 76). State 3 respiration is a maximal rate of mitochondrial respiration that is obtained at saturated ADP concentrations (75). Using

several respiratory substrates, reduced state 3 respiration and ATP synthesis were also seen in ob/ob mice (14). Besides, ob/ob hearts also showed a decrease in expression of respiratory chain complexes (14). In addition to functional mitochondrial impairment, mitochondrial structural defects and mitochondrial proliferation were reported in ob/ob hearts (14, 32). Despite these observations, the mechanisms for alterations in cardiac mitochondrial function in T2D remains poorly understood.

2.8 The Link between Impaired Myocardial Mitochondrial Function and Development of Contractile Dysfunction

Myocardial mitochondrial substrate metabolism has a key role in ATP generation, which is necessary for continuous contractile function. During diabetes, increased delivery of FA to the heart leads to an increase in FA uptake and oxidation. Compared with glucose, FA is a less efficient substrate to the heart; oxidation of FA decreases the ATP yield per atom oxygen consumed (93). As a result the ratio of cardiac work to cardiomyocyte oxygen consumption is decreased in diabetic heart. The increased cardiac oxygen consumption and decreased cardiac efficiency may contribute to the development of contractile dysfunction in diabetes and obesity; however the mechanisms are poorly understood (59).

One probable mechanism is generation of reactive oxygen species (ROS) by mitochondria. In normal metabolism, electrons derived from oxidation of substrate flow through the ETC complexes to the diatomic oxygen (O_2) which is the final electron acceptor and reduce the oxygen to water. However, some electrons leak from the respiratory chain and this leads to the production of reactive incompletely reduced forms

of oxygen such as superoxide anions and hydroxyl radicals. Mitochondrial ROS production is mainly under control of the redox state of the respiratory chain (79, 133). Even a small increase in membrane potential across the inner mitochondrial membrane above a certain threshold value, highly stimulates ROS production (71). Increased delivery of electrons to the ETC can result in elevated ROS production (97, 152). ROS can cause oxidative damage to macromolecules such as DNA, lipids and proteins including mitochondrial respiratory enzymes, matrix enzymes and membrane phospholipids (43). Overproduction of ROS and/or decreased efficiency of inhibitory scavenger system can result in the generation of oxidative stress. Under normal physiological conditions, the harmful effects of ROS are prevented by enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase, and catalase and non-enzymatic antioxidants such as tocopherols, carotenoids and ascorbate. SOD isoforms are in cytosol (Cu/Zn-SOD) or within mitochondria (Mn-SOD) (43). The mediatory role of oxidative stress in the development and progression of obesity and diabetes and their complications (50), including cardiac pathologies (24, 120) has been accepted.

The other potential mechanism for increased mitochondrial oxygen consumption and decreased cardiac efficiency in diabetic heart is mitochondrial uncoupling (14). Uncoupling proteins (UCPs) are inner mitochondrial membrane proton carriers and facilitates the proton leak across the membrane. The UCPs modulate the coupling between the respiration and ATP synthesis (73). Since a high mitochondrial membrane potential increases ROS generation, UCP dependent uncoupling is suggested to play a

role in reducing mitochondrial ROS generation. It has been shown that FA and superoxide stimulate UCP3 activity in skeletal muscle (34). FA-induced mitochondrial uncoupling in ob/ob and db/db mouse, represents a potential mechanism for increased mitochondrial oxygen consumption and decreased cardiac efficiency (15, 33).

During obesity and diabetes, enhanced FA uptake and oxidation increase delivery of reducing equivalents to ETC, resulting in enhanced production of ROS that may increase mitochondrial uncoupling activity. As a result, cardiac oxygen consumption increases that may lead to a higher rate of FA oxidation, without an appropriate increase in ATP synthesis. On the other hand, ROS-mediated oxidative damages to mitochondrial proteins, impairs mitochondrial energy metabolism and ATP generation. Both mechanisms may result in a cardiac energy deficit and contribute to development of contractile dysfunction.

2.9 Studying OXPHOS in Isolated Mitochondria and Permeabilized Fibers

To evaluate mitochondrial function one can measure activities of mitochondrial enzymes or examine the oxidative phosphorylation process. Measurements of mitochondrial oxygen consumption in the presence of various substrates and inhibitors allow a step-by-step analysis of different mitochondrial respiratory chain complexes. Measuring OXPHOS help to detect the differences and defects in mitochondrial function and allows the study of various mitochondrial compartments (78). Additionally, OXPHOS can be measured in isolated mitochondria (41), permeabilized fibers (144) and cells (145) as well as tissue homogenates (77). Using isolated mitochondria make it

possible to investigate both cardiac mitochondrial population (subsarcolemmal and interfibrillar), as a whole or separately (103). However, it has been shown that properties of mitochondria are affected at the isolation steps that include different centrifugation of tissue or cell homogenates. This outcome is of more importance when a pathological process has already damaged the heart tissue (111). To overcome these limitations, mitochondrial function can be assessed in situ, using permeabilized fibers and cells as well as tissue homogenates.

While preparing permeabilized fibers, permeabilization of the sarcolemma by mechanical separation with forceps and/or exposure to selective chemical permeabilizing agent maintains essential interactions between mitochondria, the cytoskeleton, nucleus and endoplasmic reticulum (78). As a result, normal mitochondrial interactions that have a great role for metabolic channelling and intracellular energy transfer are preserved (65, 122). Therefore, this method allows characterization of the functional parameters of mitochondria within the cellular system. Compared with the isolated mitochondria, the in situ approach is less time consuming, requires lesser amount of cells or tissue and most importantly, maintains the intracellular position of mitochondria (41). Despite these advantages, there are several limitations to this method that should be noted. Both types of mitochondrial populations (subsarcolemmal and interfibrillar) are evaluated using permeabilized tissues (144). Due to the inclusion of non mitochondrial structures, sensitivity of respiration in response to designed substrate-inhibitor titration protocols can also be different, however, the level of respiration is very low compared to mitochondrial respiration (78).

2.10 Markers for Mitochondrial Content

Citrate synthase activity is known as the nuclear indicator of mitochondrial proteins, and consequently a marker for mitochondrial content (17, 19). However, its use as a marker of mitochondrial content is not without problems. Interestingly, it has been seen that citrate synthase activity, in parallel with mitochondrial content, is increased following endurance training in healthy individuals (57, 130) and T2D (3). Similar results have also been reported with acute exercise (37, 141), after moderate-intensity physical activity combined with weight loss in sedentary obese men and women (87), whole-body insulin stimulation (136), and incubation of skeletal muscle cells with insulin for 4h in healthy adults (101). It is well known that this increased citrate synthase activity is independent of increases in mitochondrial content and emphasizes the fact that citrate synthase can be used as a marker only under basal conditions. Mitochondrial DNA (mtDNA) is another marker for mitochondrial content and is correlated to citrate synthase activity under basal conditions (147), but is not sensitive to acute altered metabolism in muscle. The level of mtDNA indicates the level of mtDNA gene expression and hence the mitochondrial content (101, 113). This measure has been used to explain how mitochondrial content and gene expression is changed in T2D (118) and other diseased states (147), as well as with interventions including exercise training (58).

Chapter Three: Methods

3.1 Animal and Diet

Insulin resistance was induced in male C57BL/6J mice following weaning. Mice were fed *ad libitum* either a chow (CH) or high fat diet (HF) for 20 weeks. Energy density (% kcal/g) for CH and HF diets was 23% protein (PRO), 21% fatty acids (FA), 55% carbohydrate (CHO) and 15% PRO, 59% FA, 26% CHO (Appendix B,C). Animals were housed in a temperature and humidity controlled room with a 12 h light/dark cycle. Animals were fasted 5 h prior to the experiment.

3.2 Echocardiography

The mice were anesthetised with isofluorane for the duration of the procedure. Mice were placed on a heated body pad. Paws were taped onto special ECG pads so that their heart rate and echocardiography (ECG) could be measured and monitored. M-mode pictures of their hearts were taken on both parasternal long-axis and parasternal short-axis for measurements. Using Vevo 770 (Visual Sonics Inc, Toronto, ON) measurements were taken on at least three consecutive cycles, more if it was possible. The interventricular septal thickness in systole (IVSs), interventricular septal thickness in diastole (IVSd), LV end-systolic dimension (LVIDs), LV end-diastolic dimension (LVIDd), LV posterior wall thickness in systole (LVPWs) and LV posterior wall thickness in diastole (LVPWd) were measured in order to get heart size and volume (in systole and diastole) and an estimate of heart mass. Measurements were done after 20 weeks. The heart function via

fractional shortening (FS), ejection fraction (EF) and LV mass were calculated as follows (Heart rate was depressed under anaesthesia and was not included in thesis) (44, 153) ;

$$FS\ (\%) = (LVIDd - - \quad LVIDs) / LVIDd \times 100$$

$$EF\ (\%) = (LVd\ area - - \quad LVs\ area) / LVd\ area \times 100$$

$$LV\ mass\ (mg) = (IVSd + LVIDd + LVPWd)^3 - - \quad (LVIDd)^3$$

3.3 Blood Collection

A 1 mL sample of fasting blood was collected via cardiac bleed. Immediately blood glucose was monitored with a one-touch blood glucose monitor (LifeScan Canada Ltd, Burnaby, BC). Following this, 500 μL blood was collected into an EDTA-coated tube. Immediately after collection, blood was centrifuged for plasma separation and plasma was placed on ice until experiment end, then was stored at -80 °C until analysis. Insulin was evaluated with an Ultra sensitive Mouse Insulin ELISA Kit (Crystal Chem Inc, Downers Grove, IL) with coefficient variation ≤ 10% and sensitivity of 5 pg/ml. Free fatty acids was quantified using HR Series NEFA-HR (128) enzymatic colorimetric kit (Wako, Richmond, VA), which detects FFA as low as 0.0014 mEq/L.

3.4 Preparation of Permeabilized Muscle Fibers

Left ventricle (LV) was obtained from anaesthetized animals (0.25 mg/gBW pentobarbital) and was placed in ice-cold working solution A containing CaK_2EGTA (2.77 mmol/l), K_2EGTA (7.23 mmol/l), $MgCl_2$ (6.56 mmol/l), imidazole (20 mmol/l),

K+/4-morpholinoethanesulfonic acid (MES) (50 mmol/l), dithiothreitol (DTT; 0.5 mmol/l), Taurine (20 mmol/l), Na_2ATP (5.3 mmol/l), phosphocreatine (15 mmol/l), pH 7.1. The muscles were cut down to fiber bundles (1-2 mm, 2-4 mg wet wt). With a pair of needle-tipped forceps fibers were gently separated from one another to maximize surface area of the fiber bundle, leaving only small regions of contact. Fibre bundles were transferred into 1 mL of working solution A plus 50 μg saponin and were gently stirred for 30 min at 4°C. The fibres were washed three times for 10 min in ice-cold working solution B containing CaK_2EGTA (2.77 mmol/l), K_2EGTA (7.23 mmol/l), $MgCl_2$ (1.38 mmol/l), imidazole (20 mmol/l), K+/4-morpholinoethanesulfonic acid (MES) (100 mmol/l), dithiothreitol (DTT; 0.5 mmol/l), taurine (20 mmol/l), K_2HPO_4 (3 mmol/l), bovine serum albumin (BSA) (2 g/l), pH 7.1. Samples were immediately blotted, weighed, and used for respirometric analysis.

3.5 Respiration Assays

Respiration was measured in a 2 mL chamber using an Oroboros (Innsbruk, Austria) (48, 49). The respiratory measurements were performed at 37°C in working solution B. Weight-specific oxygen flux (pmol O_2 (s·mg wet weight) $^{-1}$) was calculated as the time derivative of oxygen concentration using the DatLab 4 Analysis Software, (Oroboros). A standard substrate inhibitor titration protocol was developed for step-by-step measurement of respiration through different segments of the electron transport chain. The experiment was started with: 1) Addition of the substrates including glutamate (10 μM), malate (5 μM) and pyruvate (5 μM), to feed electrons into the complex I. 2) The maximal respiration capacity was then achieved by addition of the ADP (5 μM) to

the chamber. 3) The measurements were followed by adding cytochrome *c* (10 μM) to test the integrity of mitochondrial outer membrane. 4) Complex II respiration was measured by addition of complex II substrate, succinate (10 μM). 5) Uncoupled respiration was measured following addition of carbonyl cyanide *p*-(trifluoromethoxy) phenyl-hydrazone (FCCP, 0.5 μM). 6) At the end respiration was inhibited following the addition of antimycin A (2.5 μM).

.Muscle recovered from respirometry was frozen in liquid nitrogen, and stored at -80 °C for future investigation of citrate synthase activity.

3.6 Biochemical Measures

Citrate synthase (CS) activity in homogenates of all heart samples used for respiration measurements was measured spectrophotometrically at 412 nm and 25 °C by the reduction of DTNB (Adapted from the technical bulletin for citrate synthase assay Kit from Sigma (CS0720) (Sigma, Saint Louis, MO) (96).

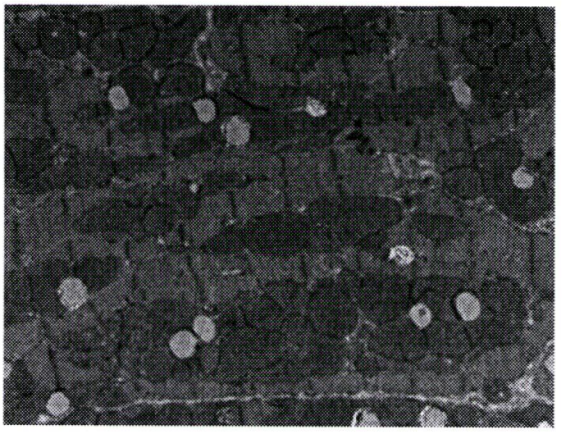
3.7 Electron Microscopy

Left ventricle myocardial fiber bundles (2×2×2 mm) were isolated and placed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer containing 2 mM CaCl2, pH 7.2 to 7.4, and stored at 47C. Then the samples were washed three times for 10 to 20 min in 0.1 M fresh cacodylate buffer at room temperature, then postfixed for 1 h in 1% osmium tetroxide in 0.1 M cacodylate buffer. Then the samples were stained with 1% aqueous uranyl acetate for 1 h at room temperature and were rinsed briefly in distilled water.

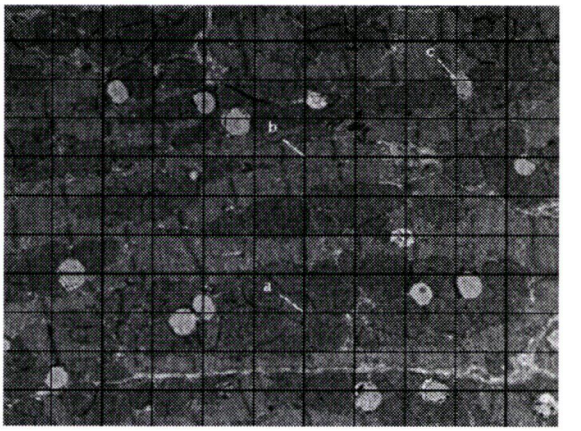
Samples were then dehydrated in serial ethanol or acetone washes (50 % 10 min, 70–95% 2×10 min, 100% 3×10 min) with a final 100% acetone wash (3×10 min). Fibres were infiltrated with Spur or Epon resin for 24 h and embedded into fresh resin moulds and cured at 60 °C for 24 h. Samples were sectioned to 1 mm and stained with toluidine blue. Ultrathin sections were cut to 70 nm and mounted on copper mesh grids and stained with uranyl acetate. A total of two muscle fibers were photographed from each muscle sample and for each of the fibers, a total of 6 images were obtained at × 3,000 magnification for mitochondria volume analysis. The volume density of mitochondria was calculated by a testing grid that was made using Adobe Photoshop (Adobe Systems, San Jose, CA). The grid contained 10×10 lines doing 100 points. It was superimposed over the electronmicrographs. Wherever mitochondria, myofibrils or lipid droplets intersected the grid point, it was calculated in the analysis. For example; if in one image 35 points of the grid were intersected with mitochondria, mitochondrial volume density of the image would be 35% (Figure 3).

Figure 3. Electron microscopy: A. Left ventricle fiber bundles of HF at × 3,000 magnification. B. The grid containing 100 points was superimposed over image A. Small arrows point to a) mitochondria, b) myofibril, c) lipid droplet.

A



B



3.8 Antioxidant Enzyme Activities

Frozen left ventricle tissues were minced into fine pieces, weighed (20-25 mg), manually homogenized and diluted at 50:1, in buffer containing 100 mM KH_2PO_4, 1 mM DL-Dithiothreitol (DTT) and 2mM ethylenediamine tetra acetic acid (EDTA) (pH=7.4, temperature=4 °C). Homogenates were then centrifuged at 5000 rpm for 5 min at 4 °C. The supernatant was transferred into separate eppendorf tube, vortexed and an aliquot of 100 µl was taken to determine protein content via BCA Protein Kit (BioRad Laboratories, Hercules, CA). Total SOD activity was determined by measuring the rate of reduction of cytochrome c by O_2^- that is directly proportional to SOD activity, via the xanthine oxidase reaction (53, 80). The activity of xanthine oxidase (0.2 U/ml) was measured on each day of the experiment to determine the volume of xanthine oxidase to be used in the experiment. Total SOD activity was determined by combining 1 ml of K_2HPO_4 buffer (50 mM with 0.1 mM EDTA, pH 7.4) containing cytochrome *c* (24.8 mg/100 ml) and xanthine (5 mM). Twenty microliters of muscle homogenate was added to the cuvette and mixed by inversion. Determined volume of xanthine oxidase was added to initiate the reaction, and absorption at 550 nm was observed every 20 s for 3 min. Mn-SOD activity was measured in a separate cuvette with addition of 10 µl of 0.1 M potassium cyanide (KCN) under identical conditions. Cu/Zn-SOD was determined by subtracting Mn-SOD activity from total SOD activity. All samples were analyzed in duplicate and activity was calculated and expressed in U g protein^{-1}.

3.9 Data Analysis

Statistical significance was tested by one way measurement of ANOVA using SigmaStat 3.5 (Systat Software Inc., San Jose, CA) followed by a Tukey's post-hoc test. Values less than 0.05 were considered significant. All values are expressed as mean ± standard error of the mean (SEM).

Chapter Four: Results

4.1 Animal Characteristics

Metabolic characteristics of animals are shown in Table 1. Body mass was significantly increased in HF animals (49.12 ± 0.73 g) compared with the control group (31.82 ± 0.95 g), which was accompanied by hyperinsulinemia ($p < 0.05$). Fasting plasma glucose for HF and CH mice were 16.56 ± 0.91 and 13.98 ± 1.45 mmol/L respectively. Fasting levels of non-esterified fatty acids (NEFA) for HF and CH mice were 1.24 ± 0.20 and 0.98 ± 0.2 mmol/L respectively.

Table 1. Physiological and biochemical parameters of male C57BL/6J mice fed chow (CH) or high fat diet (HF) for 20 weeks following weaning. Animals were fasted for 5 h prior to the experiment (n = 10 per treatment, values are expressed as mean ± SEM, *$p < 0.05$).

	Body Mass (g)	Fasting NEFA (mmol/L)	Fasting Insulin (pg/mL)	Fasting Glucose (mmol/L)
CH	31.82 ± 0.95	0.98 ± 0.2	2.55 ± 0.54	13.98 ± 1.45
HF	49.12 ± 0.73*	1.24 ± 0.2	20.25 ± 4*	16.56 ± 0.91

4.2 Cardiac Function

High fat feeding resulted in increase in left ventricular dimension and a decrease in cardiac function (Table 2). There was a significant increase in LV mass in HF (130.96 ± 7.8 mg) compared with CH (103.56 ± 4.45 mg). End-diastolic dimensions (IVSd and LVPWd) were significantly increased as a result of high fat feeding ($p < 0.05$). EF % was significantly decreased in HF animals compared with CH mice (31.412 ± 1.31 and 37.37 ± 1.39 % respectively). HF animals had significantly lower FS% than CH mice (14.77 ± 0.7 and 17.94 ± 0.73 % respectively).

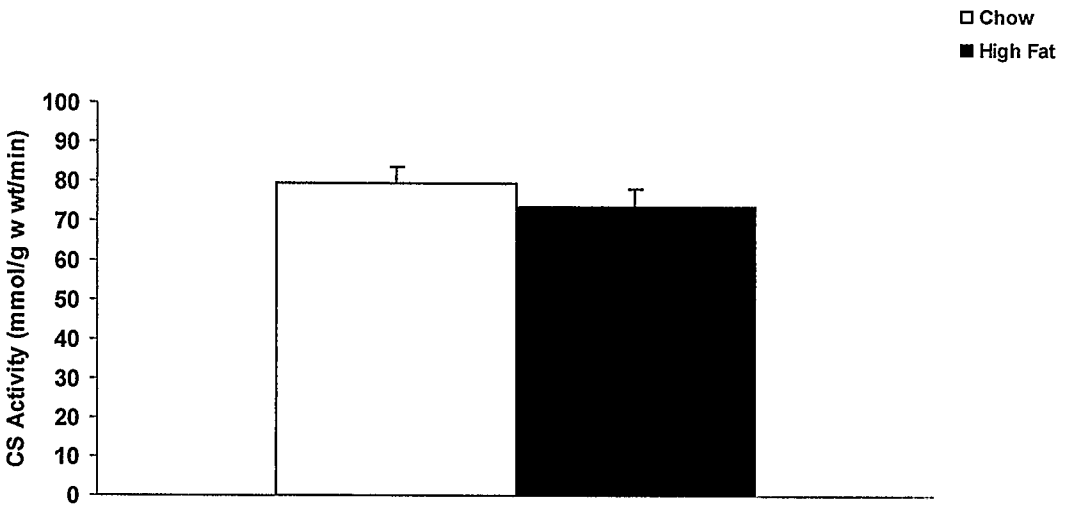
Table 2. Echocardiographic parameters of male C57BL/6J mice fed chow (CH) or high fat diet (HF) at 20 weeks. (n= 10 per treatment, values are expressed as mean ± SEM, *p < 0.05).

	Diastolic Dimensions			Systolic Dimensions			LV Mass	Contractile Function	
	IVSd (mm)	LVIDd (mm)	LVPWd (mm)	IVSs (mm)	LVIDs (mm)	LVPWs (mm)	(mg)	FS (%)	EF (%)
CH	0.74 ± 0.02	4.47 ± 0.08	0.75 ± 0.02	0.94 ± 0.04	3.68 ± 0.07	0.88 ± 0.03	103.56 ± 4.46	17.94 ± 0.73	37.37 ± 1.39
HF	0.87 ± 0.03*	4.51 ± 0.08	0.89 ± 0.04*	0.97 ± 0.04	3.84 ± 0.06	0.99 ± 0.04	130.96 ± 7.80*	14.77 ± 0.07*	31.41 ±1.31*

4.3 Citrate Synthase Activity

There was no significant difference in CS activity between HF and CH animals (73.72 ± 4.41 and 79.38 ± 4.4 μmol min^{-1} g w wt^{-1} respectively) (Figure 4).

Figure 4. Citrate synthase activity in chow and high fat heart fibres was not significantly different (n= 16 per treatment, values are expressed as mean ± SEM).

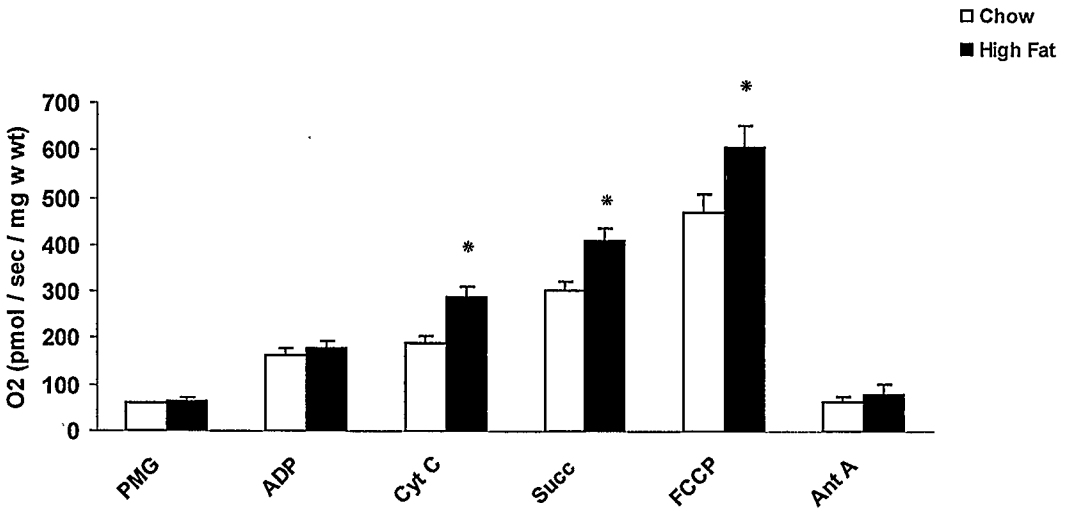


4.4 Respirometry

There was no difference in state 3 respiration with complex I substrate (179.02 ± 15.03 vs. 161.75 ± 15.83 pmol mg w $wt^{-1}s^{-1}$ for HF vs. CH respectively), however respiration was significantly higher ($P<0.05$) after the addition of each of succinate (411.17 ± 28.01 vs 306.09 ± 17.56 pmol mg w $wt^{-1}s^{-1}$), FCCP (607.26 ± 47.35 vs 472.58 ± 35.63 pmol mg w $wt^{-1}s^{-1}$), and cytochrome c (288.82 ± 24.02 vs. 188.80 ± 15.95 pmol mg w $wt^{-1}s^{-1}$) in HF mice (Figure 5 A). After normalization to CS, as a marker for mitochondrial content, mitochondrial ADP-stimulated state 3 respiration remained unchanged with values of 2.53 ± 0.26 and 2.22 ± 0.37 pmol mg w $wt^{-1}s^{-1}$ for HF and CH animals respectively. ETC complex II activity was significantly increased in HF mice (5.72 ± 0.45 pmol mg w $wt^{-1}s^{-1}$) compared with CH animals (4.13 ± 0.48 pmol mg w $wt^{-1}s^{-1}$) ($P<0.05$). Depletion of cytochrome c was detected by its stimulatory effect in HF (4.01 ± 0.37 pmol mg w $wt^{-1}s^{-1}$) compared to CH (2.59 ± 0.38 pmol mg w $wt^{-1}s^{-1}$) ($P<0.05$). Furthermore, the higher respiration rate in high fat fed animals following uncoupling by FCCP and inhibition of complex III activity by antimycin A was not significant (Figure 5 B).

Figure 5. Respiratory capacity of heart fibres per tissue weight in A and per citrate synthase activity in B. Oxidative respiration was significantly higher in HF mice rather than CH following addition of succinate and cytochrome c (PMG= pyruvate, malate, glutamate, Adenosine diphosphate (ADP), Cytochrome c (Cyt C), Succinate (Succ), carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP), Antimycin A (Ant A)). (n= 16 per treatment, values are expressed as mean ± SEM, *p < 0.05).

A



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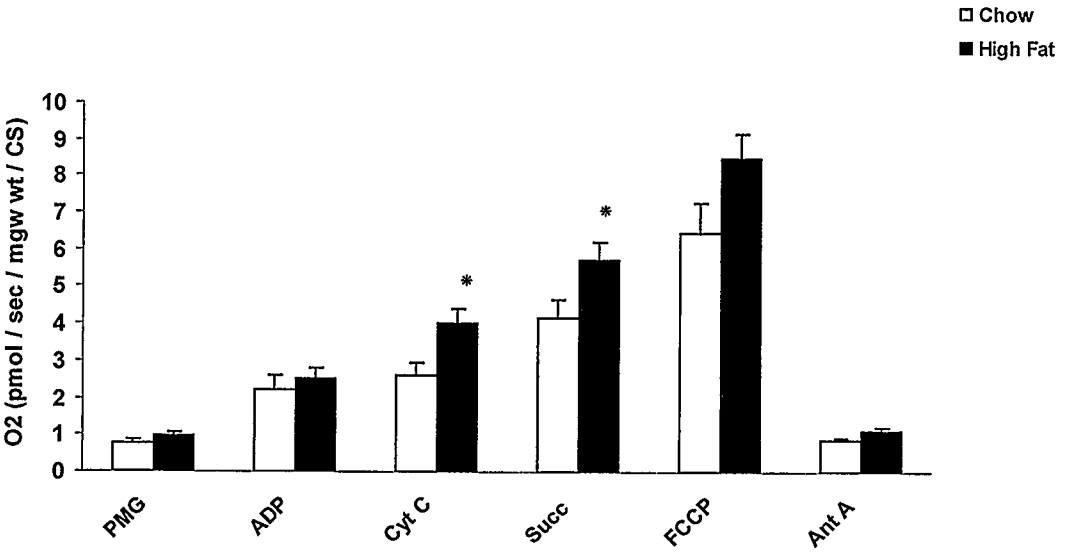


4.5 Electron Microscopy

There was a significant increase in mitochondrial volume/density of the HF animals compared with CH mice ($P<0.05$) (Figure 7). High fat diet resulted in a significant decrease in the area of the myofiber in the LV tissue of the HF mice compared with the CH animals ($P<0.05$). In addition, visual inspection of the mitochondrial images revealed mitochondria in LV tissue from CH mice (Figure 6 A) were more organised compared to HF mice (Figure 6 B). HF mitochondria generally were misshapen and had irregular cristae (Figure 6 D) compared with CH mitochondria (Figure 6 C), however, this was not statistically quantified. High fat feeding resulted in an increase in both subpopulations of the LV mitochondria with a greater influence on interfibrillar mitochondria (IFM) compared with subsarcolemmal mitochondria (SSM). The lipid content of the heart tissue was not significantly different (Figure 7).

Figure 6. Electron microscopy images of left ventricle fiber bundles of CH (A at × 3,000 magnification and C at × 15,000 magnification) and HF (B at × 3,000 magnification and D at × 15,000 magnification)

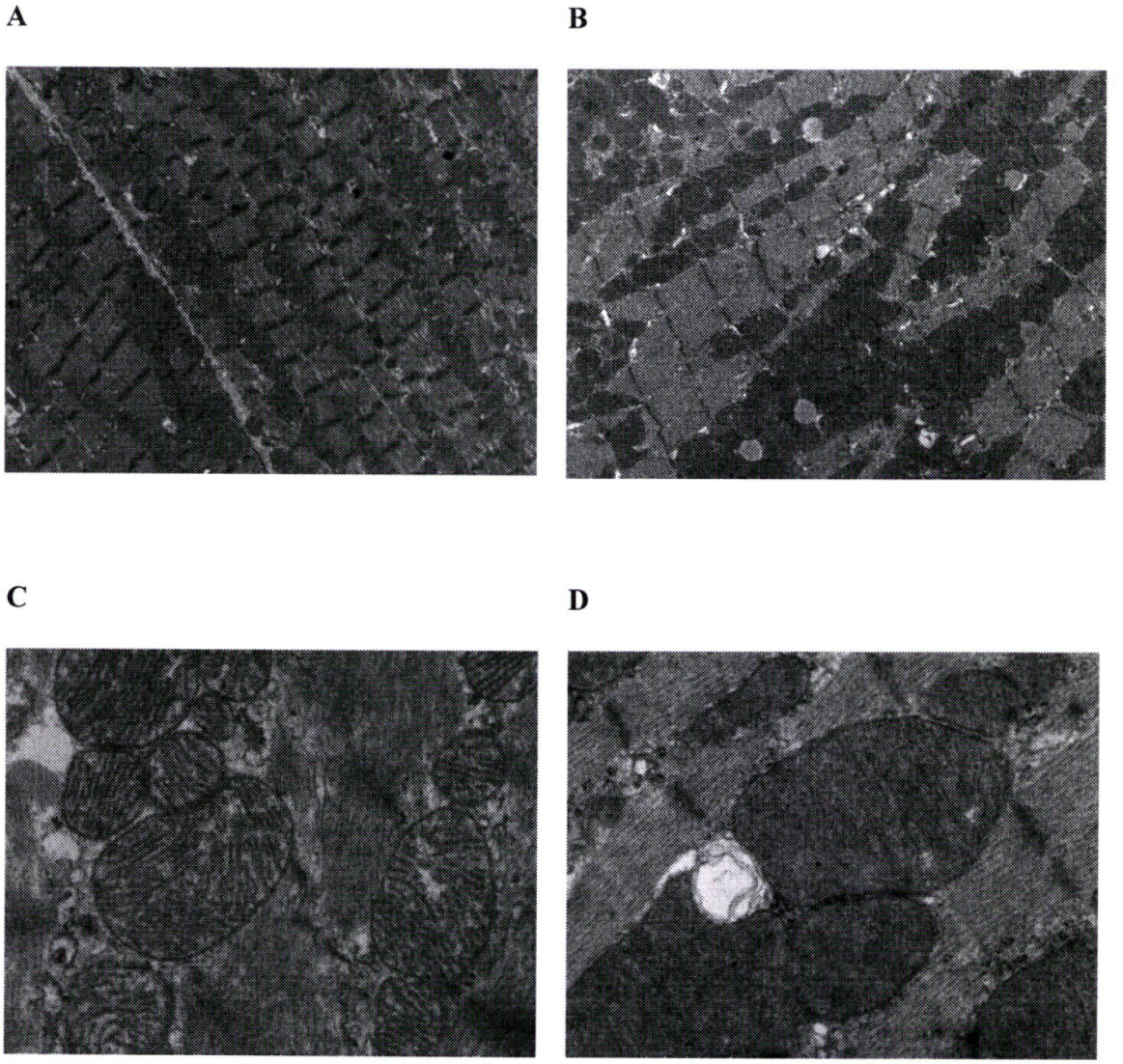
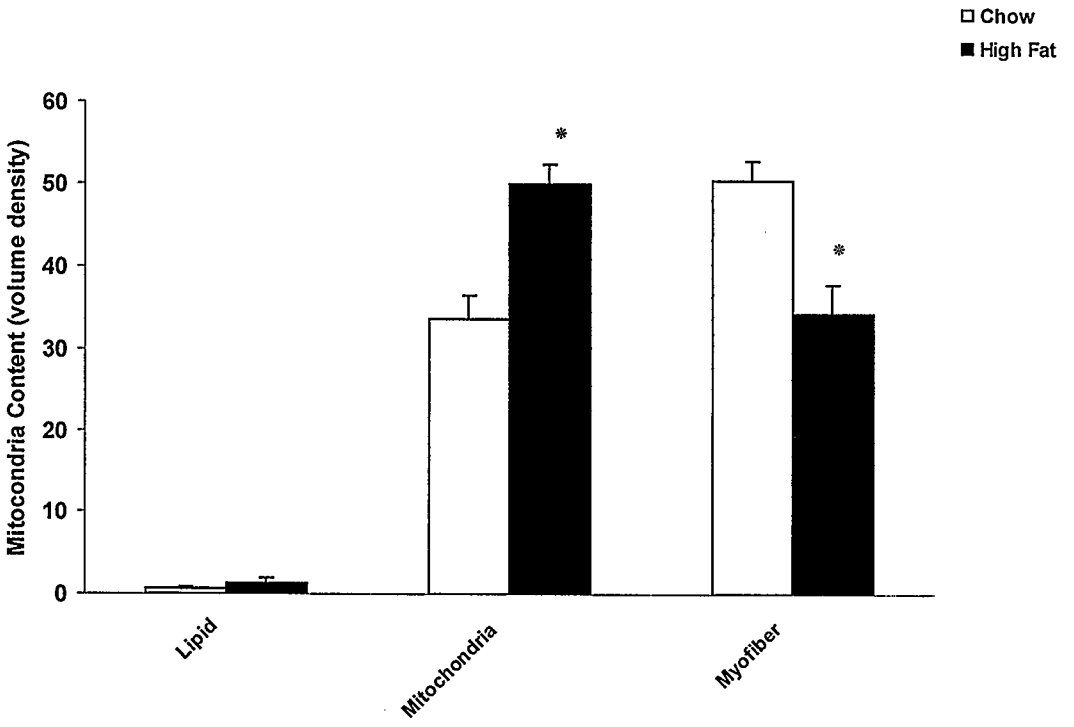


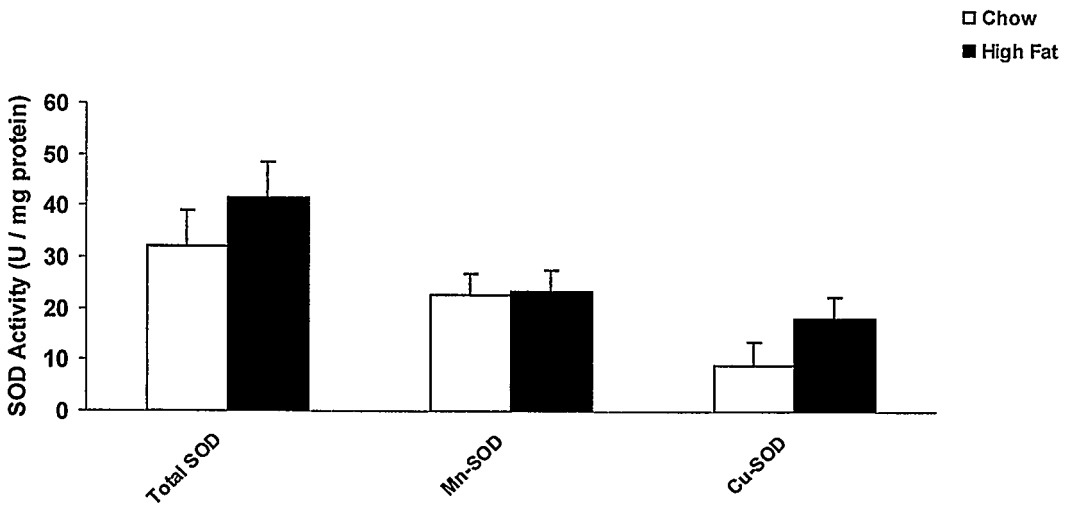
Figure 7. Lipid, mitochondria and fiber content in CH and HF heart fibers. (n= 6 per treatment, values are expressed as mean ± SEM, *p < 0.05).



4.6 Antioxidant Enzyme Activity

Changes in superoxide dismutase activity are shown in figure (Figure 8). Mitochondrial SOD activity slightly increased following HF diet. HF animals exhibited higher cytosolic SOD activities when compared to CH mice. However the difference in cytosolic SOD activity between the two groups of the study was not significant ($P = 0.07$).

Figure 8. Superoxide dismutase activity was not significantly different between the HF and CH animals. (n =10 per treatment, values are expressed as mean ± SEM, *p < 0.05).



Chapter Five: Discussion

Impaired regulation of myocardial glucose and FA metabolism are observed in insulin resistance, obesity and T2D (2, 20). Previous studies indicate that cardiac mitochondrial structure and function is disrupted in animal models of obesity and insulin resistance (14, 75, 76, 85). The mechanisms that lead to altered myocardial mitochondrial function and metabolism in T2D involve increased myocardium FA uptake and oxidation (85, 108), enhanced activation of the PPAR-α (39), increased myocardial oxygen consumption (MVO_2) and FA-induced mitochondrial uncoupling which are believed to contribute to impaired cardiac function (15, 108). These observations led to the hypothesis of the current study. The overall hypothesis of this investigation was that oxidative phosphorylation and electron transport capacity are increased in high-fat feeding. This was, in part, explained by increased delivery of reducing equivalents to the electron transport chain that leads to enhanced maximal uncoupled respiration and increased mitochondrial content. As a result, insulin resistant hearts will have impaired mitochondrial structure and function leading to reductions in myocardial efficiency.

Administration of a 20 week high fat diet resulted in obesity and insulin resistance in animals while fasting plasma levels of NEFA and glucose were not significantly higher in these animals. Therefore high fat diet led to early diabetic conditions in the animals used in this study. Left ventricular hypertrophy and impaired LV contractile function, 16 % decrease in EF and 18 % decrease in FS, were also observed following high fat diet. These results are in line with previous studies indicating cardiac dysfunction as a common outcome of diabetes (1, 4, 8, 9, 112). As observed in the present and other

studies (1, 8, 126) cardiac dysfunction can occur in early stages of diabetic conditions. It is well known that there is a correlation between altered cardiac metabolism and impaired cardiac function observed during insulin resistance. Since altered cardiac metabolism appear early in the development of diabetic conditions (26, 45), the consequences of such alterations at the cellular levels are most likely a contributing factor toward cardiac contractile dysfunction observed in this study.

In the present study, measurements of myocardial CS activity showed no difference following a high fat diet, suggesting no change in mitochondrial content. Although activity levels of CS are often used as an indirect marker for mitochondrial content, it can also reflect the mitochondrial enzyme activity. However, activity of this enzyme is only part of the mitochondrial respiration and does not provide sufficient evidence to reflect mitochondrial function. In addition, CS activity was measured on functional mitochondria, in mitochondria with permeabilized inner membrane. Using CS activity as a marker of mitochondrial content, is not without problems. Given this, electron microscopy was employed to directly examine the mitochondrial volume/density. This labour intensive technique is considered the gold standard for assessing mitochondrial density in tissues. Results of the current study showed that mitochondrial volume density increased by 49% in high fat fed mice. The increased mitochondrial volume density could be a compensatory reaction to more lipid availability to the myocardial tissues. This finding is in line with studies demonstrating that obese and insulin resistant mice exhibit increased cardiac mitochondrial biogenesis (13, 33).

Direct measurement of the OXPHOS process, is an integrated approach to evaluate mitochondrial function. In the present study, substrates derived from carbohydrate were used to feed electrons into different respiratory chain complexes to examine cardiac mitochondrial function. Measurements of mitochondrial oxygen consumption showed that ADP stimulated state 3 respiration with complex I substrates remained unchanged following high fat feeding. Respiration rate was significantly increased after addition of cytochrome c, suggesting decreased integrity of mitochondrial membrane in high fat fed animals. Mitochondrial ETC complex II activity was significantly higher in high fat fed animals when expressed per unit mass of cardiomyocytes and after normalization per unit of CS activity. FCCP-induced uncoupling respiration was also higher in high fat fed animals compared to CH, but when respiration was normalised for CS activity, the effect did not reach statistical significance ($P = 0.075$). High fat fed animals showed a tendency in increased oxygen consumption following inhibition of Complex III activity with antimycin A, both before ($P = 0.078$) and after ($P = 0.073$) normalization per unit of CS activity. Increased mitochondrial oxygen consumption following addition of succinate (complex II) could be explained by elevated electron donation into the quinone pool via flavin adenine dinucleotide as a result of increased fatty acid oxidation. Using FCCP to maximise electron flux capacity in the uncoupled state has also showed that mitochondrial potential for oxygen consumption has been increased under diabetic conditions. This later result is in line with studies indicating that uncoupled respiration is increased in the hearts of diabetic mice (15). Overall, these results provide direct evidence for increased oxidative capacity of cardiac mitochondria under diabetic conditions. The increase in mitochondrial capacity

measured in permeabilized cardiac fibers in this study, may be attributed to either an increase in mitochondrial content or an increase in mitochondrial function. Although measurements of CS activity did not support an increase in mitochondrial content, further electron microscopy measurements revealed a significant increase in mitochondrial volume density following high fat diet. On the other hand, neither measurements of the activity of CS, one of the rate limiting enzymes of the citric acid cycle, nor state 3 respiration in the presence of complex I substrates, provided enough evidence to support increased mitochondrial function in high fat fed animals. Thus, high fat feeding, in the present study resulted in increased mitochondrial content that was accompanied by increased mitochondrial oxidative capacity, however such an increase was not accompanied by increased contractile function. The observed increase in mitochondrial content in order to increase mitochondrial capacity, was likely an early mitochondrial compensatory reaction to overcome the early metabolic imbalance occurring in the diabetic progression. However over a longer period of time this adaptive response (increase mitochondrial capacity) was not sufficient to manage established metabolic imbalance, contributing to impaired cardiac function in T2D. These findings are consistent with the concept of increased mitochondrial volume density, mitochondrial DNA content and co-regulators of mitochondrial biogenesis in hearts of obese and insulin resistant animals (13-15, 33). Interestingly, as observed in this study, results of these investigations were not accompanied by increased mitochondrial function. These alterations were observed along with either unchanged ADP stimulated state 3 respiration and decreased ATP synthesis (33) or impaired state 3 respiration and ATP synthesis (13-15). The impaired mitochondrial function observed in these studies is likely due to a

more severe diabetic condition or a longer duration of hyperinsulinemia. In contrast to our findings, a reduced mitochondrial oxidative capacity has been reported by Boudina et al. (14) using ob/ob mice. They found significant decrease in mitochondrial respiratory capacity of glucose-perfused ob/ob hearts in the presence of pyruvate. One reason for these contrasting findings is that ob/ob mice do not produce leptin which is a complicating factor in the interpretation of their results. These hearts performed poorly without fatty acids because their metabolism is preconditioned to use fatty acids.

Results of direct measurements of cardiac mitochondrial oxygen consumption along with echocardiographic measurements of cardiac contractile function provided sufficient evidence for wasting of oxygen for noncontractile purposes in high fat fed animals. These results are in agreement with studies indicating that insulin resistant mice exhibited increased myocardial oxygen consumption and decreased cardiac efficiency (increased MVO2 without changing of work) (12, 59). Although the underlying mechanisms for this oxygen wasting is incompletely understood, in mouse models of obesity and insulin resistance, increased mitochondrial uncoupling was suggested as a potential candidate (13, 15). Increased fatty acid exposure to mitochondria and enhanced superoxide production are two known mechanism that can activate mitochondrial uncoupling (13, 15). In addition, increased superoxide generation can cause damage to mitochondrial structures that leads to impaired mitochondrial function (43). In the present study, activity of superoxide dismutase isoforms, one of the antioxidant defence systems, was measured to investigate the relationship between high fat feeding and the potential for excess superoxide production in the heart. These measurements indicated

that there was a trend for the high fat diet to increase cytosol superoxide dismutase (Cu/Zn-SOD) activity ($P = 0.070$) and a slight but non-significant increase in activity levels of mitochondria superoxide dismutase (Mn-SOD). An increase, considering the role of this SOD isoform to protect myocytes from oxidative damage, reflects cardiac compensation as self-defence. Since Mn-SOD is the primary defence against mitochondrial oxidative damage, this enzyme activity perhaps would have significantly altered if the treatment time would have been increased. This idea is supported by findings of the studies reporting that increased cardiac Mn-SOD activity occurs in response to long term exposure to oxidative stress (28, 110). These observations are consistent with investigations indicating that overexpression of Mn-SOD to diabetic heart provided extensive protection to both heart and mitochondria (129). Thus, enhanced delivery of reducing equivalents to the electron transport chain as a result of increased cardiac fatty acid uptake and metabolism under diabetic condition led to increased superoxide generation as was indirectly observed by increased in activity levels of Cu/Zn-SOD. However the deleterious effect of ROS was inadequate to produce higher superoxide dismutase activity at the mitochondrial level. In addition to the potential detrimental effect of increased superoxide production to mitochondrial structure, superoxide also adversely influenced mitochondrial function by activating mitochondrial uncoupling. As a result oxygen consumption was increased independent of increased cardiac contractile function.

Mitochondria are the main energy provider for cardiac function. Elevated cardiac mitochondrial content is expected to result in an increase in oxidative phosphorylation

linked to ATP synthase which in turn could increase cardiac function. However, while measurements of mitochondrial respiration in the present study showed the same rate of oxygen consumption, echocardiographic assessment indicated a significantly decrease in cardiac function. This decrease could be further explained by 32% reduction in myofibrils, as was observed by electron microscopic assessment. The increase in mitochondrial volume density has likely occupied space that could otherwise be available to myofibril population, causing a decrease in power and muscle contraction velocity (119).

In this study, the ability of mitochondria to respond with increased volume density following enhanced substrate availability was observed in the hearts from insulin resistant mice. Increased mitochondrial content led to increased oxidative capacity that was not accompanied by appropriate increase in cardiac function. This finding is supported by similar data reporting cardiac mitochondrial biogenesis without co-ordinate increase in mitochondrial function in obesity and insulin resistance (13, 15). The fact that oxygen consumption was not the same following high fat feeding indicated that cardiac mitochondrial function was impaired in these obese and insulin resistant mice. Thus altered mitochondrial function could be a contributing factor in development of impaired cardiac function under diabetic condition.

In conclusion, the results of this study provide direct evidence of impaired mitochondrial structure which could imply impaired mitochondrial function in heart following high fat feeding. Hypertrophied insulin resistant hearts exhibited mitochondria with higher volume density and increased oxidative capacity. As a result, insulin resistant

heart exhibited an incomplete mitochondrial adaptation that was associated with reductions in myocardial efficiency.

Chapter Six: Strengths, Limitations and Future Perspectives

The objectives of this research were as follows:

1. to evaluate cardiac function in mice following high fat feeding;

2. to assess the effects of high fat feeding on cardiac mitochondrial structure and function and their contribution to development of cardiac dysfunction

These objectives were achieved through a set of experiments using C57BL/6J mice fed either a chow or a high fat diet. An assessment of the strengths and limitations of the experimental procedures is outlined below:

6.1 Animals

The C57BL/6J mice used in this study are widely used as models of human disease. Similar to humans, high fat feeding in these animals results in obesity and insulin resistance compared to a low fat fed diet (42). Because these animals are resistant to atherosclerosis, it allowed us to investigate cardiac function after high fat feeding without the complications of atherosclerosis (126). In addition, these mice are not genetically modified, so all the observed alterations of mitochondrial function can be attributed to the high fat diet.

In the present study, high fat feeding led to obesity and hyperinsulinaemia that was not accompanied by increased fasting plasma levels of glucose or NEFA, indicating that perhaps a longer time or postprandial sampling was necessary to see these deleterious effects. Elevated plasma glucose, fatty acids and insulin levels associated with

obesity, insulin resistance and type 2 diabetes mellitus develop over a prolonged time. However, alterations in cardiac metabolism and function occur early, suggesting a possible metabolic damage at the cellular or subcellular levels (4). While animal models offer valuable means to examine cellular or subcellular mechanisms underlying the disease progression, we can not extrapolate these findings to those of humans. One reason for justification of this statement is as mentioned earlier: these mice do not readily experience atherosclerosis, and therefore the alteration in mitochondrial function observed in this study may not mimic human condition.

6.2 Diet

The rodent chow diet that was used in this study could be a source of discrepancies between the findings of this study and other studies. Especially since high fat diets vary in percentage of the fat composition and more importantly due to the type of fat, for example mono unsaturated fat versus poly unsaturated fat. The exact compositional analysis of the diet used in this study is presented in Appendix B and C.

6.3 Echocardiography

Echocardiography is a non-invasive technique that uses ultrasound (high-frequency sound waves) to examine in vivo cardiac function (126). Applying this method enabled us to assess alterations in cardiac function as well as heart dimension due to high fat diet. However, these measurements were performed with the mice under isofluorane. Isofluorane is known to have cardiodepressant effects (55, 121). In general, echocardiographic measurements of the conscious mice provide a more physiologically relevant values when compared with anaesthetized animals (55, 121).

6.4 Permeabilized Muscle Fibers

The permeabilized fiber method used in the present study provided the opportunity to examine mitochondria, on very small amounts of left ventricular tissue, in their cellular environment with their normal intracellular position and assembly, resulting in a situation that resembles the living cell (78, 144). This technique avoided damage of mitochondria that often occurs during isolation procedures, however, possible damage to the outer mitochondrial membrane during the mechanical permeabilization of the high fat tissues remains as a limitation of this study. Although this technique offered evaluation of total cardiac mitochondrial population, this method did not allow us to investigate IFM and SSM function separately. Furthermore, the alterations in the enzymatic activities could not be credited to either of the mitochondrial subpopulation and therefore should be considered total.

6.5 Respiration Assay

The Oroboros Oxygraph-2k (Innsbruck, Austria) with highly sensitive electrodes and the integrated design that minimizes oxygen diffusion allowed us to directly measure mitochondrial respiration in situ with high resolution and accuracy (60). This technique helped us to investigate small differences in respiratory process that may reflect adaption/maladaption in mitochondrial structure and function following high fat diet.

6.6 Electron Microscopy

Direct assessment of mitochondrial biogenesis following high fat diet was done employing electron microscopy. This method provided direct evidence regarding mitochondrial volume density, however due to the three-dimensional structure of

mitochondria this technique could not offer a comprehensive view regarding the overall alterations of mitochondrial number and structure under diabetic condition (105).

6.7 *Oxidative Stress and Antioxidant Activity*

Oxidative stress represents the imbalance between the production of reactive oxygen species and the efficiency of inhibitory scavenger system in the cell (43). Evidence for association between increased ROS production and diabetes can be achieved by direct assessment of ROS production and/or indirect evaluation of oxidative damage as well as measurements of oxidative stress biomarkers, such as superoxide dismutase, catalase and glutathione reductase. Due to the defensive role of superoxide dismutase activity against production of reactive oxygen species, total and mitochondrial SOD was measured in LV fibers. These measurements helped us to indirectly assess the potential increase in oxygen free radicals following high fat feeding. However, measurements of ROS could have added more valuable data to the present investigations, due to the potential role of overproduction of ROS in impaired mitochondrial structure and function (71). Unfortunately, ROS could not be assessed due to limitations in tissue availability.

In summary, the findings of the present study and those of others on altered cardiac mitochondrial function under diabetic conditions do not provide clear evidence to enable us to understand the involvement of mitochondria in progression and development of the impaired cardiac function. The use of various animal models, diet compositions as

well as different methods have produced inconsistent data that makes it difficult to explain the effect of high fat diet on altering mitochondrial functions or its enzymatic activities and their association with impaired cardiac function. Considering the above mentioned limitations, further studies are needed to evaluate the impact of high fat diet on altered mitochondrial structure and function as the contributors to impaired cardiac function under obesity and insulin resistance conditions. Early detection of mitochondrial by-products can potentially be used to develop preventive and therapeutic approaches for population at risk.

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APPENDIX A: ETHICAL APPROVAL

UNIVERSITY OF CALGARY

Protocol BI 2008-40

Certification of Animal Protocol Approval

Applicant: Jane Shearer

Faculty/Department: Kinesiology

Project Title: Glycogen Induced Insulin Resistance

Sponsoring Agency(s): CIHR, AHFMR,

Effective: April 1, 2009 Expires: March 31, 2010

The Animal Care Committee,
having examined the animal care and treatment protocol,
approves the experimental procedures proposed and certifies
with the applicant that the care and treatment of animals
used will be in accordance with the principles
outlined in the most recent policies and
"Guide to the Care and Use of Experimental Animals"
By The Canadian Council on Animal Care.

Applicant — Date: June 29/09

Chair of Animal Care Committee or University Veterinarian — Date: March 30, 2009

Original - ACC File

APPENDIX B: CHOW DIET COMPOSITION

Mouse Diet 9F 5020*

DESCRIPTION

Mouse Diet 9F is a complete life-cycle diet containing 9% fat. This diet is formulated using the unique and innovative concept of Constant Nutrition, paired with the selection of highest quality ingredients to assure minimal inherent biological variation in long-term studies. Many mouse strains vary in their nutritional needs depending upon their genetic background. Mouse Diet 9F is specially formulated for those strains that require less energy to fulfill their metabolic needs than is provided by Mouse Diet 5015.

Features and Benefits

- Constant Nutrition content for minimal nutritional variation
- High-energy diet that supports post-partum reproduction where females are under stress of lactation and reproduction
- High quality animal protein added to create a superior balance of amino acids for optimum performance
- Wide ingredient spectrum
- Economical for breeder colonies

Product Forms Available

- Oval pellet, 10 mm x 16 mm x 25 mm length (3/8"x5/8"x1")
- Meal (ground pellets), special order

Other Versions Available

- 5021 Autoclavable Mouse Breeder Diet

GUARANTEED ANALYSIS

Crude protein not less than	20.0%
Crude fat not less than	9.0%
Crude fiber not more than	3.0%
Ash not more than	6.5%

INGREDIENTS

Ground wheat, ground corn, dehulled soybean meal, wheat germ, fish meal, brewers dried yeast, corn gluten meal, porcine animal fat preserved with BHA, soybean oil, calcium carbonate, salt, DL-methionine, choline chloride, menadione dimethylpyrimidinol bisulfite, dicalcium phosphate, monocalcium phosphate, cholecalciferol, pyridoxine hydrochloride, vitamin A acetate, biotin, dried whey, folic acid, dl-alpha tocopheryl acetate, thiamin mononitrate, calcium pantothenate, vitamin B supplement, lecithin, riboflavin, nicotinic acid, casein, manganous oxide, zinc oxide, ferrous carbonate, copper sulfate, zinc sulfate, calcium iodate, cobalt carbonate, sodium selenite.

FEEDING DIRECTIONS

Mouse Diet 9F should be fed to breeders and lactating mice on a free-choice basis. Plenty of fresh, clean water should be available to the animals at all times.

Mice-Adult mice will eat up to 5 grams of pelleted ration daily. Some of the larger strains may eat as much as 8 grams per day per animal, especially during heavy lactation. Feed should be available on a free choice basis in wire feeders above the floor of the cage.

12/11/09

CHEMICAL COMPOSITION[1]

Nutrients[2]

Protein, %	**21.6**	Sulfur, %	0.27
Arginine, %	1.14	Sodium, %	0.25
Cystine, %	0.31	Chlorine, %	0.42
Glycine, %	0.93	Fluorine, ppm	12
Histidine, %	0.50	Iron, ppm	200
Isoleucine, %	1.01	Zinc, ppm	120
Leucine, %	1.81	Manganese, ppm	115
Lysine, %	1.12	Copper, ppm	17
Methionine, %	0.67	Cobalt, ppm	0.55
Phenylalanine, %	0.96	Iodine, ppm	1.5
Tyrosine, %	0.63	Chromium, ppm	0.56
Threonine, %	0.78	Selenium, ppm	0.24
Tryptophan, %	0.25	***Vitamins***	
Valine, %	1.02	Carotene, ppm	0.8
Serine, %	1.06	Vitamin K (as menadione), ppm	3.1
Aspartic Acid, %	2.11	Thiamin Hydrochloride, ppm	15
Glutamic Acid, %	4.45	Riboflavin, ppm	8.0
Alanine, %	1.33	Niacin, ppm	90
Proline, %	1.53	Pantothenic Acid, ppm	21
Taurine, %	0.02	Choline Chloride, ppm	2200
Fat (ether extract), %	**9.0**	Folic Acid, ppm	2.9
Fat (acid hydrolysis), %	**9.1**	Pyridoxine, ppm	8.0
Cholesterol, ppm	210	Biotin, ppm[3]	0.30
Linoleic Acid, %	2.31	B$_{12}$, mcg/kg	51
Linolenic Acid, %	0.21	Vitamin A, IU/gm	15
Arachidonic Acid, %	0.02	Vitamin D (added), IU/gm	3.3
Omega-3 Fatty Acids, %	0.32	Vitamin E, IU/kg	57
Total Saturated Fatty Acids, %	2.71	Ascorbic Acid, mg/gm	—
Total Monounsaturated Fatty Acids, %[4]	2.87	***Calories provided by:***	
Fiber (Crude), %	**2.2**	Protein, %	23.066
Neutral Detergent Fiber, %	10.9	Fat (ether extract), %	21.570
Acid Detergent Fiber, %	3.0	Carbohydrates, %	55.364
Nitrogen-Free Extract (by difference), %	**52.0**	*Product Code	
Starch, %	39.4		
Glucose, %	0.16		
Fructose, %	0.16		
Sucrose, %	0.69		
Lactose, %	0.77		
Total Digestible Nutrients, %	**85.2**		
Gross Energy, kcal/gm	**4.60**[5]		
Physiological FuelValue, kcal/gm	**3.75**		
Metabolizable Energy, kcal/gm	**3.56**		
Minerals			
Ash, %	**5.0**		
Calcium, %	0.81		
Phosphorus, %	0.60		
Phosphorus (non-phytate), %	0.34		
Potassium, %	0.69		
Magnesium, %	0.16		

1. Formulation based on calculated values from the latest ingredient analysis information. Since nutrient composition of natural ingredients varies and some nutrient loss will occur due to manufacturing processes, analysis will differ accordingly.
2. Nutrients expressed as percent of ration except where otherwise indicated. Moisture content is assumed to be 10.0% for the purpose of calculations.
3. NDF = approximately cellulose, hemicellulose and lignin.
4. ADF = approximately cellulose and lignin.
5. Physiological FuelValue (kcal/gm) = Sum of decimal fractions of protein, fat and carbohydrate (use Nitrogen Free Extract) x 4,9,4 kcal/gm respectively.

LabDiet®
www.labdiet.com

APPENDIX C: HIGH FAT DIET COMPOSITION

AIN-76A w/58% Fat Energy/Sucrose/Red 58R3

DESCRIPTION

AIN-76A Semi-Purified Diet 5800-B Surwit Diet with 58% Fat Energy, Sucrose and Dyed Red.

Storage conditions are particularly critical to TestDiet® products, due to the absence of antioxidants or preservative agents. To provide maximum protection against possible changes during storage, store in a dry, cool location.
Storage under refrigeration (2° C) is recommended. Maximum shelf life is six months.
(If long term studies are involved, storing the diet at -20° C or colder may prolong shelf life.)
Be certain to keep in air tight containers.

*Product Forms Available**	*Catalog #*
1/2" Pellet, Irradiated	1810835
1/2" Pellet	58864

**Other Forms Available By Request*

INGREDIENTS (%)

Hydrogenated Coconut Oil	33.3467
Casein - Vitamin Free	22.7977
Sucrose	17.4981
Maltodextrin	16.9983
AIN-76 Mineral Mix	3.9996
Soybean Oil	2.4998
Sodium Bicarbonate	1.0499
AIN-76A Vitamin Mix	0.9999
Potassium Citrate, Tribasic Monohydrate	0.4000
Choline Bitartrate	0.2000
DL-Methionine	0.2000
Red Dye	0.0100

FEEDING DIRECTIONS

Feed ad libitum. Plenty of fresh, clean water should be available at all times.

CAUTION:
Perishable - store properly upon receipt. For laboratory animal experimental use only, NOT for human consumption.

4/25/2

NUTRITIONAL PROFILE

Protein, %	**20.7**
Arginine, %	0.80
Histidine, %	0.59
Isoleucine, %	1.09
Leucine, %	1.97
Lysine, %	1.66
Methionine, %	0.79
Cystine, %	0.08
Phenylalanine, %	1.09
Tyrosine, %	1.15
Threonine, %	0.88
Tryptophan, %	0.25
Valine, %	1.30
Alanine, %	0.63
AsparticAcid, %	1.47
GlutamicAcid, %	4.66
Glycine, %	0.44
Proline, %	2.69
Serine, %	1.26
Taurine, %	0.00
Fat, %	**35.8**
Cholesterol, ppm	0
Linoleic Acid, %	1.28
Linolenic Acid, %	
Arachidonic Acid, %	0.20
Omega-3 Fatty Acids, %	0.00
Total Saturated Fatty A	0.20
Total Monounsaturated Fatty Acids, %	0.37
Polyunsaturated Fatty Acids, %	0.53
	1.47
Fiber (max), %	0.0
Carbohydrates, %	35.0
Energy (kcal/g) [2]	5.49

From:	kcal	%
Protein	0.827	15.2
Fat(ether extract)	3.226	59.2
Carbohydrates	1.401	25.7

Minerals	
Calcium, %	0.59
Phosphorus, %	0.64
Phosphorus (available), %	0.64
Potassium, %	0.56
Magnesium, %	0.06
Sodium, %	0.40
Chlorine, %	0.18
Fluorine, ppm	0.0
Iron, ppm	40
Zinc, ppm	41
Manganese, ppm	67
Copper, ppm	6.9
Cobalt, ppm	0.0
Iodine, ppm	0.24
Chromium, ppm	2.3
Molybdenum, ppm	0.00
Selenium, ppm	0.12

Vitamins	
Vitamin A, IU/g	4.0
Vitamin D-3 (added), IU/g	1.0
Vitamin E, IU/kg	50.0
Vitamin K (as menadione), ppm	0.50
Thiamin Hydrochloride, ppm	6.0
Riboflavin, ppm	6.0
Niacin, ppm	30
Pantothenic Acid, ppm	15
Folic Acid, ppm	2.0
Pyridoxine, ppm	5.8
Biotin, ppm	0.2
Vitamin B-12, mcg/kg	10
Choline Chloride, ppm	1,000
Ascorbic Acid, ppm	0.0

1. Based on the latest ingredient analysis information. Since nutrient composition of natural ingredients varies, analysis will differ accordingly. Nutrients expressed as percent of ration on an As Fed basis except where otherwise indicated.
2. Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9,4 kcal/gm respectively.