UNIVERSITY OF CALGARY

Substrate Metabolism in the Diabetic Heart – Relationship to the Observed Cardiac Dysfunction and the Significance of Putative Mechanisms in the Control of Lipid Utilization

by

Andrew Neil Carley

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Substrate Metabolism in the Diabetic Heart – Relationship to the Observed Cardiac Dysfunction and the Significance of Putative Mechanisms in the Control of Lipid Utilization" submitted by Andrew Neil Carley in partial fulfillment of the requirements for the degree of PhD. Medical Science.

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ABSTRACT

Diabetic individuals are at increased risk for the development of cardiovascular disease. Not all of the increased risk can be accounted for by atherosclerosis and coronary artery disease. The existence of a diabetic cardiomyopathy has been postulated. Hearts from diabetic *db/db* mice (a type 2 diabetic model) display an increased reliance on fatty acids for cardiac energy generation and it has been suggested that this is related to the impaired myocardial function of both *in vivo* and *ex vivo* perfused *db/db* hearts. Therefore it was undertaken to determine if chronic manipulation of substrate supply to the heart through the use of an insulin sensitizer could lead to alterations in cardiac metabolism of isolated *db/db* hearts. Corrections in the diabetic status of *db/db* mice led to normalization of their cardiac metabolism; however this was without effect on cardiac function measured either *in vivo* through echocardiography, or via *ex vivo* perfusion. These observations were similarly confirmed with the use of another pharmacological agent. The increased reliance on FA by isolated *db/db* hearts was not evident in mitochondria isolated from db/db mouse hearts. Uncoupling protein 3, which has been postulated to be involved in FA metabolism, was not found to be elevated in hearts from *db/db* mice.

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DEDICATIONS

I dedicate this thesis to my parents who have supported me in all that I do. If it were not for them I wouldn't have much of what I have today. Most significantly I wouldn't have the computer I used to prepare my thesis. For that gift and everything that has come before that I thank you. I also dedicate this thesis to my sisters who visited often and made Calgary feel more like home when I first moved out here.

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ABBREVIATIONS

ACC	acetyl CoA carboxylase
ACS	acyl CoA synthetase
ADP	adenosine diphosphate
AGE	advanced glycation end-products
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine 3',5'-monophosphate
cGMP	cyclic guanosine 3',5'-monophosphate
CM	chylomicrons
CoA	Coenzyme A
COOH	2-(2-(4-Phenoxy-2-propylphenoxy)ethyl)indole-5-acetic acid
CPT	carnitine palmitoyl transferase
E/A ratio	ratio of early to late filling phases of the left ventricle
EF	ejection fraction
ETC	electron transport chain
FA	fatty acids
FABPpm	plasma membrane fatty acid binding protein
$FADH_2$	flavin adenine dinucleotide, reduced form
FAT/CD36	fatty acyl translocase/CD36
FATP	fatty acid transport protein
FS	fractional shortening
GLUT	glucose transporter
hFABP	intracellular fatty acid binding protein
HK	hexokinase
HR	heart rate
HSL	hormone sensitive lipase
ID	internal diameter
KHB	Krebs Henseleit bicarbonate
LPL	lipoprotein lipase
LV	left ventricle
LVmass	left ventricular mass

LVEDP	left ventricular end diastolic pressure
LVIDd	left ventricular dimension in diastole
LVIDs	left ventricular dimension in systole
MCD	malonyl CoA decarboxylase
mCPT-1	muscle isoform of CPT-1
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide, reduced form
NO	nitric oxide
NOS	nitric oxide synthase
OD	outer diameter
P_i	inorganic phosphate
PCr	phosphocreatine
PDE	phosphodiesterase
PDH	pyruvate dehydrogenase
PDK	pyruvate dehydrogenase kinase
PDP	pyruvate dehydrogenase phosphate phosphatase
PFK-1	phosphofructokinase-1
PFK-2/FBPase	phophofructokinase-2/fructose 2,6-bisphosphatase
PKC	protein kinase C
PKG	protein kinase G
PPAR	peroxisome proliferator-activated receptor
PSP	peak systolic pressure
PWT	posterior wall thickness
SWT	septal wall thickness
STZ	streptozotocin
TCA	tricarboxylic acid
TG	triacylglycerol
TGLPR	triacylglycerol-lipoprotein receptor
UCP	uncoupling protein
Vcf	velocity of circumferential fibre shortening
VLDL	very-low density lipoproteins
ZDF	Zucker Diabetic Fatty rat

CHAPTER 1 – GENERAL INTRODUCTION

Diabetes mellitus has become one of the more significant health concerns of the new millennium. The incidence of diabetes is expected to increase explosively in the coming years[1, 2]. The increase in the prevalence of type 2 diabetes is primarily responsible for the overall increase in the diabetic population[1]. Type 2 diabetes compromises 90% of the diabetic population. Type 2 diabetes, sometimes referred to as non-insulin dependent diabetes, results from insulin resistance in combination with a β-cell secretory defect. The remainder of the diabetic population is made up of type 1 diabetics, sometimes referred to as insulin dependent diabetes, resulting from the autoimmune destruction of the β-cell and requiring hormone replacement therapy.

1.1 Diabetic Cardiomyopathy

1.1.1 Human Studies

Diabetes is a significant predictor for a cardiovascular event and heart disease is the leading cause of death in diabetic individuals[3]. Type 2 diabetics specifically exhibit many of the classical risk factors for cardiovascular disease such as obesity, hyperlipidemia, and hypertension[3]. However, the risk for the development of cardiovascular disease that is present in the diabetic condition cannot be explained solely by traditional risk factors. The Framingham heart study was one of the first longitudinal studies to present data demonstrating the unique risk that is inherent in the diabetic condition[4, 5]. Controlling for traditional risk factors for cardiovascular disease, diabetic individuals are still at a 3-5 fold higher risk for heart failure[4, 5]. Diabetics are also at a heightened risk for developing heart failure after a myocardial infarction[6].

A number of factors may be responsible for increased rates of heart failure in diabetics. The presence of a number of common co-morbidities together may act synergistically to worsen the statistical outcome. Traditional risk factors such as hypertension act synergistically with hyperglycemia to elevate the risk of developing complications in individuals in which both conditions are superimposed[7]. The increased risk is greater than would be expected by combining the individual risks of the two conditions[7], however. Another possibility is that the diabetic heart is somehow compromised directly by diabetes leading to a so-called primary cardiomyopathy or diabetic cardiomyopathy.

The first group to propose the existence of a primary cardiomyopathy in diabetic patients was Rubler and colleagues[8], although the term "diabetic cardiomyopathy" was first used by Hamby et al.[5, 9]. In the greater than 30 years since these initial observations, the existence of a primary diabetic cardiomyopathy has remained controversial. The strongest evidence in favour of a diabetes-induced cardiomyopathy in humans come from echocardiographic studies on patients who have had diabetes for only a short duration and are argued to be asymptomatic[10-15]. Studies such as these have identified diastolic dysfunction in diabetics despite the absence of any clinically measurable microvascular disease or elevations in blood pressure. The most common presentation is a disruption of the normal diastolic filling pattern in the absence of any systolic dysfunction or clinically evident vascular disease. Diastolic dysfunction can be documented even in those individuals with well-controlled diabetes[16]. The absence of systolic dysfunction may be misleading. The development of more sensitive methods for assessing systolic function

has led to the suggestion that early signs of systolic dysfunction may also be present in otherwise asymptomatic diabetic individuals[17, 18].

The absence of clinically measurable ischemic heart disease does not exclude its presence. Diabetes is associated with a number of significant factors that strongly predict the presence of microvascular disease. Coronary artery disease may not be evident using current techniques but may nonetheless exist, and such an argument has been used to dispute the existence of a primary diabetic cardiomyopathy. Recent work by Moir et al.[19] supports this suggestion as more sensitive techniques were able to document both early signs of ischemic heart disease (decreased myocardial blood flow) along with the symptoms of diabetic cardiomyopathy, suggesting that in human subjects the exclusion of sub clinical ischemia cannot be made. Interestingly, a correlation between the symptoms attributed to a diabetic cardiomyopathy and reductions in myocardial blood flow could not be made, suggesting that they may be unrelated and independent in etiology. Therefore it is difficult to exclude the presence of coronary artery disease in supposedly asymptomatic diabetics and separating the contributions of coronary artery disease and diabetic cardiomyopathy in humans is a difficult prospect. There may also be some interplay between the two disorders. Diabetics are more sensitive to ischemic insult[6, 20, 21], which may relate to specific alterations in the myocardium, compromising the ability of the diabetic heart to respond to changes in myocardial blood flow.

1.1.2 Animal Studies

The analysis of cardiac function in animal models of diabetes allows for greater separation of the relative roles of coronary artery disease and other diabetic co-

morbidities from a diabetic cardiomyopathy. Rodents specifically are resistant to atherosclerosis and the development of coronary artery disease, a resistance that is not impaired by the introduction of diabetes[22, 23]. Much of the early work in this field has focused on insulin deficient models such as streptozotocin(STZ)-treated or alloxan-treated animals. STZ is an antibiotic from *Streptomyces achromogenes* that results in β-cell specific necrosis and degranulation within 1 h of treatment in a dose dependent manner[24-27]. Although these models are easily generated, they are more related to type 1 diabetes than to type 2 diabetes as they fail to recreate the insulin resistance that is a hallmark of type 2 diabetes. Nonetheless early work clearly indicated that STZ-treated rodents exhibited both *in vivo* and *ex vivo* cardiac dysfunction in the absence of any coronary artery disease[28-30]. More recent work from our laboratory and others has begun to examine the cardiac phenotype of type 2 diabetic rodent models to more closely address questions that are relevant to a greater percentage of the human diabetic population.

Type 2 Diabetes/Obesity Models

The pathogenesis of type 2 diabetes is multifactorial with both genetic and environmental (diet, physical activity) contributions. High-fat diets administered to mice produce insulin resistance but only mild hyperglycemia[31, 32]. On the other hand, monogenic models in both mice and rats[33] exhibit severe features of type 2 diabetes with marked obesity, hyperglycemia and dyslipidemia. The following monogenic models of type 2 diabetes are the result of mutations in either leptin receptors or leptin itself and

have been widely used to study cardiac metabolism and cardiac function in diabetes/obesity.

The *db/db* mouse is a mutant leptin receptor inbred strain; a point mutation in the long form of the leptin receptor (*Leprdb*), the signaling form of the receptor[33], leads to hyperphagia and obesity[34]. There are measurable increases in plasma insulin levels as early as day 10, with beta cell hyperplasia and hypertrophy[34]. Thus, despite early insulin resistance, compensatory hyperinsulinemia maintains euglycemia. However, notwithstanding enhanced insulin secretion, hyperglycemia develops due to progressive increases in insulin resistance so that *db/db* mice transition to overt diabetes at 6 weeks of age[35]. Plasma cholesterol, triacylglycerols (TG) and fatty acids (FA) are also elevated[34, 35], along with increased body weight which plateaus at about 2 months of age at 40–50 g, almost double the weight of control mice. There is a fall in plasma insulin levels beginning at 14 weeks of age, due to increased beta cell apoptosis[34, 35]. Thus, the general metabolic features of *db/db* mice, with initial insulin resistance followed by an insulin secretory defect, are very similar to the pathogenesis of type 2 diabetes in humans[36, 37]. Interestingly, *db/+* heterozygotes with one mutant copy of the leptin receptor are phenotypically normal with respect to body weight and blood concentrations of glucose and lipids. The severity of the diabetic phenotype is influenced by the background strain in which the receptor mutation is maintained. The *db/db* mouse is typically maintained on the Ks/J background; mice maintained on a BL/6 background develop a less severe phenotype[34] that more resembles the *ob/ob* mouse (discussed below).

Cardiac dysfunction in the *db/db* mouse has been demonstrated both *in vivo* and *ex vivo*. Echocardiographic assessment of mice at 6 wks of age shows only minor

differences in cardiac function, while at 12 wks clear evidence of both diastolic (reduced E/A ratio for trans-mitral flow) and systolic (reduced fractional shortening and heart rate) dysfunction is observed[38]. Isolated hearts perfused with glucose and fatty acids with and without insulin also show reductions in systolic and diastolic function[39-41]. Belke et al. [39] has shown that isolated hearts from *db/db* mice have elevated left ventricular end diastolic pressure (LVEDP) and reduced cardiac power. Aasum et al.[40] presented similar data on the progression of contractile dysfunction in the *db/db* mouse as has been encountered *in vivo*. At 6 wks of age, only females had significant cardiac dysfunction, while at 12 wks both male and female *db/db* mice demonstrated reduced cardiac output. The *ob/ob* mouse is a leptin mutant (*Lepob*) that fails to secrete leptin[33, 34]. Thus, *ob/ob* mice are leptin-deficient, in contrast to leptin-resistant *db/db* mice. Similar to the *db/db* mouse, the *ob/ob* model shows hyperphagia, obesity and insulin resistance. Interestingly, the *ob/ob* mouse is more severely insulin resistant but less hyperglycemic compared to the *db/db* mouse[34, 42]. There is some increase in plasma glucose levels evident at 35 days of age[34, 42-44]. Therefore, on the BL/6 background, *ob/ob* mice provide an obese, insulin resistant model with transient hyperglycemia. When the *Lepob* mutation is maintained on the genetic background (KsJ) used for *db/db* mice, the *ob/ob* phenotype is more severe[34].

There is not a significant body of research on the contractile function of the *ob/ob* mouse heart. Buchanan et al.[45] documented some modest reductions in cardiac function, however this was less than found in *db/db* hearts in the same study. Mazunder et al.[43] have suggested that there is measurable dysfunction in isolated *ob/ob* hearts only in the presence of insulin, compared to *ob/+* hearts also perfused with insulin. The study of Buchanan et al.[45] also was conducted with insulin in the perfusate. *In vivo* assessment of

cardiac function has also been undertaken however no significant cardiac dysfunction was documented[46]; this study did include the use of anesthesia however, which can mask systolic dysfunction in mouse hearts.

The leptin receptor mutant rat models are the Zucker fatty rat (*fa/fa*) and the Zucker diabetic fatty rat (ZDF). These two models develop from the same mutation (*leprfa*); a single amino acid substitution results in defective leptin receptor folding and translocation to the plasma membrane[47]. The Zucker fatty rat is an outbred strain that develops modest and transient hyperglycemia while the ZDF rat is an inbred strain that develops overt diabetes (hyperglycemia)[48, 49]. Both models show obesity and extreme insulin resistance. Male ZDF rats before 6 weeks of age are hyperinsulinemic but euglycemic; hyperglycemia develops at about 6 weeks of age and increases to a relatively stable level of hyperglycemia at 10–12 weeks[50]. These rat strains also tend to develop dramatically greater dyslipidemia than that presented in mouse models of obesity and diabetes[35,48,49].

Cardiac function in the ZDF rat has been studied in a more detailed manner than has occurred with the Zucker fatty rat. Zhou et al.[51] presented a similar pattern of echocardiographic dysfunction in the ZDF rat as was measured by Semeniuk et al. [38] with *db/db* mice. Diastolic and systolic dysfunction was present at 20 wks of age. Isolated perfused hearts from ZDF rats show reductions in cardiac power as early as 9 weeks of age[52]. *In vivo* assessment of Zucker obese rats has not been completed, however cardiac function has been assessed in isolated perfused hearts[53]. Cardiac dysfunction has been documented in some studies while others have suggested that there is an absence of dysfunction but have found increased sensitivity to ischemia/reperfusion injury[53, 54].

In summary the *db/db* mouse and the ZDF rat display impaired diastolic and systolic dysfunction that presents early in the development of diabetes and occurs in the absence of coronary artery disease. Cardiac dysfunction has been documented in both *ex vivo* perfused hearts and through *in vivo* echocardiography. In the obese, insulin resistant models, cardiac dysfunction has been documented, however it is generally more modest and the degree of dysfunction evident in *ex vivo* perfused hearts may be influenced by the presence of insulin in the perfusate.

1.1.3 Diabetic Cardiomyopathy - Mechanisms

A number of different mechanisms have been suggested to be responsible for the development of a diabetic cardiomyopathy. Cardiac fibrosis, ventricular remodeling, impairments in calcium handling, disruptions in ion channel function, and altered oxygen consumption have all been suggested to be factors that could be responsible for the functional deficits of the diabetic heart[29, 30, 55-60]. Many of these mechanisms have been argued to be a consequence of altered cardiac metabolism[61, 62].

1.2 Cardiac Metabolism

The heart must maintain an appropriate level of cardiac function in the face of variations in energy supply and demand: hormones, nutrients (fasting, satiety), and oxygen (physical exertion). Cardiac metabolism must adequately meet the metabolic demands of the heart under these variable conditions. The role of cardiac metabolism is to provide adequate levels of ATP for the beating heart[63]. The heart is a very omnivorous organ and can utilize a number of substrates to meet its metabolic needs[64, 65]. Under

physiologic conditions the heart is believed to rely heavily on exogenous FA. As much as 70% of its metabolic needs may be met by exogenous FA. The heart can also utilize glucose, both from exogenous or endogenous (glycogen) sources, lactate, pyruvate, and ketones.

1.2.1 Lipid Metabolism

The importance of FA for myocardial metabolism was established 50 years ago by the classic studies of Bing[66]. The conventional view has been that FA oxidation accounts for 60–70% of cardiac energy production[67]. However, much of the experimental evidence for the preferential utilization of FA by the myocardium has come from isolated hearts perfused with only two radiolabeled exogenous substrates, glucose and a FA (usually palmitate). Recently, the use of stable isotopic techniques has permitted the utilization of up to four ^{13}C-labeled substrates to be examined simultaneously. In the presence of physiological concentrations of glucose, lactate and pyruvate, FA remain the predominant substrate for myocardial utilization [64, 68].

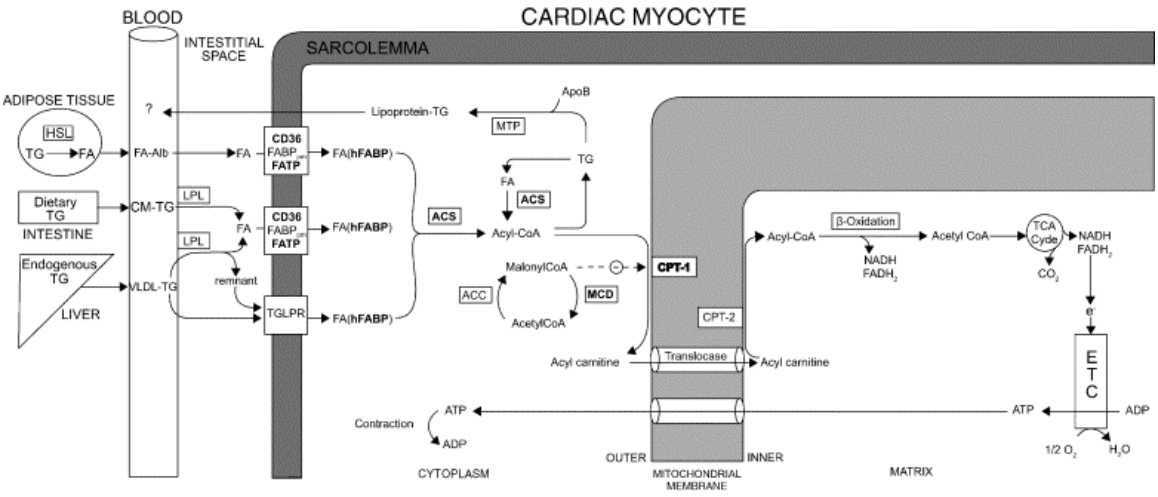

Fig. 1.2.1.1 - Cardiac FA metabolism. There are two exogenous FA sources in the circulation that are available for cardiac utilization: (a) adipose tissue lipolysis (catalyzed by hormone sensitive lipase, HSL) produces circulating FA complexed to plasma albumin (Alb); and (b) uptake of triacylglycerols (TG) in circulating lipoproteins, either by the hydrolytic action of an enzyme, lipoprotein lipase (LPL) located on the endothelium of the coronary vasculature, producing LPL-derived FA, or by a lipoprotein receptor-mediated pathway (TGLPR). Chylomicrons (CM) transport dietary TG, whereas very-low-density lipoproteins (VLDL) transport endogenous TG formed in the liver. Intracellular FA metabolism involves formation of fatty acyl CoA and then either esterification to TG or entry into mitochondria (CPT-1-dependent) for β-oxidation and energy production by the electron transport chain (ETC) and oxidative phosphorylation. Activity of CPT-1 is regulated by malonyl CoA content, determined by the net effects of synthesis (ACC) and degradation (MCD). Some cardiac PPARα targets (enhanced expression) are shown in bold.

In vivo, there are two sources of FA for myocardial metabolism (Fig. 1.2.1.1): (i) circulating FA bound to plasma albumin (FA-Alb), derived from adipose tissue lipolysis[69, 70]; and (ii) hydrolysis of TG-rich lipoproteins, intestinal chylomicron (CM-TG) particles and hepatic very-low-density lipoproteins (VLDL-TG), by an enzyme, lipoprotein lipase (LPL), located on the surface of endothelial cells in the coronary vasculature[71-73]. In addition to cardiac FA uptake by LPL-catalyzed hydrolysis of TG-rich lipoproteins, plasma TG may be taken up by a lipoprotein receptor-dependent endocytic pathway[74]. Uptake of CM-TG is mediated predominantly by LPL-catalyzed

degradation; in contrast, VLDL-TG uptake is dependent on both LPL action and by the TG-lipoprotein receptor (TGLPR) pathway (Fig. 1.2.1.1). In addition to TGLPR-mediated uptake of the intact VLDL particle, the VLDL remnant produced by the catalytic action of LPL on core TG can also be taken up by the lipoprotein receptor pathway, increasing the efficiency of VLDL degradation by the heart.

FA bound to albumin in the circulation and LPL-derived FA must traverse endothelial cells in the coronary vasculature[75, 76] to reach the interstitial space for uptake and metabolism by cardiac myocytes (Fig. 1.2.1.1). FA uptake by cardiac myocytes is due to two transport processes: simple diffusion[77] and protein-mediated transport[78, 79] which has been suggested to account for about 80% of total FA uptake into cardiac myocytes[80-82]. An 88-kDa FA translocase/CD36 (FAT/CD36) and a 43-kDa plasma membrane FA binding protein (FABPpm) appear to be the predominant cardiac sarcolemmal FA transport proteins[79, 83], although FA transport proteins (FATP, multiple isoforms) may also contribute[84]. Interestingly, FAT/CD36 can recycle between an intracellular membrane compartment and its functional site in the sarcolemmal membrane[79, 82]. Furthermore, insulin can stimulate the translocation of FAT/CD36 to the sarcolemma of cardiac myocytes[82, 85], much like insulin-stimulated translocation of glucose transporter-4 (GLUT4)[79].

After sarcolemmal uptake, intracellular FA are activated to form fatty acyl-CoA (Fig. 1.2.1.1) by the action of the acyl-CoA synthetases (ACS). Fatty acyl-CoAs are extremely reactive and so intracellular FA binding proteins (hFABP) within the cell control movement and concentrations of both FA and fatty acyl-CoA[76, 86, 87]. Fatty acyl-CoA at this point can either be transported into mitochondria for beta oxidation or

undergo esterification to form intracellular TG[88]. The fate of fatty acyl-CoA is likely determined in part by localization of different ACS isoforms or enzyme complexes, and the energy demand of the heart.

Intracellular TG stores can be mobilized by hydrolysis to provide an endogenous FA source; even in the presence of exogenous FA, hydrolysis of endogenous TG (lipolysis) in cardiac myocytes can provide 10% of total FA utilization[89].

FA oxidation requires fatty acyl-CoA entry into mitochondria (Fig. 1.2.1.1) which is dependent on the activity of carnitine palmitoyl transferase-1 (CPT-1)[90-92]. CPT-1 is located in the outer mitochondrial membrane and catalyzes the conversion of fatty acyl-CoA to acyl carnitine[93]. The activity of CPT-1 is inhibited by malonyl CoA[93-95]. The myocardial content of malonyl CoA is regulated by the actions of two enzymes[96]: acetyl CoA carboxylase (ACC) catalyzes the formation of malonyl CoA from cystolic acetyl CoA, whereas malonyl CoA decarboxylase (MCD) degrades malonyl CoA. There are two major isoforms of CPT-1, one that is expressed at high levels in the liver, and a second that is expressed at high levels in skeletal muscle and heart (mCPT-1)[97]. The predominant mCPT-1 isoform in heart is very sensitive to inhibition by malonyl CoA [94, 95, 98]. The regulation of cardiac FA oxidation by malonyl CoA inhibition of mCPT-1[96] is complicated by intracellular compartmentation. Some intracellular malonyl CoA may be intramitochondrial[99], where it is unlikely to be in contact with CPT-1. Recent data indicate that a substantial portion of the malonyl CoA within the cytoplasm that is responsible for CPT-1 inhibition in the heart is derived from acetyl CoA generated in peroxisomes[100].

After translocation into mitochondria, acyl carnitine is reconverted to fatty acyl-CoA via the action of CPT-2 on the inner mitochondrial membrane, at which point, intramitochondrial fatty acyl-CoA can enter the beta-oxidation pathway[92]. Following the β-oxidation pathway, FA-derived acetyl CoA enter the same pathway as that used for acetyl CoA generated from metabolism of glucose, lactate or ketone bodies, namely the TCA cycle with entry of reducing equivalents to the electron transport chain and oxidative phosphorylation forming ATP.

1.2.2 Carbohydrate Metabolism

The heart is capable of utilizing a number of carbohydrate sources to meet its metabolic needs (Fig. 1.2.2.1). Most cardiac research has centred on the uptake and metabolism of glucose, however the heart is capable of utilizing lactate and pyruvate[64, 65, 68]. Glucose uptake in the adult heart occurs through the glucose transporters GLUT1 and GLUT4[79]. GLUT1 participates in basal glucose uptake and is primarily localized to the sarcolemma[101, 102], while GLUT4 is responsible for both a portion of basal and the majority of insulin-stimulated glucose transport[101, 102]. GLUT4 protein is localized within endosomes in the intracellular compartment and translocates to the plasma membrane in response to insulin[101] and increased cardiac work[103]. The effect insulin has on glucose transport is not as significant as that which occurs in skeletal muscle, with only a 4 fold increase in glucose uptake with physiologic concentrations of insulin[101]. In skeletal muscle, the presence of insulin produces a more robust increase in glucose uptake. This may relate to the higher expression of GLUT1 in the heart compared to skeletal muscle and the increased ratio of GLUT1 to GLUT4[104].

Following the uptake of glucose into the cardiomyocyte, glucose is phosphorylated through the actions of hexokinase[105, 106] (Fig. 1.2.2.1). Glucose 6-phosphate can then enter the glycolytic pathway or be used in the formation of glycogen. The activity of hexokinase is not believed to be a significant barrier to glucose utilization[105, 106]. The first committed step in the glycolytic pathway is catalyzed by PFK-1, which converts fructose 6-phosphate to fructose 1,6-bisphosphate. PFK-1 is inhibited by ATP and stimulated by AMP. PFK-1 is also influenced by the concentration of fructose 2,6-bisphosphate. The enzyme responsible for the concentration of fructose 2,6-bisphosphate, PFK-2 also can exert significant control over the glycolytic rate[107]. PFK-2 is responsive to insulin and AMP-activated protein kinase (AMPK), and therefore represents a significant control point in glycolysis. The end product of the glycolytic

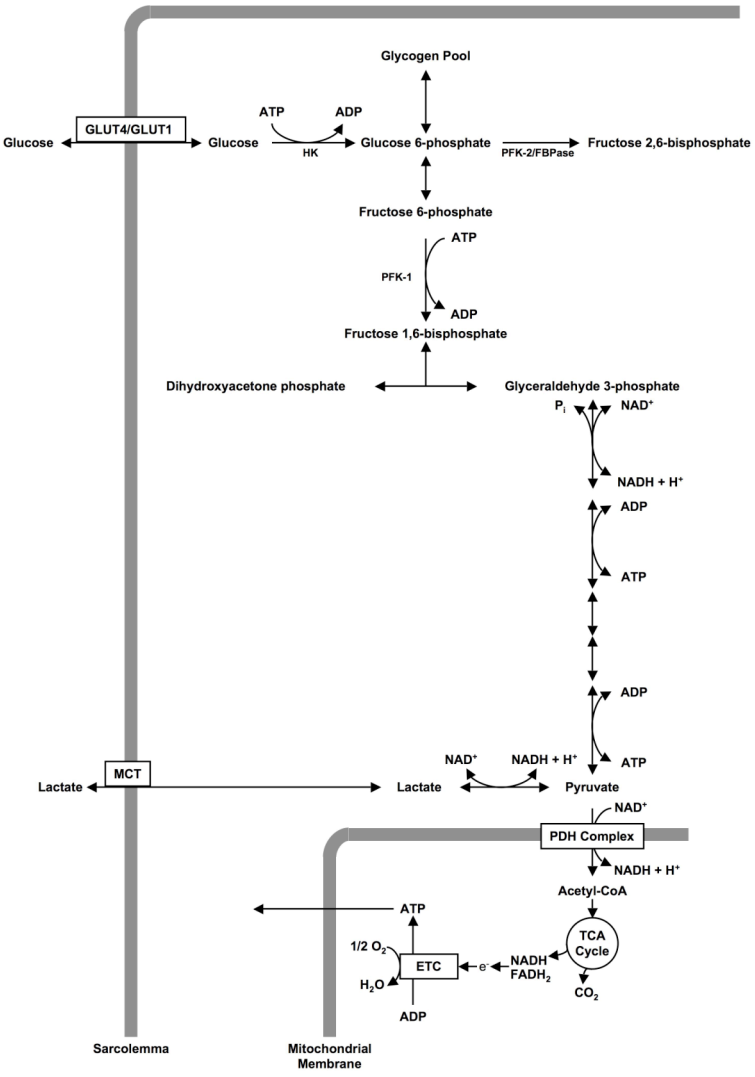

Fig. 1.2.2.1 - Cardiac glucose and lactate metabolism. Intracellular glucose metabolism involves formation of pyruvate and then either decarboxylation by the pyruvate dehydrogenase complex (PDH) to tricarboxylic acid (TCA) intermediates and transport into the mitochondria or reduction to lactate. Lactate can be transported across the sacrolemma via monocarboxylate transporter (MCT). Reducing equivalents generated in the TCA cycle are then passed through the electron transport chain (ETC) resulting in the oxidative phosphorylation of ATP.

pathway is pyruvate that can undergo decarboxylation by mitochondrial pyruvate dehydrogenase (PDH) and conversion to TCA intermediates, or be reduced to lactate.

The PDH complex is made up of 5 components, 3 which are responsible for the decarboxylation of pyruvate to acetyl CoA, and 2 regulatory components, pyruvate dehydrogenase phosphate phosphatase (PDP) and pyruvate dehydrogenase kinase (PDK)[108, 109]. PDP catalyzes the dephosphorylation of the PDH complex and increases catalytic activity, while PDK phosphorylates the PDH complex leading to its inactivation[109]. The flux of pyruvate through the PDH complex reflects the activity of the two regulatory enzymes. The acetyl CoA that is generated by PDH undergoes the same fate as the acetyl CoA generated by the metabolism of fatty acids, such that the end result in the formation of ATP (Fig. 1.2.1.1).

1.2.3 Regulation of cardiac metabolism

Cardiac metabolism is carefully controlled to permit metabolic switching (Randle cycle) when the substrate supply to the heart is altered.

Insulin

Elevated FA concentrations inhibit glucose and lactate oxidation by the heart as a consequence of reductions in glucose uptake and glycolytic flux, and pyruvate dehydrogenase inhibition[110-112]. Thus, insulin promotes myocardial glucose utilization by directly stimulating glucose uptake (GLUT4 translocation)[79], glycolysis and glucose oxidation; and indirectly by reducing circulating FA (inhibition of adipose tissue lipolysis), thus, relieving FA inhibition of glucose utilization. Furthermore, increased

production of acetyl CoA from glucose results in the ACC-catalyzed formation of malonyl CoA that inhibits CPT-1 and thus reduces FA oxidation[111]. The reciprocal utilization of glucose or FA by the heart represents metabolic flexibility[113], a key physiological mechanism (homeostatic adaptability) for any organism with a discontinuous supply of nutrients (fuels).

Insulin also has direct effects on cardiac FA metabolism. In rat cardiac myocytes, insulin induced the translocation of FAT/CD36 to the sarcolemmal membrane[82]; FA uptake and esterification to TG was increased by insulin but FA oxidation was unchanged when FA were the sole substrate provided[114]. On the other hand, insulin reduced rates of FA oxidation by perfused hearts from rats[115] and mice [43, 116] in the presence of glucose. It must be acknowledged that metabolic effects of insulin on quiescent cardiac myocyte preparations may be different than beating perfused hearts because energy demand influences translocation of fatty acid transporters [79]. Inclusion of glucose in the perfusion buffer to either isolated myocytes or perfused hearts significantly increases the ability of insulin to influence FA oxidation.[43, 116, 117]

AMPK

AMP-activated protein kinase (AMPK) has been proposed to act as a metabolic energy sensor[118-120]. Activation of AMPK occurs in response to reduction in the ratio of ATP/AMP. AMPK activation inhibits ATP-consuming biosynthetic pathways and stimulates ATP-generating pathways such as FA oxidation[118, 120] and glycolysis[121]. Although AMPK activation *in vivo* by infusion of a nucleoside analog into rats increased cardiac FA clearance[122] and cardiac glucose uptake[123], recent studies with transgenic

mice expressing a kinase-dead form of AMPK through dominant-negative knockdown strategies has concluded that the effects of AMPK on the heart are restricted to regulation of post-ischemic metabolism[121, 124].

PPARs

Cardiac energy metabolism can also be regulated at the level of gene expression through nuclear receptor signaling mechanisms[125] that permit rates of FA and glucose utilization to be adjusted under conditions of chronic changes in substrate delivery. Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily that function as ligand activated transcription factors[126]. PPARs heterodimerize with retinoid X receptors and then bind to specific DNA response elements in the promoter region of target genes. Three PPAR isoforms (α, β/δ, γ) can be distinguished by expression patterns and activation by relatively selective ligands[126]. PPARα is highly expressed in tissues with high capacity for FA oxidation, including the heart[127]. Studies with cultured cardiomyocytes[125, 128, 129] incubated with selective PPARα ligands have revealed specific target genes that regulate FA uptake (FAT/CD36, FATP-1, LPL), intracellular FA binding (hFABP) and cellular FA metabolism (ACS, mCPT-1, and both medium- and long-chain mitochondrial acyl-CoA dehydrogenases)(Fig. 1.2.1.1). Activity of mCPT-1 may also be increased by PPARα activation through an upregulation of MCD[130]; the resulting fall in intracellular inhibitory malonyl CoA concentrations will increase FA oxidation. PPARα may also directly affect the metabolism of glucose; PDK-4 is argued to be a PPARα target[108]. Increased PDK-4 activity will lead to a

decrease in the ratio of active to inactive PDH and therefore a decreased flux through the PDH complex.

The function of cardiac PPARα has also been investigated with genetically engineered mice. PPARα null mice do not show the typical fasting-induced upregulation of genes involved in FA oxidation[131]. On the other hand, cardiac-specific over-expression of PPARα leads to increased expression of FA metabolizing genes with an increase in FA oxidation by perfused hearts[132,133].

Because FA are endogenous ligands for cardiac PPARα, oxidative capacity of the heart can be regulated according to FA delivery and the demand for FA oxidation. For example, cardiac PPARα expression is increased during post-natal development in the heart, an adaptation to permit enhanced FA oxidation in response to dietary fat provided by suckling[134]. Chronic activation of cardiac PPARα in diabetic hearts, in part in response to elevated plasma FA, may produce some of the metabolic changes detailed below.

PPARδ is also abundantly expressed in cardiac tissue[128]. Addition of PPARδ activating ligands to cultured cardiac myocytes increased FA oxidation and the expression of FA metabolizing genes, results that are very similar to PPARα activation. On the other hand, cardiac PPARγ expression was very low[128], suggesting that effects of PPARγ ligands on cardiac metabolism will be indirect, due to insulin sensitization and altered delivery of substrates in the circulation to the heart.

1.3 Cardiac Metabolism in Diabetes

1.3.1 Animal Models

Using a working mouse heart perfusion system in which metabolism of radiolabeled glucose and palmitate (complexed to albumin) can be measured along with parameters of contractile function[135], elevated rates of FA oxidation and reduced rates of glucose oxidation and glycolysis were observed with perfused *db/db* mouse hearts[39, 40, 136-138]. Increased FA oxidation was the earliest metabolic alteration in perfused *db/db* hearts, evident at 6–8 weeks of age before glucose utilization was changed[40]. If insulin is included in the perfusate, a reduction in glucose oxidation along with increased fatty acid oxidation can be seen at 4 wks of age[45]. Enhanced FA oxidation was also observed when *db/db* hearts were perfused with radiolabeled CM in the presence of glucose[137], indicating that FA oxidation was increased in *db/db* hearts irrespective of the source. Functional endothelium-bound (heparin-releasable) LPL activity was unchanged in perfused hearts[137], suggesting that the elevated rate of FA oxidation in *db/db* hearts perfused with CM was due to an altered intracellular metabolic fate without a change in FA supply, although receptor-mediated uptake (TGLPR) has not been investigated with *db/db* hearts. The increased FA oxidation in perfused *db/db* hearts is related to workload (energy demand) since FA (palmitate) oxidation was not increased in quiescent cardiac myocytes isolated from *db/db* hearts whether glucose or insulin were present or absent[117].

Results with perfused hearts from Zucker and ZDF rats have been less conclusive in demonstrating an increased level of cardiac fatty acid oxidation. In an isolated working heart study when oleate was used as the exogenous FA, there was no difference in rates of FA oxidation in hearts from fed Zucker rats versus control hearts[53]. In fact, there was a reduction in FA oxidation in hearts from fasted Zucker rats[53] due to an inability of fasting to increase cardiac FA oxidation in the Zucker rat hearts. In isolated cardiac myocytes

from Zucker rat hearts using palmitate as the fatty acid source, there was no difference in basal rates of fatty acid oxidation compared to control cardiac myocytes[114], similar to results with *db/db* cardiac myocytes[117]. However, incubation of Zucker cardiac myocytes with oligomycin to simulate contraction resulted in significantly higher rates of FA oxidation compared to oligomycin-stimulated control cells[114]. Recently, Chatham et al.[139] have studied the metabolism of perfused hearts from ZDF rats; palmitate oxidation was increased using perfusates that also contained physiological concentrations of glucose, lactate and pyruvate. Previously, it had been suggested that FA oxidation was reduced in ZDF hearts, an inference based on reduced expression of some FA oxidation enzymes (acyl CoA oxidase, a peroxisomal enzyme, and the liver isoform of CPT-1)[51]. A similar metabolic phenotype was observed with *ob/ob* hearts perfused with palmitate complexed to albumin, with an increase in FA oxidation of 1.5- to 2-fold[43].

Direct measurements of cardiac FA uptake in type 2 diabetic rodent models have been performed, using both giant sarcolemmal vesicles and cardiomyocytes from Zucker rat hearts[114, 140]. The use of giant vesicles allows measurement of FA uptake without the confounding effects of FA metabolism; however, whole cell uptake measurements are necessary to examine the role of transport vesicle cycling mechanisms[79]. FA uptake was elevated 2-fold in giant vesicles from obese Zucker fatty rat hearts[114, 140]. Although this enhanced FA uptake was associated with an increase in mRNA for both FAT/CD36 and FABPpm, there was no significant difference in total protein expression for either of these transporters in giant vesicles or cardiac myocytes[140]. Rather, there was a redistribution of both FAT/CD36 and FABPpm to the plasma membrane in obese Zucker rat hearts. The response to insulin and oligomycin (to simulate contraction) was

examined in isolated cardiac myocytes from obese Zucker fatty rat hearts[114]. Oligomycin increased FA uptake into cardiac myocytes from both Zucker and control rat hearts; FA uptake was higher in Zucker cardiac myocytes incubated with oligomycin versus control cells plus oligomycin. The addition of insulin led to increased FA uptake into control cardiac myocytes but had no effect in cardiac myocytes from Zucker rat hearts[114]; insulin also no longer had a stimulatory effect on TG esterification. The distribution of FAT/CD36 was essentially insensitive to manipulation by either insulin or oligomycin, indicating a permanent redistribution to the plasma membrane. Although there is strong experimental evidence for increased FA uptake into Zucker rat hearts [114, 140], FA oxidation was not increased[53] suggesting that intracellular FA are incorporated into TG stores[114]. Recently, Wang et al.[139] observed increased FA uptake into sarcolemmal vesicles from ZDF rat hearts at 12 weeks of age, consistent with increased FA oxidation. As noted above, enhanced PPARα expression in ZDF hearts[62] could be a mechanism to increase FAT/CD36 mRNA levels.

Cardiac FA uptake has not been measured directly in mouse models of obesity or type 2 diabetes; however, there has been some quantification of protein and transcript levels of the fatty acid transporters. In *ob/ob* mouse hearts, mRNA for FAT/CD36, FATP1 and FATP4 was elevated, along with hFABP[44]. FA uptake into the mouse heart is most likely increased since both fatty acid oxidation[39, 141] and intramyocardial TG levels[136, 142] are increased.

1.3.2 Humans

Both obesity and diabetes have been shown to affect cardiac metabolism in humans. In a study of obese women, insulin resistance was associated with increased myocardial FA acid uptake and oxidation[143]. In a similar study the concentration of circulating TG and FA in type 2 diabetics was inversely correlated to myocardial glucose uptake[144]. A histological study of patients with heart failure undergoing heart surgery showed that individuals with diabetes had significantly higher myocardial TG levels than nondiabetics with heart failure[145]. The presence of diabetes also prevented the decrease in myocardial PPARα targets that is normally associated with heart failure[145]. Early diabetics also show a reduction in myocardial PCr/ATP ratios that is directly related to the early diastolic dysfunction[15]. More recently FA oxidation was assessed in hearts from type 1 diabetics[146]. FA oxidation, FA uptake and the percentage of exogenous FA oxidized was increased in diabetics, while myocardial glucose uptake was decreased.

1.4 Cardiac Metabolism – Potential Role in a Diabetic Cardiomyopathy

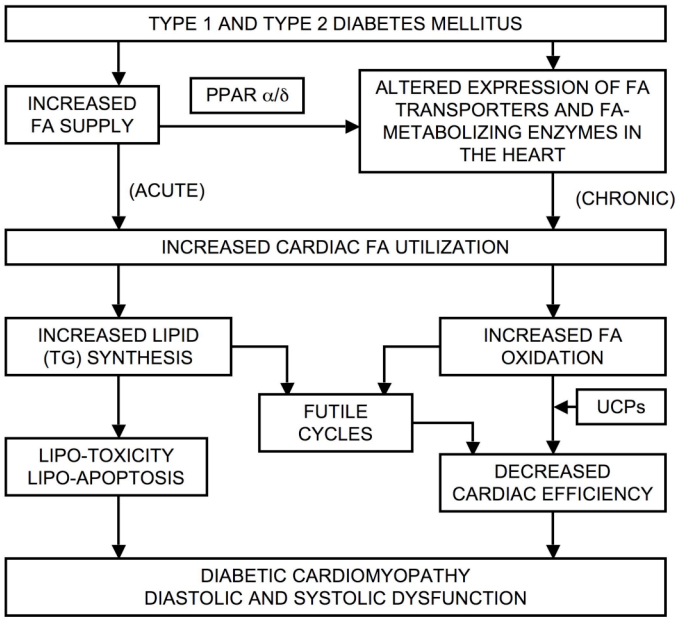

Fig. 1.4.1 – Mechanisms whereby enhanced cardiac FA utilization can produce contractile dysfunction. Abbreviations: FA, fatty acid(s); PPARα/δ, peroxisome proliferators-activated receptors-α/δ; TG, triacylglycerols; UCPs, mitochondrial uncoupling proteins. Increased FA utilization in diabetic hearts can produce contractile dysfunction by two mechanisms: (a) increased lipid synthesis and lipotoxicity (lipoapoptosis); (b) decreased cardiac efficiency because of elevated rates of FA oxidation and futile cycles.

1.4.1 Lipotoxicity

Lipotoxicity refers to situations in which FA uptake exceeds oxidative capacity of a cell[147, 148]. As a result, intracellular lipids accumulate with deleterious consequences on cellular function (Fig. 1.4.1). For example, elevated intracellular palmitate can promote

apoptosis through *de novo* ceramide formation[51, 149, 150] and other mechanisms[151, 152]. Intracellular TG accumulation is often used as a marker of lipotoxicity, but TG are unlikely to be a direct mediator. Fatty acyl-CoA may be the true culprit[153]; potential changes in ACS isoform activity need to be evaluated in diabetic hearts. The limited capacity of the heart to store TG could contribute to lipotoxicity susceptibility. The activity of a number of ion channels have been shown to be influenced by the presence of fatty acyl CoA, through direct interaction with the channel and through indirect mechanisms such as binding to channel agonists/antagonists[154-157].

Hearts from *db/db*, *ob/ob* mice and ZDF rats exhibit increased TG stores[51, 136, 142, 145] and reduced cardiac contractile function (altered diastolic filling pattern, reduced fractional shortening)[38, 39, 44, 51, 158]. Results with a transgenic *db/db* mouse model support a link between enhanced FA utilization (lipotoxicity) and contractile dysfunction[39]. Over-expression of the insulin-stimulated GLUT4 glucose transporter in *db/db* mice resulted in reduced rates of cardiac FA oxidation and complete normalization of contractile function[38, 39]. Genetically-engineered mice have been used to test the linkage between altered cardiac FA metabolism (TG accumulation) and cardiac dysfunction[158]. Increased FA utilization as the consequence of cardiac-specific expression of PPARα[159, 160] or ACS[161] resulted in cardiac contractile dysfunction (reduced fractional shortening). Curiously, over-expression of an LPL construct that resulted in targeting of the enzyme to cardiomyocytes promoted TG uptake and lipid accumulation in transgenic hearts, along with contractile dysfunction (reduced fractional shortening)[162]. Thus genetically-engineered mice with enhanced cardiac FA utilization have contractile dysfunction that resembles a diabetic cardiomyopathy[158].

The lipotoxicity model[147, 148] argues that defects in cellular function are a result of a mismatch between lipid oxidation and lipid uptake. However, lipotoxicity is not solely dependent on TG stores and lipid deposition; increases in FA oxidation can also contribute. Mitochondria can be a substantial source of reactive oxygen species[163]. Zucker hearts show increases in lipid peroxidation[164]; antioxidant defenses are up-regulated[165] but clearly this up-regulation is insufficient to protect the heart from lipid peroxidation. Lipid peroxidation could be occurring through two mechanisms, increased production of reactive oxygen species as a result of excessive FA oxidation and an elevated mitochondrial membrane potential, or increased peroxidation due to the presence of a large quantity of lipid that is able to undergo peroxidation. Lipid peroxidation in the Zucker rat heart was proportional to the concentration of myocardial lipid[164]. The role of mitochondria-derived reactive oxygen species in models with confirmed increases in cardiac FA oxidation has yet to be determined.

1.4.2 Cardiac Efficiency

Theoretical calculations of the yield of ATP per oxygen atom consumed show that FA is a less efficient fuel when compared to glucose. In other words, more oxygen is required for ATP production when hearts are metabolizing FA (2.33 P/O) compared to glucose (2.58 P/O) utilization, producing a reduction in cardiac efficiency (work/O2 consumption)(Fig.1.4.1)[166]. Perfusion of isolated rat hearts with FA increased myocardial oxygen consumption relative to glucose only perfusions[167]. In contrast, elevating the FA concentrations in working rat heart perfusions reduced cardiac efficiency (cardiac work/O_2 consumption) even though O_2 consumption was unchanged[168]. The clinical

significance of lower cardiac efficiency has been examined during ischemia/reperfusion studies. In a number of isolated heart studies, increasing the utilization of glucose and decreasing the utilization of FA led to increased contractile recovery following ischemia/reperfusion with an increase in efficiency[169-172].

The theoretical maximal difference in efficiency based on P/O ratios for fat and glucose metabolism should only be 11%. However, a 30% reduction in cardiac efficiency has been observed when lipid utilization is increased[167]. This discrepancy feeds the argument that additional mechanisms besides metabolic switching with a reduced P/O ratio for FA utilization may be involved. First, an intracellular TG↔FA futile cycle can increase ATP demand through continued ACS activity (Fig. 1.2.1.1)[173]. Second, FA can induce uncoupling of mitochondria[174], perhaps by upregulation of uncoupling protein 3 (UCP3) expression and activity or through some other mechanism[175]. Evidence for reduced cardiac efficiency in type 2 diabetic hearts is beginning to accumulate. Mazumder et al.[43] reported that perfused *ob/ob* mouse hearts exhibited increased myocardial oxygen consumption under all perfusion conditions, with reduced efficiency and decreased cardiac output in perfusions with low concentrations of palmitate and normal glucose concentrations. However, there was no difference in efficiency when *ob/ob* hearts were perfused with a high concentration of palmitate, compared to control hearts perfused with high palmitate. Control hearts perfused with high palmitate showed a dramatic deterioration in function whereas *ob/ob* mouse hearts did not deteriorate any further. Cardiac efficiency has recently been investigated with *db/db* mouse hearts[176]. Unloaded MVO_2 was increased in isolated working *db/db* hearts at low fatty acid concentrations. Increasing the fatty acid concentration in the perfusate failed to increase

unloaded MVO_2 in diabetic hearts but did so in control hearts. However even at high fatty acid concentrations, the unloaded MVO_2 of the *db/db* heart remained significantly elevated versus nondiabetic hearts. Contractile efficiency was reduced in *db/db* hearts with the increase in FA concentration in the perfusate. On the other hand, cardiac efficiency was normal in ZDF rat hearts despite elevated FA oxidation[139] and the recovery of contractile function after ischemia was improved[50].

1.4.3 Glucotoxicity

The sustained hyperglycemia that is part of the diabetic condition may also contribute to alterations in the diabetic myocardium. The incubation of cardiomyocytes with supraphysiologic glucose concentrations in the media leads to impaired calcium handling[177] and apoptosis[149]. Although there is a significant reduction in the oxidation of glucose in *db/db* hearts[39], there is a more modest reduction in the glycolytic rate[39, 45]. It would appear that the inhibition of pyruvate flux through the PDH complex is greater than the defect in glucose uptake and/or glycolytic flux. This has led some to suggest that there may be a shunting of glycolytic pathway intermediates into the hexosamine biosynthetic pathway[59]. This could lead to o-linked glycosylation of proteins, which has been related to alterations in calcium cycling in cardiomyocytes[177]. Hyperglycemia has also been linked to increases in protein kinase Cβ (PKCβ) activation[178-180]. Overexpression of PKCβ2 selectively in the myocardium results in a cardiomyopathy[181]. Advanced glycation end-products (AGEs)[182], originating through non-enzymatic irreversible glycosylation, may also contribute to the development of a diabetic cardiomyopathy[55-57]. The myocardial stiffness that is a hallmark of diabetes may be the

result of collagen cross-linking. Agents that serve to break apart AGE-based linkages help to reduce the severity of cardiac dysfunction in STZ-induced diabetic rats[56].

1.5 Summary and Objectives

A number of studies have suggested that there is a link between the altered substrate utilization pattern (increased reliance on FA for the generation of ATP) and the development of a diabetic cardiomyopathy. It is therefore important to clearly understand the linkages between cardiac metabolism and cardiac function and the perturbations that are induced by the diabetic condition. Therefore the following objectives have been undertaken:

1)To determine if chronic manipulation of substrate supply to diabetic *db/db* hearts, through corrections in their diabetic status, leads to alterations in cardiac metabolism.

2)To determine the relationship between chronic changes in cardiac metabolism and contractile function.

3)Examination of novel mechanisms proposed to control lipid utilization by the mitochondria and what role these mechanisms may have in the upregulation of FA utilization by the *db/db* mouse heart.

CHAPTER 2 - THE ISOLATED WORKING PERFUSED HEART

2.1 Introduction

The workload imposed on cardiac muscle is both significant and continuous, therefore it is important to measure cardiac metabolism under loaded conditions. The relationship between cardiac energy demand and cardiac metabolism is tightly controlled[63]. As cardiac function increases so too does the synthesis of ATP. Although it is possible to justify the use of non-contracting skeletal muscle cells or tissue homogenates in the measurements of skeletal muscle metabolism, the difference between quiescent cardiomyocytes or non-contracting cardiac tissue and the beating heart is quite substantial making the justification for the use of nonworking cardiac tissue more difficult. The rates of substrate oxidation in isolated perfused working hearts can be 100 times greater compared to quiescent cardiomyocytes[39, 117, 138]. Therefore it is important to access cardiac metabolism at relevant workloads, especially when relating metabolism and cardiac function. It must also be acknowledged that the intact heart is not composed solely of a single cell population and therefore the metabolism of isolated cardiomyocytes may not translate directly to the intact heart[183].

2.2 *Ex vivo* Perfused Working Heart

The isolated working perfused heart[184-186] provides a number of significant advantages over the traditional Langendorff perfusion model and *in vivo* methodologies for measuring cardiac metabolism. Originally developed by Drs. Morgan and Neely[184], the isolated working perfused heart (Fig. 2.2.1) is a left-side only model involving cannulation of the pulmonary vein and aorta allowing the heart to undergo loading and

work against physiological preload and afterload conditions. Unlike the traditional Langendorff model, the heart is working (left ventricular ejection). The removal of the heart from the animal allows for the metabolism of the heart to be assessed without the confounding effects of circulating hormones and neurotransmitters. It also allows exogenous substrate delivery to the heart to be controlled. The concentration of circulating metabolites in the nondiabetic *db/+* mouse and the diabetic *db/db* mouse are dramatically different[35]. By perfusion of an isolated heart, the provision of substrates can be controlled and the response of the heart can be assessed using similar substrate profiles.

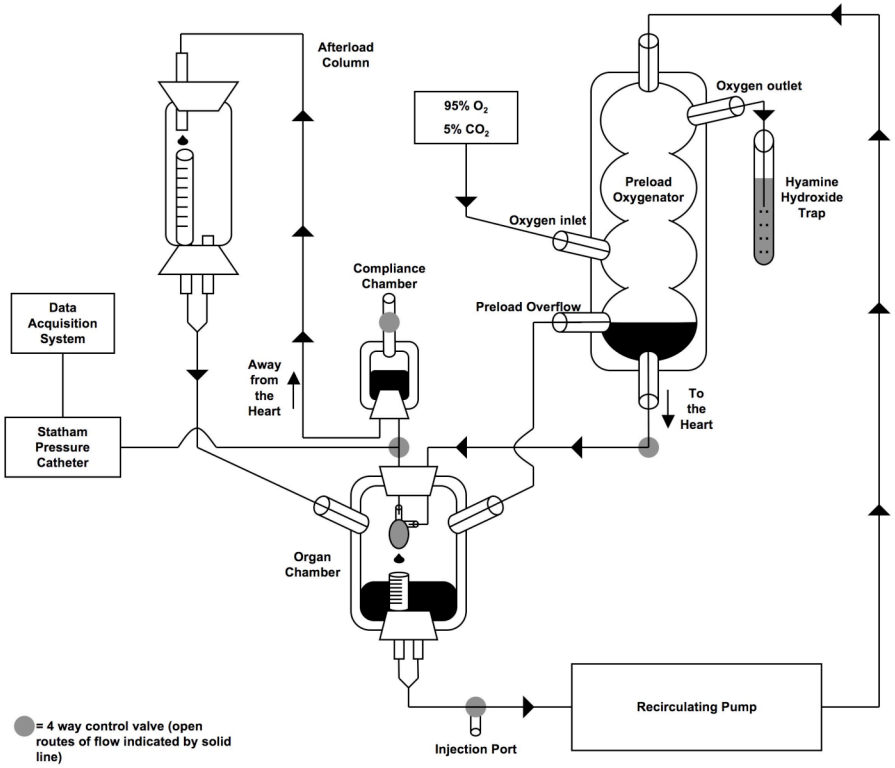

Fig. 2.2.1 – Diagram of the isolated working perfused heart apparatus. Preload and afterload pressures are set by the height of the preload reservoir and afterload column respectively. Perfusate enters the heart through the pulmonary vein and exits the heart through the aorta. A fluid-filled pressure transducer is placed in the aortic line to measure aortic pressures. Aortic and coronary flows are determined by trapping the effluent and measuring the rate of flow via inline graduated cylinders. All gases leaving the system are trapped in hyamine hydroxide. An injection port is used to remove buffer samples at designated time points. 4-way control valves placed at various positions allowed for the control of flow through the system.

The perfusion of isolated hearts has been discussed elsewhere[135, 185-188] but the process from removal of the heart to commencement of experimentation will be

discussed here. Following sacrifice, the heart is removed from the animal and placed in ice-cold Krebs Henseleit bicarbonate (KHB) buffer consisting of (in mM): 118.5 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 0.5 EDTA, 11 mM glucose. Extraneous tissues are removed and the aorta is cannulated via an 18-gauge plastic cannula (0.95 mm ID, 1.30 mm OD). The heart is perfused with KHB warmed to 37^{o}C (~60 mmHg perfusion pressure) in a retrograde fashion to wash the coronary circulation of blood. The time between removal of the heart from the animal and introduction of retrograde flow is minimized (~ 2 min). As the heart is being washed the left atrium is cannulated via the pulmonary vein with a steel 16-gauge cannula that has been bent into an L-shape to permit entry into the pulmonary vein (1.14 mm ID, 1.52 mm OD). The heart is then switched into working mode and the buffer is switched to KHB supplemented with 3% BSA and 0.7 mM palmitate. The preload oxygenator, inflow line to the heart, organ chamber, outflow line to the compliance chamber, and the compliance chamber itself are all water-jacketed. This insures that the heart is maintained at 37^{o}C and all perfusate entering the heart is 37^{o}C. Sutherland et al.[186] have shown that there is not a significant effect on heart rate as long as the heart is maintained at 35-41^{o}C. Following ejection from the ventricle the flow exiting the heart enters the compliance chamber into which 2 mL volume of air has been injected. The compliance chamber is pre-filled with perfusate prior to experimentation and air is injected into the top of the compliance chamber. This introduces some compliance into an otherwise rigid perfusion system. In the absence of a compliance chamber the heart is unable to maintain function. Flow through the system then travels up the afterload column, which has also been pre-filled prior to commencement of the experiment to minimize the chance of bubbles

entering the system. Perfusate drains into organ chamber after ascending the afterload column and is returned to the preload oxygenator via a recirculating pump. Flow through the system is controlled at various points via 4-way stop cock valves. Permanently in-dwelling graduated cylinders allow for the measurement of coronary and aortic flows by temporary occlusion of flow below the graduated cylinders. A second line remains open so that pressure does not build up in the system. A fluid-filled statham pressure transducer is introduced into the aortic line directly above the heart for the measurement of aortic pressures.

The perfusate is continually being gassed with 95% O_2-5% CO_2 via the preload oxygenator. The system can be operated in a closed or open state. When the system is closed all gases exiting the system do so through the oxygen outlet and all CO_2 is trapped in hyamine hydroxide. An injection port is located below the organ chamber to allow for the removal of buffer samples.

2.2.1 Measurement of Cardiac Metabolism in the Ex Vivo Perfused Working Heart

The use of radioactive isotopes allows the accumulation of metabolic end-products to be followed. Through the following Chapters the use of radioisotopes to measure cardiac metabolism has been undertaken. Specifically [U-^{14}C]glucose and [9,10-^{3}H]palmitate have been used to measure glucose and FA oxidation respectively. The perturbation in glycolytic rates is minor in the diabetic *db/db* mouse heart[39, 45, 136] and therefore it has not been measured.

In all isolated working heart perfusions the buffer is a modified KHB supplemented with 3% BSA and 0.7 mM palmitate. Palmitate is prebound to BSA

through the use of Na_2CO_3. 15 mL of H_2O is mixed with 10 mL of 95% ethanol. The designated concentration of free palmitate is added as the H_2O/ethanol mixture is brought to a boil along with excess Na_2CO_3. If 0.4 mmoles of palmitate are being added then a minimum of 0.45 mmoles of Na_2CO_3 is added. The excess Na_2CO_3 ensures adequate binding of palmitate to BSA. If radiolabelled palmitate is necessary it is added at this point. After the ethanol is boiled off, the Na-palmitate mixture is added to rapidly stirring solution of BSA solubilized into KHB without $NaHCO_3$ or glucose. The concentration of BSA in this solution (12%) is increased to further maximize binding. After the binding of palmitate to BSA in this concentrated solution it is dialyzed overnight at 4^{o}C against 10-20 volumes of glucose and $NaHCO_3$ free KHB. The next day the solution is diluted to its intended volume and the correct amount of $NaHCO_3$ and glucose is added to generate an 11 mM glucose and 0.7 mM palmitate solution. The palmitate in the buffer is made up of two pools, 0.4 mM is from added palmitate as part of the Na-palmitate mixture and the remaining 0.25-0.3 mM palmitate is already prebound to the BSA. The BSA used is not FA free. The buffer is then aliquoted and stored at -80^{o}C until the day of perfusions. Radiolabelled glucose is added on the day of perfusion directly to the aliquot as required.

[U-^{14}C]glucose is used to measure the oxidation of glucose through monitoring the accumulation of $^{14}CO_2$. CO_2 is released during the decarboxylation of pyruvate and flux through the TCA cycle (Fig. 1.2.2.1). Two sites of CO_2 accumulation are monitored – release of CO_2 from the perfusion apparatus into 1 M hyamine hydroxide, and diffuse CO_2 within the system in the form of HCO_3^-. Hyamine hydroxide samples are taken at designated time points and counted directly, whereas the diffuse CO_2 needs to be

liberated from solution and trapped. At designated time points 750 mL buffer samples are withdrawn from the perfusion apparatus and injected immediately into metabolic flasks. The flasks consist of a test tube connected by a hollow rubber stopper to a scintillation vial filled with presoaked filter paper. The filter paper in the scintillation vial is presoaked with 375 uL of hyamine hydroxide. The metabolic flasks are briefly shaken and left over-night. The scintillation vials are then removed and Ultima Gold Rx (Sigma Aldrich) scintillation fluid is added. Due to the basic nature of hyamine hydroxide a scintillant specifically resistant to quench is required when samples contain hyamine hydroxide.

[9,10-^{3}H]palmitate is used to measure FA oxidation through the accumulation of $^{3}H_2O$ in the perfusate. $^{3}H_2O$ is the end product of the ETC (Fig. 1.2.1.1). At designated time points buffer samples are removed from the perfusion apparatus and kept at -80^{o}C until analysis. On the day of analysis 0.5 mL of sample is added to 13x100 mm test tubes. The following reagents are added with vortexing between each step: 1.8 mL 1:2 chloroform:methanol (by volume), 0.625 mL chloroform, 0.625 HCl:KCl (0.9:1.1 M). The samples are then centrifuged at 1000g for 10 min and the lower organic phase is removed. The following reagents are added with vortexing between each step: 2 mL 1:1 chloroform:methanol (by volume), 0.9 HCl:KCl (0.9:1.1 M). The samples are again centrifuged at 1000g for 10 min. 0.5 ml of the upper aqueous phase is removed and scintillation fluid is added and the samples counted for the determination of radioactivity. The accumulation of $^{3}H_2O$ is compared to the specific activity of palmitate within the perfusate to determine the utilization of palmitate. This method of isolation of $^{3}H_2O$ has been shown to yield greater than 99% extraction of $^{3}H_2O$ from [9,10-^{3}H]palmitate[89].

CHAPTER 3 – CHRONIC AND ACUTE TREATMENT OF DIABETIC *DB/DB* MICE WITH A PPARγ AGONIST

3.1 Introduction

A link between diabetes-induced alterations in cardiac metabolism and cardiac dysfunction has been proposed. In insulin-deficient models, long term treatment with insulin has proven successful in correcting contractile function[189]. Acute administration of inhibitors of fatty acid oxidation or activators of glucose metabolism have altered the metabolism of isolated hearts from STZ-treated rats[190, 191] and influenced the recovery of hearts undergoing ischemia-reperfusion[169, 171, 192]. The ability of acute manipulation of cardiac metabolism to alter contractile function in isolated diabetic hearts remains controversial[190, 191].

In our laboratory, work by Belke et al.[39] demonstrated that a transgenic approach, GLUT4 over-expression, was successful in normalizing both the metabolism and contractile function of *db/db* hearts. This approach represented a life-long alteration of the diabetic animal and does not speak to the ability to correct or reverse the contractile function of the *db/db* heart through manipulation of the substrate oxidation pattern by a pharmacological intervention.

PPARγ agonists have shown the ability to correct both the hyperglycemia and hyperlipidemia of *db/db* mice and other rodent models of type 2 diabetes[193-195]. The corrections in circulating metabolites are associated with increased insulin sensitivity[195-197]. Unlike insulin-deficient models, insulin treatment is not a first choice for metabolic correction as the *db/db* mouse remains insulin resistant and increases in circulating

insulin increase the mitogenic effects of insulin leading to negative cardiovascular side effects[198, 199].

PPARγ is an important mediator of adipocyte differentiation from preadipocytes to mature adipocytes[200, 201,202]. PPARγ is selectively expressed in adipose tissue, however expression is evident in other tissues, most notably the spleen and digestive tract[203, 204]. PPARγ is also expressed at high levels in macrophages and plays a role in cholesterol efflux and atherogenesis[205, 206]. The expression of PPARγ is induced early in the adipocyte differentiation process and the absence or knockdown of PPARγ expression inhibits differentiation[200, 202, 207]. The adipose tissue selective expression of PPARγ has led to a "trapping hypothesis" to explain the insulin sensitizing effects of PPARγ ligands. The administration of PPARγ ligands leads to a reduction in plasma lipids through increased expression of LPL and putative fatty acid transport proteins in adipose tissue[208, 209]. Adipose tissue lipolysis is also reduced.[201] FA are argued to be causative in the development of insulin resistance. PPARγ ligands also reduce TNF-α mRNA and plasma concentrations, another insulin resistance mediator[210]. The ability of PPARγ agonists to induce insulin sensitization in vivo is proportional to their ability to bind to the PPARγ receptor[194].

The first class of PPARγ agonists to be extensively studied were the thiazolidinediones. Of this class, troglitazone was the first to enter clinical usage for the treatment of diabetes. It is not the most potent of the thiazolidinediones and has been shown to have a number of PPARγ independent actions[211, 212]. In some instances it has shown the ability to block calcium channels and lead to increases in liver weight that is

not evident when more selective ligands are used[211]. The realization of side-effects related to the liver led to the search for more selective ligands that retain the anti-diabetic efficacy without the effects on liver function. Once such ligand is COOH, which is a nonthiazolidinedione PPARγ agonist.

PPARγ expression is extremely low if not absent in the heart[204203] and therefore administration of a PPARγ agonist represents a strategy whereby the effect of substrate delivery to the heart can be chronically altered[128]. Thus, potential changes in cardiac metabolism and performance will be the result of indirect actions rather than direct manipulation of cardiac gene expression. Therefore, the effect of the chronic and acute manipulation of substrate supply on the cardiac metabolism and contractile function of the type 2 diabetic *db/db* mouse heart was examined.

3.2 Methods

3.2.1 Three week treatment with COOH

Male C57BL/KsOlaHsd-lepr^{db}/lepr^{db} *db/db* mice were purchased from Harlan Laboratories (Denmark). Mice arrived at 8-9 wks of age and were allowed to acclimatize for 1 wk. Animals were given *ad libitum* access to food and water and housed under a 12 h light/dark cycle. All experiments were approved by the University of Tromsø Animal Welfare Committee.

Treatment Protocol

Following the 1 week acclimatization period, mice were randomly divided into 2 groups. *Db/db* mice received PPARγ ligand COOH via oral gavage. COOH (2-(2-(4-

Phenoxy-2-propylphenoxy)ethyl)indole-5-acetic acid) is an indole-acetic acid derived PPARγ agonist (Fig. 3.1) from Merck: (transactivation EC50s on PPARγ/δ/α=180/>3000/>3000 nM). The drug was suspended in 0.05% methylcellulose to attain a daily dosage of 30 mg/kg body weight. Untreated mice received the same volume of methylcellulose as vehicle control. Animals were treated for 3 wks (to 12 weeks of age) at which time they were sacrificed for *ex vivo* heart perfusions. The purpose of this pilot study was to establish the effectiveness of orally-administered COOH as an anti-diabetic treatment for diabetic *db/db* mice.

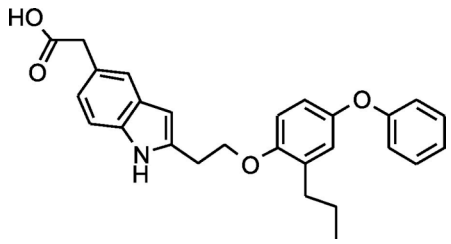

Fig. 3.2.1.1- Structure of 2-(2-(4-phenoxy-2-propylphenoxy)ethyl)indole-5-acetic acid (COOH), a nonthiazolidinedione peroxisome proliferator-activated receptor-γ (PPARγ)-selective ligand.

Isolated Heart Perfusions

Mice received 100 U of heparin via ip injection 20 min prior to ip injection of 10 mg sodium pentobarbital. Blood was collected from the chest cavity following removal

of the heart and blood glucose concentrations were measured using a commercially available kit. Hearts were mounted on a perfusion apparatus and placed in working mode by the protocol of Belke et al.[39, 135]. Briefly, this involved cannulation of the left atrium to control preload and cannulation of the aorta to set afterload in working (LV ejecting) mode. Hearts were perfused with a modified Krebs Henseleit bicarbonate (KHB) buffer consisting of (in mM): 118.5 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 0.5 EDTA, containing 0.4 mM [9,10-^{3}H]palmitate (specific activity = 2.2 x 10^9 dpm/mmol) bound to 3% BSA and 11 mM [U-^{14}C]glucose (specific activity = 9.1 x 10^6 dpm/mmol). The total fatty acid concentration in the perfusate was approximately 0.7 mM due to the fact that the BSA used was not essentially fatty acid free (endogenous fatty acid content was 0.25-0.3 mM). The perfusate was continually gassed with 95% O_2 and 5% CO_2.

Hearts were perfused for 40 min (preload pressure of 15 mmHg; afterload pressure of 50 mmHg) with functional measurements and the withdrawal of perfusate samples (2.5 mL) for metabolic analysis occurring every 10 min. Coronary and aortic flows were determined through the use of graduated cylinders placed within the working heart apparatus; heart pressures were measured via a pressure transducer placed in the aortic afterload line and analyzed using a pressure acquisition system develop in house at the University of Tromsø. The pressure signal was used for calculation of heart rate (HR) and peak systolic pressure (PSP). Perfused hearts were allowed to beat spontaneously. The sum of aortic and coronary flows was used to determine cardiac output.

The oxidation of glucose and palmitate was measured simultaneously in each heart during working heart perfusions. Perfusates contained [U-^{14}C]glucose and [9,10-

^{3}H]palmitate, as described by Belke et al.[39, 135]. Trapping of $^{14}CO_2$ in the perfusate was used to determine the rate of glucose oxidation; the release of $^{3}H_2O$ into the perfusate was used to determine the rate of palmitate oxidation. Steady-state rates of metabolism were determined by averaging the results from perfusate samples removed at the 4 time points (0-10, 10-20, 20-30, 30-40 min) for each heart perfusion. At the end of the perfusion protocol, the atria were removed and hearts were frozen and stored at –80^{o}C for the determination of ventricular dry weight.

3.2.2. Chronic 6 wk Treatment with COOH

Treatment Protocol

Male C57BL/KsOlaHsd-lepr^{db}/lepr^{db} and their lean non-diabetic heterozygote littermates (*db/+*) were purchased from Harlan Laboratories (Indianapolis, Indiana); mice arrived at 5-6 wks of age.

Following the 1 week acclimatization period, *db/+* and *db/db* mice were randomly divided into 4 groups (Fig. 3.2.2.1). A group of *db/+* and *db/db* mice each received powdered chow with and without the PPARγ ligand COOH as a food admixture. The drug was formulated into powdered chow (Prolab RMH 2500/5P14; PMI International Inc., Brentwood, MO) to attain a daily dosage of 30 mg/kg body weight/day. Mice were given ad libitum access to food and water and housed under a 12 h light/dark cycle. All experiments were approved by the University of Calgary Animal Welfare Committee. Food intake was monitored daily and body weight was monitored weekly. Content of drug in the powdered chow was adjusted as needed to maintain the desired dosing, and

ranged from 0.2-0.3 mg/g chow. Untreated *db/+* and *db/db* groups received regular mouse chow. Animals were treated for 6 wks (to 12 wks of age). *In vivo* assessment of cardiac function was undertaken at the end of the 6 wk treatment protocol (12 wks, Fig. 3.2.2.1). At the end of the feeding protocol, mice were sacrificed and blood glucose and lipid concentrations were determined using commercially available kits. Results were compared to those obtained following oral gavage of COOH in methylcellulose. (Methods; 3.2.1). Hearts were removed for *ex vivo* perfusions.

In summary (Fig. 3.2.2.1), the treatment period was commenced at 6 weeks of age, when *db/db* mice exhibit normal function, and concluded at 12 weeks when altered metabolism and contractile dysfunction is evident in hearts from untreated *db/db* mice[38, 40].

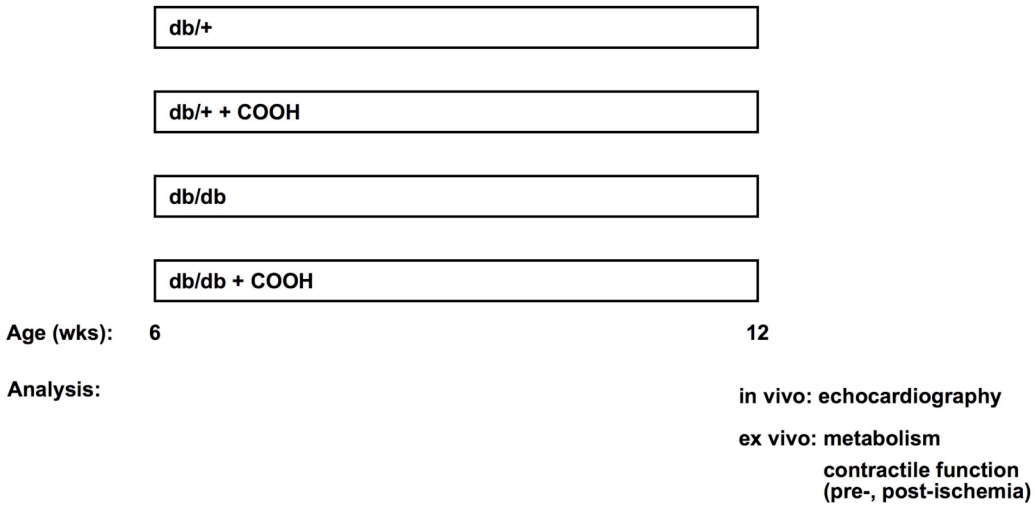

Fig. 3.2.2.1 – Treatment protocol. Mice were divivded into 4 groups and received powered chow or powdered chow supplemented with COOH (30 mg/kg body weight/day) as indicated. After 6 wks of treatment (at 12 wks of age) mice underwent echocardiography and were sacrificed for isolated working heart perfusions.

Assessment of Cardiac Function by Echocardiography

Echocardiograms (M-mode measurements) to assess systolic function were obtained from conscious mice, as described by Semeniuk et al.[38]. Septal wall thickness (SWT), posterior wall thickness (PWT), left ventricular (LV) internal dimensions in systole (LVIDs) and diastole (LVIDd) were determined from LV M-mode scans, using a Hewlett Packard Sonus 5500 ultrasound machine with a 15 MHz linear transducer. Values for heart rate (HR) were obtained from Doppler measurements of LV outflow tract velocities.

Fractional shortening (FS) and the velocity of circumferential fibre shortening (Vcf) were calculated as indices of systolic function: FS(%) = [LVIDd-LVIDs)/LVIDd] x 100; Vcf = FS/ET. LV mass was calculated from the following equation (30): LV mass (mg) = $[(LVIDd + SWT + PWT)^3 + LVIDd^3]$ x 1.055, where 1.055 is the density of the myocardium.

Isolated Heart Perfusions

Mice received 100 U of heparin via ip injection 20 min prior to ip injection of 10 mg sodium pentobarbital. The tail prick method was then used to draw blood for blood glucose determination via the ONETOUCH Ultra glucose meter (LIFESCAN). A subset of mice were sacrificed for measurement of lipid parameters without receiving heparin, as heparinization artificially alters lipids in the blood (decreased TG; increased FA) through cleavage of LPL into the circulation. Hearts were mounted on a perfusion apparatus and placed in working mode by the protocol of Belke et al.[39, 135], as described in detail in Chapter 2.

Hearts were perfused for 40 min (preload pressure of 15 mmHg; afterload pressure of 50 mmHg) with functional measurements and the withdrawal of perfusate samples (2.5 mL) for metabolic analysis occurring every 10 min. Coronary and aortic flows were determined through the use of graduated cylinders placed within the working heart apparatus; heart pressures were measured via a pressure transducer placed in the aortic afterload line[135] using CV Works (University of Calgary). The sum of aortic and coronary flows was used to determine cardiac output. The pressure signal was used for calculation of heart rate (HR). Perfused hearts were allowed to beat spontaneously.

The oxidation of glucose and palmitate was measured simultaneously in each heart during working heart perfusions. Perfusates contained [U-^{14}C]glucose and [9,10-^{3}H]palmitate, as described by Belke et al.[39, 135] and in Chapter 2. At the end of the perfusion protocol, the atria were removed and hearts were frozen and stored at –80^{o}C for the determination of ventricular dry weight, which was used to normalize metabolic and flow data to correct for small variations in heart size.

Ischemia-Reperfusion Protocol

To assess the ability of nondiabetic and diabetic hearts to recover following a brief period of ischemia, isolated hearts underwent an ischemia-reperfusion protocol. Following the initial 40 min aerobic perfusion period, perfusate flow to the heart and away from the heart was shut-off. The hearts remained in a period of global no-flow ischemia for 12.5 min but were kept warm by keeping the perfusion rig sealed. At the end of 12.5 min hearts were reperfused for 10 min via retrograde Langendorff perfusion

(50 mmHg) and then were switched back into working mode (15 mmHg preload, 50 mmHg afterload). Functional recovery was followed over 40 min of reperfusion.

Measurement of Myocardial TG

A subset of mice were sacrificed without undergoing heparinization and the hearts removed without undergoing perfusion. Hearts were flash-frozen and stored at -80^{o}C. The determination of TG was adapted from the protocols of Frayn and Maycock[213] and Gauthier et al.[214]. Frozen hearts were powdered in a pre-cooled mortar and pestle on dry ice. ~15 mg of powdered heart was added to 3 mL of ice-cold 2:1 chloroform:methanol (v/v) and homogenized using a hand-held tissue homogenizer. The samples were left overnight at 4^{o}C. 1.5 mL of 4 mM $MgCl_2$ was added and the samples were vortexed and centrifuged at 1000 g at 4^{o}C for 1 hr. The upper phase was aspirated and 1.5 mL of the lower phase was removed and evaporated to dryness. The dried lipids were resuspended in 0.25 mL of 5 N ethanolic-KOH and incubated at 60^{o}C for 1 hr. 0.5 mL of 0.15 M $MgSO_4$ was added to the samples and the samples were vortexed. Following 1 hr of centrifugation at 1000 g and 4^{o}C the upper phase was removed for the determination of free glycerol concentration fluorometrically, based on the protocol of Laurell and Tibbling[215]. The glycerol assay was linear (R^2=0.97) over a concentration range of 0-400 uM glycerol.

3.2.3 Acute Treatment with COOH

PPARγ treatment of *db/db* mice leads to a restoration of blood glucose levels within 3 days[193, 194]. Therefore, it was of interest to determine if an acute 1 wk

treatment period with COOH could alter the metabolic phenotype of *db/db* hearts, in comparison to the chronic 6 wk protocol.

Treatment Protocol

At 10-11 weeks of age, mice were randomly divided into 2 groups. Diabetic mice each received powdered chow with and without the PPARγ ligand COOH as a food admixture as described above (3.2.2) Briefly, the drug was formulated into powdered chow (Prolab RMH 2500/5P14; PMI International Inc., Brentwood, MO) to attain a daily dosage of 30 mg/kg body weight. Food intake was monitored daily and body weight was monitored weekly. Animals were treated for 7 d (to 11-12 weeks of age), at which time they were killed for *ex vivo* heart perfusions. The treatment period was commenced at 10-11 weeks of age, when mice exhibit impaired contractile function and altered cardiac metabolism and are overtly diabetic.

Isolated Heart Perfusions

Hearts from untreated and COOH-treated *db/db* mice were perfused as described previously (section 3.2.2), for measurements of contractile function and FA oxidation.

Statistics Analysis

Data are expressed as means±standard error. Differences in means within groups were determined by students t-test, differences in means across groups were determined by ANOVA with the Student-Neuman-Keuls test for pairwise comparison. Means were considered to be statistically significant when the p value was less than 0.05.

3.3. Results

3.3.1 Three week treatment with COOH

Table 3.3.1.1. Characteristics of control *db/db* and COOH treated *db/db* mice.

Mouse Type	Body Weight, g	Body Weight Increase, %	Ventricle Dry Weight, mg	Blood Glucose, mM	Blood FFA, mM
db/db	38.8±1.4	1.5±1.0	26.5±0.6	42.9±1.6	1.68±0.16
db/db + COOH	51.2±0.8*	14.8±0.6*	29.9±0.5*	22.0±1.3*	0.86±0.07*

Data are presented as means±SE for control db/db (n=8) and COOH treated db/db (n=12); serum samples where collected from fed mice following heparinization. FFA, free fatty acids; *Significantly different than db/db control mice ($p<0.05$).

At the end of the 3 wk treatment period, *db/db* mice treated with COOH were significantly less hyperglycemic and hyperlipidemic than untreated *db/db* mice (Table 3.3.1.1.). Blood FFA levels are artificially elevated by the use of heparin as an anticoagulant in the advance of perfusions. COOH treated *db/db* mice gained more body weight and weighed significantly more than their untreated counterparts. There was a significant increase in the ventricular dry weight in the hearts of COOH treated *db/db* mice.

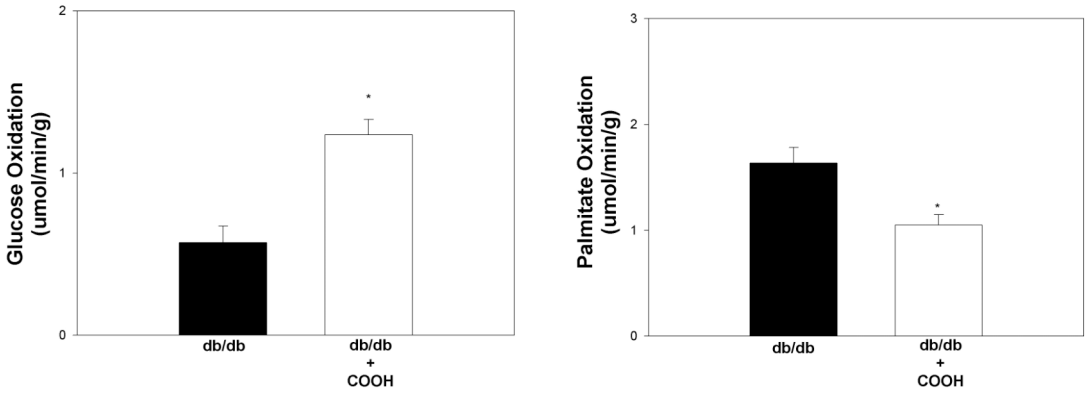

Fig. 3.3.1.1 – Substrate metabolism of isolated hearts from *db/db* (n=8) mice or *db/db* (n=10) mice treated with COOH (30 mg/kg body weight/day) via oral gavage. Hearts were perfused with 11 mM ^{14}C-glucose and 0.7 mM ^{3}H-palmitate. Average rates of substrate oxidation over the 40 min perfusion period are shown. *$p<0.05$ vs. *db/db*.

The substrate utilization pattern of isolated hearts from *db/db* mice and COOH treated db/db mice can be seen in Fig. 3.3.1.1. The oxidation of glucose was increased and the oxidation of palmitate was decreased in hearts from COOH treated *db/db* mice relative to the cardiac metabolism of untreated *db/db* mice. Despite these changes in cardiac metabolism there was not a significant difference in the contractile function of isolated hearts between treated and untreated mice (Table 3.3.1.2)

Table 3.3.1.2. Ventricular function in perfused hearts from control *(db/db*) and COOH treated (*db/db*) mice

	db/db (n=8)	db/db + COOH (n=10)
PSP, mmHg	63±1	63±1
HR, bpm	295±7	265±12
Cardiac Output, ml/min	10.2±0.6	9.9±0.5
Coronary flow, ml/min	2.5±0.2	2.9±0.1
Aortic flow, ml/min	7.8±0.6	7.0±0.5

Data are presented as means±SE for control *db/db* and COOH treated *db/db*. PSP, peak systolic pressure, was determined in the aortic line.

3.3.2 6 wk treatment with COOH

At 9 wks of age, the *db/db* mouse is already overtly diabetic[35]. Beginning at 6 wks of age the *db/db* mouse transitions from a pre-diabetic stage to an overtly diabetic stage. Aasum et al.[40] have suggested that in male mice there is an increase in cardiac fatty acid oxidation at this point but not a reduction in cardiac glucose oxidation. Work I have completed has also corroborated these findings. Other attempts to use PPARγ ligands to correct cardiac function in rat models of type 2 diabetes have proven successful when the treatment protocol began at the transition point from a pre-diabetic stage to overt diabetes, but clearly in advance of full-fledged diabetes[51, 52]. Based on these observations, a second study involving COOH treatment was undertaken. A more chronic treatment period of 6 wks beginning at 6 wks of age was undertaken to further examine the reversibility of contractile dysfunction in *db/db* hearts and the relation to cardiac metabolism. Additional measures of cardiac function, ischemia-reperfusion and *in vivo* echocardiography, were assessed to provide further indices of cardiac function.

The formulation of COOH in powdered chow was an effective drug delivery system for COOH. At the end of the pilot 2-wk treatment period, *db/db* mice treated with COOH were no longer hyperglycemic.

Table 3.3.2.1. Characteristics of *db/+* and *db/db* mice fed powdered chow +/- COOH (30 mg/kg body weight) for two weeks.

	db/+		db/db	
	treated	untreated	treated	untreated
Plasma glucose (mM)	6.9±0.2	7.9±0.1	8.3±0.6*	28.5±1.8
Body weight (g)	29.5±0.3	28.5±0.0	50.3±1.0*	45.3±0.7

*Significantly different than db/db untreated ($p<0.05$). Data are presented as means±SE.

Based on the successful 2-wk pilot experiment, a more chronic 6 wk treatment protocol was performed (Fig. 3.2.2.1). All *db/db* mice consumed significantly more food than nondiabetic mice (Fig. 3.3.2.1). Over the first 10 d of treatment, no differences in food intake were evident between treated and untreated *db/db* mice. However after 10 d of treatment *db/db* mice fed chow supplemented with COOH consumed significantly less food than those mice receiving control chow (Fig. 3.3.2.1).

At the end of the 6 wk protocol, *db/db* mice treated with COOH had significantly reduced blood glucose concentrations, compared to *db/db* mice fed control chow (Table 3.3.2.2). Untreated *db/db* mice did not display significant increases in plasma TG and FA, however *db/db* mice treated with COOH had significantly lower plasma FA than chow-fed *db/db* mice and *db/+* mice fed chow. Similarly, COOH-treated *db/+* mice exhibited significant reductions in plasma TG and FA. COOH treatment of *db/+* mice

did not lead to significant change in body weight, but body weight was reduced significantly in COOH-treated *db/db* mice (Table 3.3.2.2). Heart weight was increased in both *db/+* and *db/db* mice treated with COOH (Fig. 3.3.2.2). The concentration of myocardial TG in unperfused hearts of *db/db* mice was increased in comparison to *db/+* mice (Fig. 3.3.2.3), while *db/db* mice treated with COOH for 6 wks showed no elevation in myocardial TG levels.

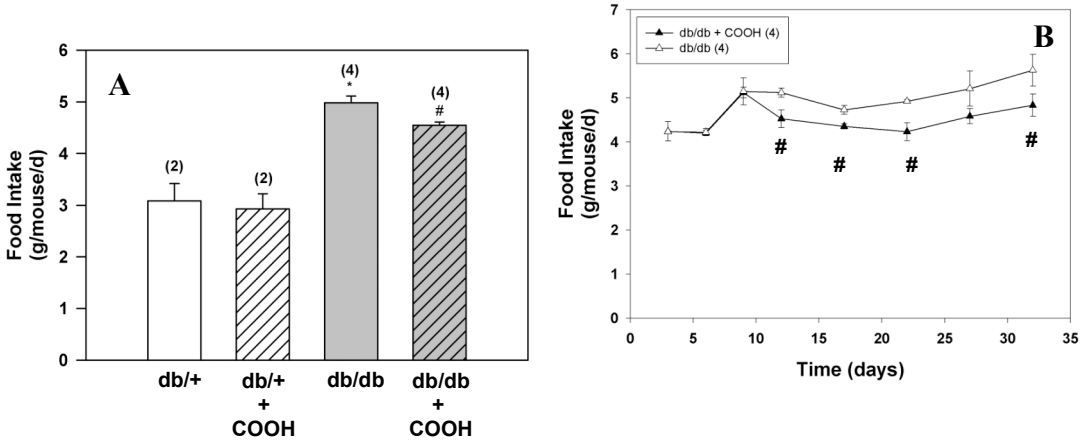

Fig. 3.3.2.1 – Food intake by mice fed powdered chow and powdered chow supplemented with COOH, over 6 weeks. Data are presented at mean±S.E. n value represents number of mouse cages. A: Average food intake over the 6 wk period; B: time course of food intake over the 6 wk period. *p<0.05 vs. *db/+*, #p<0.05 vs. *db/db*.

Table 3.3.2.2 – Characteristics of mice fed powdered chow or powdered chow supplemented with COOH for 6 weeks.

	Experimental Group			
	db/+	*db/+* + COOH	*db/db*	*db/db* + COOH
Body Weight (g)	27.5 ± 1.2(20)	30.2 ± 0.6(13)	51.2 ± 0.9(20)*	48.2 ± 1.0(24)*#
Plasma Glucose (mM)	10.0 ± 0.4(19)	9.0 ± 0.3(13)	27.2 ± 1.1(20)*	10.1 ± 0.6(24)#
Plasma TG (mM)	0.56 ± 0.12(4)	0.15 ± 0.00(2)	0.46 ± 0.13(4)	0.40 ± 0.04(4)
Plasma FFA (mM)	0.50 ± 0.06(4)	0.17 ± 0.00(2)*	0.39 ± 0.04(4)	0.25 ± 0.03(4)*#

Data are fed mice at 12 wks of age, mean ± SE. TG, triglycerides; FFA, free fatty acids. *Significantly different from *db/+*, #significantly different from *db/db*.

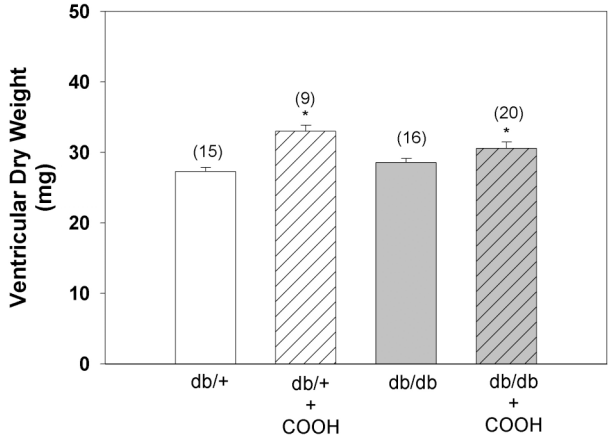

Fig. 3.3.2.2 – Ventricular dry weight of hearts from *db/+* and *db/db* mice. Mice received powdered chow or powdered chow supplemented with COOH over 6 wks. Data are presented at mean±S.E. n value in brackets. *$p<0.05$ vs. corresponding control group.

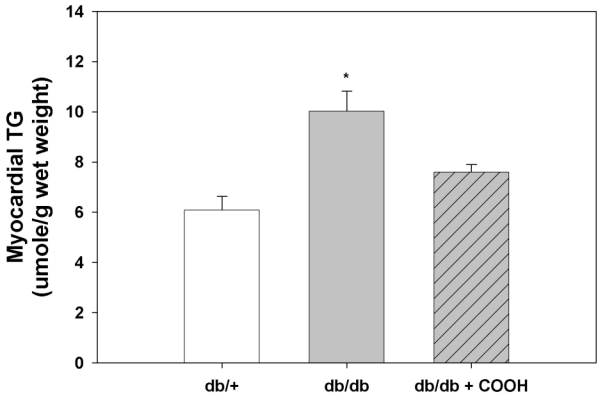

Fig. 3.3.2.3 – Myocardial TG levels from the unperfused hearts of *db/+*, *db/db, and COOH* treated *db/db* mice. Mice received powdered chow or powdered chow supplemented with COOH over 6 wks. Data are presented at mean±S.E. n=4 for all groups. *p<0.05 vs. *db/+*.

3.3.3 Assessment of Cardiac Function *In vivo* by Echocardiography

Table 3.3.3.1 – Echocardiographic assessment of *db/+* and *db/db* hearts fed powdered chow or powdered chow supplemented with COOH (30 mg/kg body weight/day).

	Experimental Group			
	db/+ (12)	db/+ + COOH (6)	db/db (12)	db/db + COOH (12)
HR (beats/min)	707 ± 10	704 ± 10	654 ± 6*	624 ± 16*
SWT (mm)	0.7 ± 0.02	0.7 ± 0.02	0.7 ± 0.02	0.7 ± 0.02
PWT (mm)	0.7 ± 0.02	0.7 ± 0.02	0.7 ± 0.02	0.7 ± 0.02
LVIDs (mm)	1.5 ± 0.05	1.6 ± 0.1	2.0 ± 0.1*	2.0 ± 0.1*
LVIDd (mm)	3.6 ± 0.06	3.7 ± 0.12	3.7 ± 0.05	3.8 ± 0.05*
LV mass (mg)	84.1 ± 2.7	79 ± 5	86.9 ± 2.9	94.8 ± 3.8
FS (%)	58.5 ± 1.4	58.0 ± 1.8	45.5 ± 2.3*	46.9 ± 2.5*
Vcf (circs/sec)	15.5 ± 0.7	14.8± 1.0	11.0 ± 0.5*	9.9 ± 0.6*
ET (sec)	0.04 ± 0.001	0.04 ± 0.003	0.04 ± .002	0.05 ± 0.003*

HR, heart rate; SWT, septal wall thickness; PWT, posterior wall thicknes; LVIDs, systolic left ventricular internal dimension; LVIDd, diastolic left ventricular internal dimension; FS, fractional shortening; Vcf, velocity of circumferential fibre shortening; ET, ejection time. Results are mean ± SE (number of mice in parentheses). *, p<0.05 relative to *db/+* control mice.

All diabetic mice showed impaired systolic function regardless of COOH treatment. A significant reduction in HR, FS, and Vcf was observed. Unlike what was observed during isolated perfusions, HR was not significantly lower in *db/db* mice receiving COOH versus *db/db* mice receiving normal chow. A suggestion of hypertrophy was evident in *db/db* mice receiving powdered chow (increased ventricular dimensions during both diastole and systole) however this was not shown in the ventricular dry weight of hearts at the end of *ex vivo* perfusion. COOH treatment of *db/+* mice was without effect on *in vivo* cardiac function.

3.3.4 Heart Perfusions

Following the 6 wk treatment protocol, cardiac metabolism and contractile function was assessed via isolated working heart perfusions. As can be seen in Fig. 3.3.4.1 (A and B), linear rates of cardiac metabolism were attained over 40 min of isolated perfusion time for both glucose and fatty acid oxidation. *Db/db* mice receiving powdered chow displayed an increase in fatty acid oxidation and a reduction in glucose oxidation compared to *db/+* chow-fed mice, consistent with previous metabolic studies with perfused *db/db* hearts[39, 40]. The addition of COOH to the powdered chow of *db/db* mice led to a reduction in the rate of fatty acid oxidation and an increase in the glucose oxidation of isolated hearts, such that metabolic rates for hearts from COOH treated *db/db* mice were no longer significantly different in their substrate oxidation pattern compared to control *db/+* mice(Fig. 3.3.4.1). Treatment of control *db/+* mice with COOH had no effect on either glucose oxidation or FA oxidation.

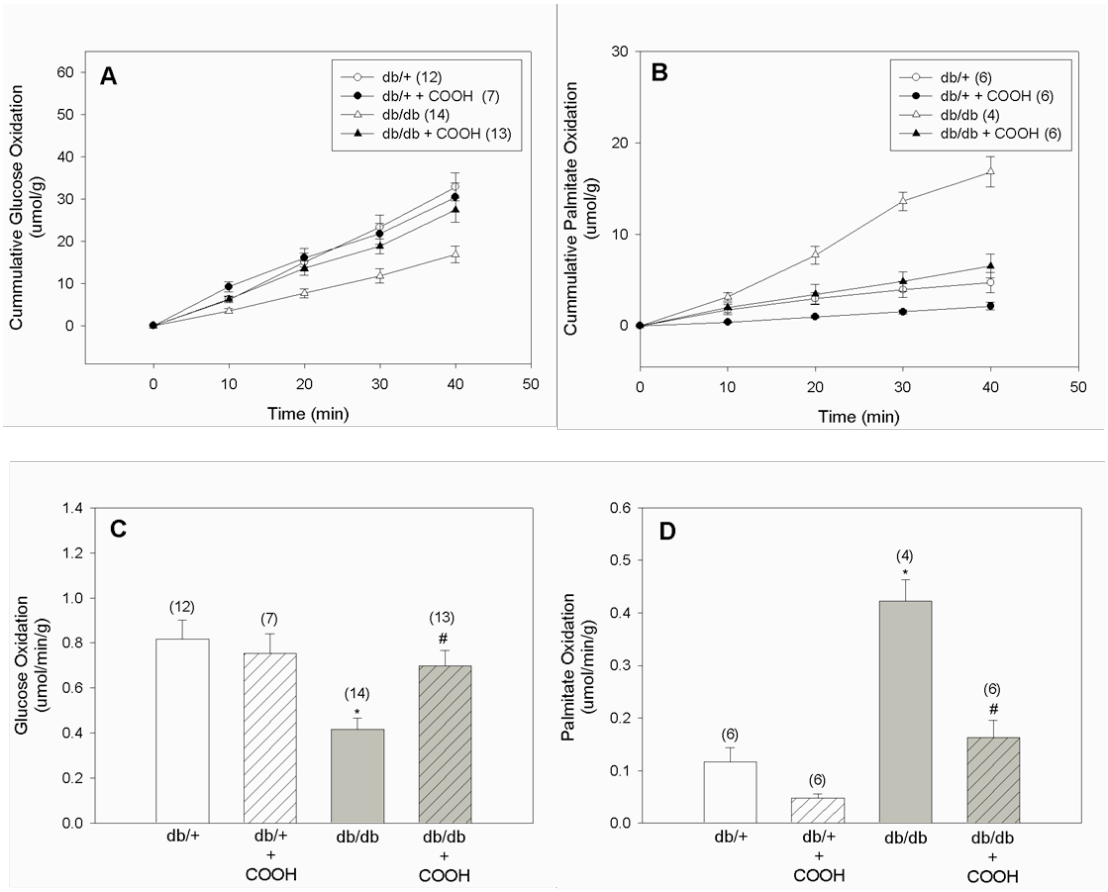

Fig. 3.3.4.1 – Substrate metabolism of isolated hearts from *db/+* and *db/db* mice fed chow or chow supplemented with 30 mg/kg body weight/day COOH. Hearts were perfused with 11 mM ^{14}C-glucose and 0.7 mM 3H-palmitate. Cumulative rates of substrate oxidation (A and B); average rates of substrate oxidation over the 40 min perfusion period (C and D). *p<0.05 vs. *db/+*, #p<0.05 vs. *db/db*.

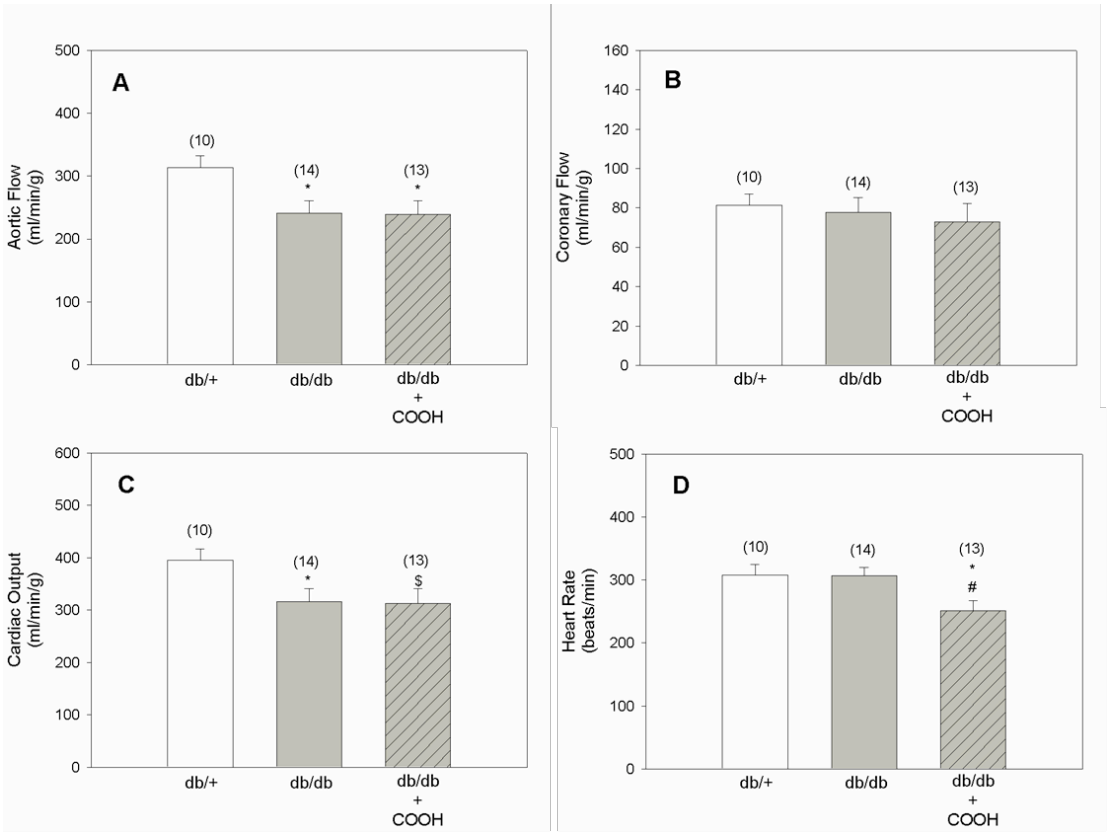

Fig. 3.3.4.2 – Cardiac function of *ex vivo* perfused hearts from *db/+* mice fed control chow and *db/db* mice fed control chow or chow supplemented with COOH (30 mg/kg body weight/day). Hearts were perfused for 40 min with 11 mM 14C-glucose and 0.7 mM 3H-palmitate for metabolic measurements. *p<0.05 vs. *db/+*, $p<0.052 vs. *db/+*, #p<0.05 vs. *db/db*.

Perfused hearts from untreated *db/db* mice exhibited features of contractile dysfunction, with decreased aortic flows and cardiac output (Fig. 3.3.4.2, A and C), consistent with previous studies[39, 40, 136]. No differences in coronary flow were documented between any experimental groups. Despite the corrections in substrate metabolism by isolated hearts from COOH-treated *db/db* mice, cardiac function of *ex vivo* perfused hearts was not improved in *db/db* hearts after treatment with COOH versus

either chow-fed *db/+* and *db/db* mice(Fig. 3.3.4.2). Cardiac output in untreated *db/db* hearts relative to *db/+* control hearts was significantly reduced at a p value of less than 0.05 while hearts from *db/db* mice receiving COOH for 6 wks displayed a reduction in cardiac output that was significant at a p value of 0.052 (Fig.3.3.4.2, C). The significant reduction in aortic flow in COOH-treated *db/db* hearts clearly indicates that the contractile function of hearts from COOH treated *db/db* mice remain compromised. Interestingly *db/db* mice treated for 6 wks with COOH exhibited a lower spontaneous heart rate than all other mice(Fig.3.3.4.2, D). COOH treatment for 6 wks did not significantly alter the cardiac function of *db/+* mice (HR, 322 ± 24 beats/min; Cardiac output, 366 ± 26 ml/min/g).

Recovery of contractile performance after ischemia and reperfusion may provide a more sensitive index of cardiac function than that from aerobic perfusions. Following 12.5 min of global no-flow ischemia, hearts from *db/db* mice demonstrated a significant reduction in functional recovery compared to control hearts (Fig. 3.3.4.3). The recovery of aortic flow was significantly impaired in untreated *db/db* hearts. Treatment of *db/db* mice for 6 wks with COOH led to a partial reduction in ischemia-reperfusion sensitivity, ie. percent recovery of aortic flow was no longer significantly less than what occurred with hearts from *db/+* mice. However, there was also not a significant difference between *db/db* hearts from COOH treated mice and *db/db* mice receiving normal chow (Fig. 3.3.4.3).

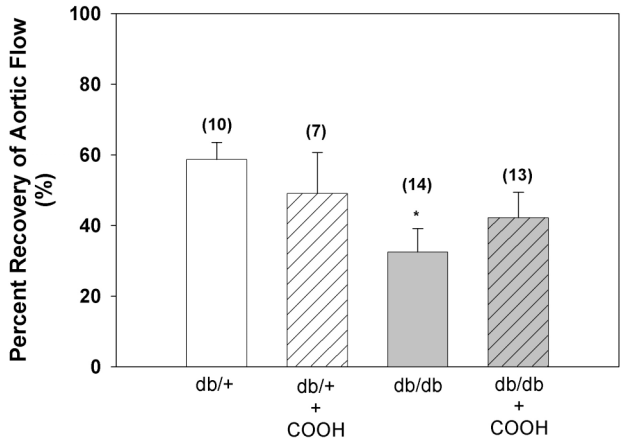

Fig. 3.3.4.3 – Percent recovery of aortic flow of isolated hearts from *db/+* and *db/db* mice fed powdered chow with or without COOH (30 mg/kg body weight/day). Hearts under went 12.5 min of global no-flow ischemia. Percent recovery aortic flow was measured over a 40 min reperfusion, calculated relative to pre-ischemic function. *$p<0.05$ vs. *db/+*.

3.3.5 Acute 1 wk Treatment with COOH

Oral administration of COOH to *db/db* mice for 3 or 6 weeks was able to improve diabetic status and restore cardiac metabolic rates to normal. Therefore, the response to an acute 1 wk treatment protocol was investigated. Treatment of *db/db* mice for 1 wk with COOH (30 mg/kg body weight/day) was sufficient to result in a significant reduction in blood glucose to normal values compared to control *db/+* mice (Fig. 3.3.5.1). No differences in body weight were evident between *db/db* mice regardless of COOH treatment; all *db/db* mice had significantly higher body weights compared to *db/+* mice. The 1 wk treatment period with COOH was associated with a reduction in the rate of FA oxidation by isolated *db/db* hearts.

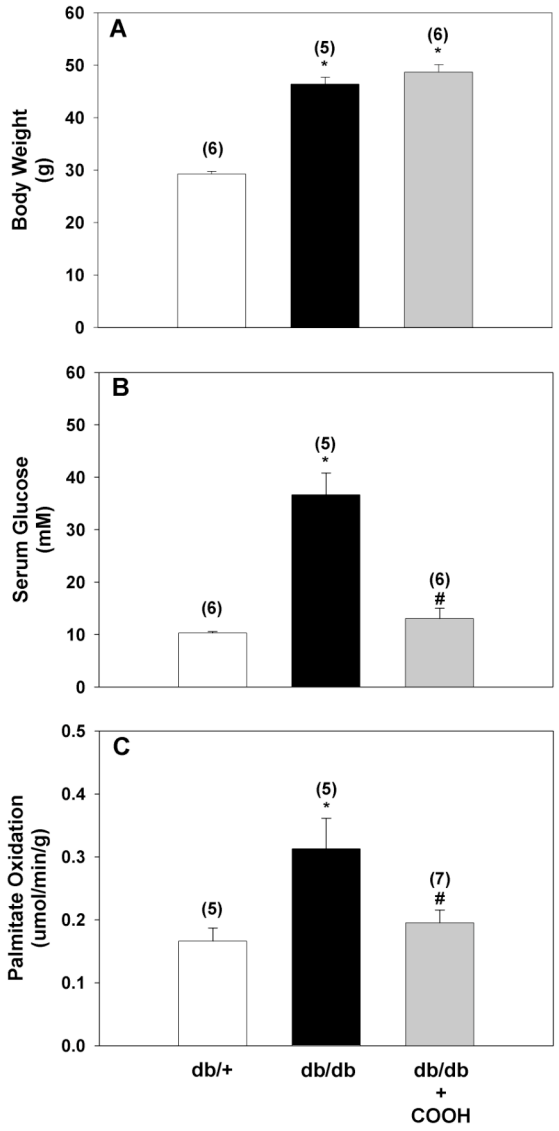

Fig. 3.3.5.1 – Characteristics control, untreated *db/db* and COOH-treated (1wk) *db/db* mice, and rates of palmitate oxidation for perfused hearts from these groups. Data are presented as mean±S.E. n values indicated. *p<0.05 relative to *db/+*; #, p<0.05 relative to *db/db*.

3.4 Discussion

Treatment of *db/db* mice with a non-thiazolidinedione COOH as briefly as 1 wk or as chronically as 6 wks led to a reduction in their diabetic status and a correction in the substrate utilization patterns of isolated hearts. Despite the changes in both glucose and fatty acid oxidation, there was no improvement in cardiac function measured either *in vivo* via echocardiography or *ex vivo* with isolated perfused hearts.

3.4.1 Corrections in the diabetic status of *db/db* mice

The reduction in the hyperglycemia in *db/db* mice by COOH was similar to other reports on the anti-diabetic capabilities of PPARγ agonists[193, 194]. COOH also led to reductions in lipid parameters. Surprisingly, the *db/db* mice fed powdered chow for 6 wks were not hyperlipidemic relative to chow-fed *db/+* mice (Table 3.3.2.2). The chronic oral administration of COOH led to a reduction plasma FA levels in the circulation of both *db/db* and *db/+* mice when provided as a food ad mixture for 6 wks. The absence of hyperlipidemia in normal chow fed *db/db* mice is an unexpected result. A recent study by Oakes et al.[216] showed no difference in plasma TG levels between *db/db* and *db/+* mice, however increased plasma FA in *db/db* mice was evident. Buchanan et al.[45] have shown that the dysregulation in cardiac metabolism is not dependent on plasma lipid levels, as there is disruption in the advance of plasma metabolite perturbations. Nonetheless, COOH decreased circulating lipids.

Treatment of *db/db* mice with COOH led to an increase in body weight compared to untreated *db/db* mice when administered over periods of less than or equal to 3 wks. However, chronic treatment with COOH for 6 wks resulted in a reduction in body weight

in treated vs. untreated *db/db* mice. This is somewhat unexpected as PPARγ agonists are believed to induce adipogenesis and should therefore lead to an increase in body weight in treated *db/db* mice[200, 201,202]. The decrease in body weight is most likely the result of a reduction in food intake initially evident following 10 d of treatment with COOH. The reduction in food intake is likely associated with the corrections in diabetic status of the *db/db* mice as a result of COOH treatment. Over the 6 wk treatment period the chronic decrease in food intake in the COOH treated *db/db* mice was able to result in a modest decrease in body weight. The reduction in body weight does not appear to be an effect of drug formulation into the chow as *db/+* mice treated with COOH did not demonstrate either a decrease in food intake or body weight during 6 wks of COOH treatment.

3.4.2 Cardiac Metabolism

Treatment with COOH for 6 wks significantly altered the substrate utilization profile of *db/db* hearts (Fig. 3.3.4.1). Both the oxidation of glucose and FA was altered such that hearts from COOH treated mice resembled untreated *db/+* hearts in their substrate utilization profile. This result is similar to results attained by Aasum et al.[136] and Golfman et al.[52] using other PPAR ligands. PPARγ is not expressed at significant levels in the hearts of rodents[128]. The addition of PPARγ agonists to isolated cardiomyocytes did not influence the rate of FA oxidation, unlike PPARα agonists that led to increased oxidation of FA[128]. Therefore the effects of COOH treatment on cardiac metabolism are likely to be secondary to the corrections in the diabetic status of *db/db* mice. Specifically, this most likely involved an action on adipose tissue to increase the

uptake of FA. FA have been proposed to be involved in the development of insulin resistance at the tissue level. PPARγ treatment of Zucker obese rats[208, 209] was associated with an increased deposition of FA into adipose tissue pools and away from nonadipose tissue. As noted above, there was not a significant elevation in circulating lipids found in *db/db* mice fed normal chow. Although this was a somewhat confusing result, it does not necessarily affect the mechanism whereby PPARγ treatment altered cardiac metabolism. The administration of PPARγ ligands to normal rats at high doses, 100 times the therapeutic dose, also changed the substrate utilization pattern of normal hearts that was dependent on a reduction in circulating FA[217]. The dosages of COOH used in the present study are at or near the therapeutic range. Administration of COOH to *db/+* mice for 6 wks had no significant affect on cardiac metabolism, suggesting that the alterations in cardiac metabolism in COOH-treated *db/db* hearts were related to corrections in the diabetic status of *db/db* mice. When isolated *db/+* and *db/db* hearts are perfused with the same concentration of FA within the perfusate, the *db/db* mouse heart shows increases in both the oxidation and esterification of FA[135, 137], suggesting that even at equal concentrations of FA the *db/db* heart experiences an oversupply of FA at the cellular level. Therefore, in the diabetic rodent, the circulating plasma FA concentrations are not necessarily indicative of the influence of exogenous FA on the heart. Similarly, *db/db* mice at 12 wks of age displayed increases in myocardial TG levels. This elevation was corrected by chronic treatment with COOH.

3.4.3 Cardiac Function of *In Vivo* and *Ex Vivo* Perfused Hearts

The cardiac function of both *in vivo* and *ex vivo* perfused *db/db* hearts remained depressed after COOH administration chronically for 6 wks. Thus, treatment of either *db/+* or *db/db* mice with COOH failed to improve cardiac function either *in vivo* or *ex vivo* perfused hearts. A number of mechanisms have been proposed to link the development of a diabetic cardiomyopathy with the metabolic profile of the diabetic heart[61, 62, 141]. However, in this study any relationship between cardiac metabolism and contractile function in the *db/db* heart could not be demonstrated. The initial pilot 3 wk study was undertaken at a stage when *db/db* mice are overtly diabetic and signs of cardiac dysfunction are already apparent[35, 40, 45]. One could argue that this point of intervention is too late in the progression of the diabetic condition to produce any improvement in contractile function despite normalization of the diabetic phenotype. However, beginning treatment of *db/db* mice at 6 wks of age, at which point the mice are just transitioning into the diabetic state, was also unsuccessful. The acute 1 wk treatment of *db/db* mice also showed that normalization of cardiac metabolism occurs rapidly following the commencement of treatment. It is anticipated that after 1 wk during the chronic treatment protocols, cardiac metabolism has been corrected and therefore the contractile phenotype should not worsen and presumably improve. It must be noted that following 16 d of treatment of obese Zucker rats with rosiglitazone, the most potent of the thiazolidinediones, although myocardial TG levels were decreased the heart maintained a similar ability to hearts from untreated Zucker rats to take up FA[208].

In diabetic rats, treatment with PPARγ agonists has proven successful in altering both the metabolism and substrate utilization of diabetic hearts. Zhou et al.[51] and

Golfman et al.[52] treated ZDF rats with different PPARγ agonists. Zhou et al. [51] treated ZDF rats with troglitazone, a thiazolidinedione PPARγ agonist, for 14 wks, substantially longer than was undertaken here. Troglitazone-treated ZDF rats showed improvements in cardiac function measured *in vivo* via echocardiography. Golfman et al.[52] treated ZDF rats with a non-thiazolidinedione for only 1 wk and showed improved cardiac metabolism and function of isolated hearts. The hyperglycemia of the type 2 diabetic ZDF rat model is more modest than that observed with *db/db* mice[35, 39, 49, 71], however they are substantially more hyperlipidemic[35, 39, 51]. There is also some controversy concerning the substrate utilization of the ZDF rat. Golfman et al.[52] only presented a reduction in glucose oxidation and no change in oleate oxidation, while recent work using stable isotopes and more physiologically relevant provision of substrates has clearly shown an increased reliance on FA in ZDF perfused hearts[139]. Conversely, Aasum et al[136]. showed no improvement in cardiac function in *db/db* hearts following chronic treatment with a PPARα agonist, despite similar corrections in cardiac metabolism that was observed here.

Reduced cardiac efficiency is evident in the *db/db* mouse and this has been argued to be related to its metabolic profile (enhanced FA utilization), and a key player in the observed contractile dysfunction[176]. The reduction in FA oxidation and the increase in glucose oxidation should increase the efficiency of COOH-treated *db/db* hearts and therefore this mechanism should not remain an issue for the *db/db* heart. Indeed, the observation that recovery of contractile function after ischemia and reperfusion was improved (Fig. 3.3.4.4) is consistent with an improvement in cardiac efficiency. Hearts from *db/db* mice treated with COOH are also no longer "lipotoxic" as cardiac TG was no

longer elevated following COOH treatment. This is not a direct measure of the concentration and localization of the reactive intermediates of FA metabolism, but rather an indication of the lipid loading of the heart. Therefore reactive FA intermediates could still be elevated in the *db/db* heart.

The cardiac metabolism of glucose was also corrected in COOH treated *db/db* mice. Although this chapter only presents an increase in the oxidation of glucose in isolated hearts from COOH treated *db/db* mice, the insulin-stimulated uptake of glucose was also increased in freshly isolated cardiomyocytes from treated *db/db* mice[138]. Similarly to the rapid effect of COOH treatment on FA oxidation, insulin-stimulated glucose uptake is also improved after 1 wk of treatment with COOH in *db/db* cardiomyocytes[117]. Combined with the expected reductions in interstitial glucose concentrations, the pathways contributing to glucotoxicity, one would argue, have also been corrected.

A key feature of the work completed here was the combination of methods to measure both *in vivo* and *ex vivo* contractile function. The isolated working heart system is a difficult technique to master and does require a certain level of skill. As well there are significant external constraints that can interfere with heart function. It is therefore significant that functional measurements were also made using echocardiography. These two independent measurements of cardiac function produced similar results, raising questions over the ability of metabolic treatments to affect cardiac function in diabetic hearts

CHAPTER 4 – EFFECT OF SILDENAFIL ON CARDIAC METABOLISM AND FUNCTION OF DIABETIC *DB/DB* HEARTS

4.1 Introduction

Cyclic guanosine 3',5'-monophosphate (cGMP) is an intracellular second messenger that regulates cellular function through several signaling mechanisms[218, 219]. Generation of cGMP occurs either as a result of stimulation of receptor guanylyl cyclases by natriuretic peptides[218, 220] or by the action of NO-activated soluble guanylyl cyclases[218, 221]. Many of the actions attributable to cGMP formation are mediated by protein kinase G (PKG). In the vasculature PKG phosphorylates targets resulting in vasodilation while in the heart the activation of PKG prevents pathological hypertrophy induced by aortic banding and acts as a brake mechanism following β-adrenergic stimulation[222]. Catabolism of cGMP is accomplished by the actions of specific phosphodiesterases (PDE). Sildenafil elevates tissue levels of cGMP through inhibition of PDE5A.

There has been a great deal of interest in the cardiac consequences of sildenafil. β-adrenergic stimulation of the heart leads to increases in both cAMP and cGMP levels[222, 223]. While cAMP leads to the activation of protein kinase A and an increase in contractility, cGMP leads to the activation of PKG and a reduction in contractility. The expression of PDE5A is low in the heart and therefore under basal conditions, inhibition of PDE5A does not lead to an increase in cGMP or alterations in contractile function[224]. Nevertheless in heart failure there is a down-regulation of PDE5A expression and a blunted response to β-adrenergic stimulation, which is argued to be the result of increased

cGMP[222]. Conversely sildenafil has been shown to be beneficial for the hypertrophic heart which has been remodeled due to underlying pathology such as increased afterload[224]. Chronic treatment of mice with sildenafil prevents or corrects pathological cardiac hypertrophy in the presence of hypertrophic stimuli. Cardiomyocyte-specific overexpression of the natriuretic peptide receptor-associated guanylyl cyclase has also been demonstrated to be anti-hypertrophic[225], while the cardiomyoycte-specific knockdown of guanylyl cyclase expression leads to pressure-independent cardiac hypertrophy[226].

The response of the diabetic heart to sildenafil administration warrants investigation. Diabetes is associated with erectile dysfunction[227-229], therefore the response of the diabetic heart to chronic sildenafil supplementation is an important question to be answered. Other phosphodiesterase inhibitors have been proposed as anti-diabetic agents, however these have been cAMP-specific phosphodiesterase inhibitors chosen for the ability of cAMP to potentiate insulin release by the pancreas[230-235]. Evidence is accumulating that cGMP may influence the metabolism of glucose by skeletal muscle[236]. In skeletal muscle, manipulation of nitric oxide signaling alters the metabolism of glucose. The effects of cGMP on cardiac metabolism are controversial and have not been extensively studied in the diabetic heart.

This study attempts to determine the chronic effects of sildenafil administration on the function of the diabetic *db/db* mouse heart. Particular focus will be paid to the cardiac function both *in vivo* and *ex vivo*. Sildenafil's effect on the diabetic status and cardiac metabolism will also be considered. Although Chapter 3 was not able to demonstrate any linkage between cardiac metabolism and cardiac function, a greater

understanding of cardiac metabolism in diabetes is required. Sildenafil treatment significantly corrects the cardiac function of hypertrophic hearts[224], another disorder which has been suggested to have a metabolic connection[169, 237]. Hypertrohpic hearts show an increased ratio of glycolysis/glucose oxidation[169, 237], similar to *db/db* hearts and therefore the addition of sildenafil can be postulated to also impact cardiac metabolism in diabetic hearts. As the current evidence suggests sildenafil's effects are most likely to be in glucose metabolism, the oxidation of glucose alone will be the primary focus in terms of cardiac metabolism.

4.2 Methods

4.2.1 Experimental animals

Male mice were used in all experiments. C57Bl/KsJ-lepr^{db}/ lepr^{db} *db/db* mice and their lean, nondiabetic heterozygote littermates (*db/*+) were purchased from Jackson Laboratories (Bar Harbour, ME). Mice arrived at 5-6 wk of age and were allowed to acclimatize for 1 wk before experimentation. Mice were maintained on a 12/12 h light/dark cycle and had ad libitum access to food and water. After 1 wk of acclimatization, mice were divided into three groups (Fig. 4.2.1.1): *db/*+ receiving powdered chow, *db/db* mice receiving powdered chow, and *db/db* mice receiving powdered chow supplemented with Sildenafil (500 mg/ kg body weight/day). Mice were maintained on this diet regimen for 6 weeks until 12 weeks of age, at which time animals were sacrificed for heart perfusion studies. Mice used for studies of the acute effects of sildenafil were studied at 12 weeks of age.

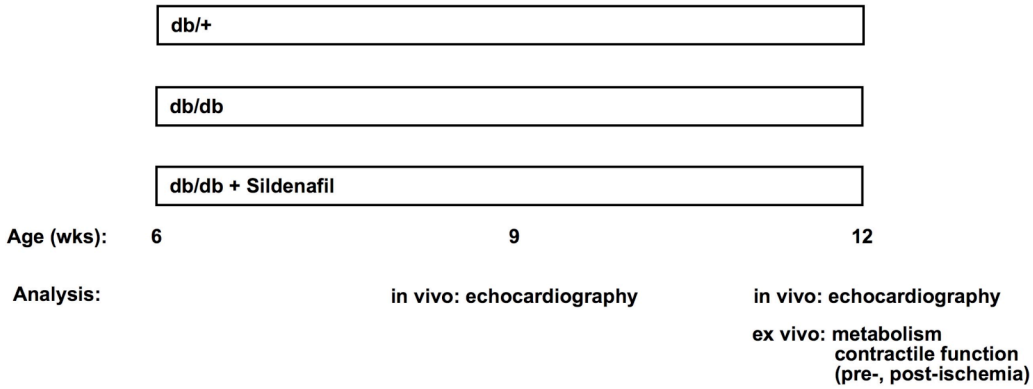

Fig. 4.2.1.1 – Treatment Protocol. Mice were divided into three groups: *db/+* and *db/db* mice receiving powdered chow, or *db/db* mice receiving powdered chow containing sildenafil (500 mg/kg body weight/day). After 3 and 6 wks of treatment mice underwent echocardiographic assessment. At the end of the 6 wks of treatment mice were sacrificed and underwent isolated working heart perfusions.

4.2.2 Echocardiography

At 9 and 12 weeks of age, the three groups of mice underwent echocardiographic assessment of cardiac function, as described previously[38] and as outlined in Chapter 3 (Section 3.2.2).

4.2.3 Heart Perfusions

At 12 wk of age, mice were sacrificed and hearts underwent *ex vivo* perfusion, essentially as described previously[135, 138], and as described in detail in Chapter 2. Mice received 100

U of heparin via intraperitoneal injection 20 min prior to sacrifice. Mice were sacrificed by a lethal dose of sodium pentobarbital (10 mg) via intraperitoneal injection. Blood was collected from the chest cavity and blood glucose concentrations were determined using a glucose assay kit (Sigma).

Hearts were perfused with a modified Krebs-Henseleit buffer consisting of (in mM): 118.5 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 0.5 EDTA, 11 mM [U-^{14}C]glucose, and 0.7 mM palmitate bound to 3% BSA. Two perfusion protocols were used. To test the chronic effects of sildenafil administration, hearts from *db*/+, *db*/*db* mice and sildenafil-treated *db*/*db* mice underwent a 60 min aerobic perfusion period, followed by a brief period of global no-flow ischemia (12.5 min), 10 min of retrograde Langendorff reperfusion, and a 60 min working heart reperfusion period. In a second set of perfusions, *db*/*db* hearts were perfused with vehicle or sildenafil in the perfusate (50 nM) to test the acute effect of sildenafil on heart function and cardiac metabolism. Hearts were perfused for 100 min; perfusate conditions were the same in both perfusion groups.

Statistics Analysis

Data are expressed as means±standard error. Differences in means were determined by ANOVA with the Student-Neuman-Keuls test for pairwise comparison. Means were considered to be statistically significant when the p value was less than 0.05.

4.3 Results

4.3.1 Animal Characteristics

Diabetic *db/db* mice weighed significantly more than their nondiabetic littermates and were markedly hyperglycemic (Table 4.3.1.1). Treatment for 6 wk with sildenafil did not alter the body weight of *db/db* mice, however blood glucose concentrations were reduced

from 41.1±1.9(13) to 33.2±2.7(13) mM. Sildenafil-treated *db/db* mice remained hyperglycemic versus *db/+* mice 11.8±1.2(10) mM, however.

Table 4.3.1.1 - Characteristics of 12 week old *db/+* and *db/db* mice or 12 week old *db/db* mice fed sildenafil (500 mg/kg body weight daily) for 6 weeks.

	db/+	***db/db***	***db/db* + Sildenafil**
Body weight (g)	27.5±0.7(10)	46.0±1.0(12)*	47.3±1.1(11)*
Blood Glucose (mM)	11.8±1.2(10)	41.1±1.9(13)*	33.3±2.7(11)*#
Ventricular wet weight (mg)	136±2(12)	127±3(11)	124±4(9)
Ventricular dry weight (mg)	26.5±0.5(12)	25.0±0.6(11)	25.3±0.8(9)

Data are from fed mice and are presented as means±SE(n). *p<0.05 vs. *db/+* control mice. #p<0.05 vs. *db/db* untreated mice.

4.3.2 Echocardiography

At 9 and 12 wk of age, cardiac function was assessed *in vivo* through echocardiographic assessment of cardiac function (Tables 4.3.2.1 and 4.3.2.2). All mice were conscious during assessment. Diabetes led to a reduction in systolic function at both 9 and 12 wk of age, which was not altered by sildenafil treatment. This was evident by the reduction in heart rate (HR) and a decrease in Vcf. LV ejection time was significantly prolonged in all diabetic animals at 9 wk of age. At 12 wk of age, the difference in LV ejection time between diabetic and nondiabetic mice did not reach significance. Treatment with sildenafil did not affect LVmass, however there was a significant reduction in the dimension of the septal wall in sildenafil-treated *db/db* mice at 9 wk of age. Mice at 9 wk

had been receiving sildenafil daily for 3 wks. Following 6 wk of treatment with sildenafil (12 wk old), there was a significant reduction in both the septal and posterior wall dimensions versus both untreated *db/db* and *db/+* mice.

Table 4.3.2.1 – Echocardiographic assessment of 9 week old *db/+*, *db/db* or 9 week old *db/db* mice fed sildenafil (500 mg/kg body weight daily) for 3 weeks.
Mice were analyzed under fed conditions and were conscious.

	***db/+*(13)**	***db/db*(14)**	***db/db* + Sildenafil(13)**
SWT (mm)	0.74±0.01	0.71±0.01	0.69±0.01*
PWT (mm)	0.74±0.01	0.71±0.01	0.71±0.02
LVIDd (mm)	3.37±0.07	3.35±0.03	3.33±0.05
LVIDs (mm)	1.43±0.04	1.42±0.03	1.43±0.03
LVmass	79.9±6.1	78.2±3.2	72.7±2.5
FS (%)	57.6±0.6	57.6±0.6	57.1±0.8
Vcf (circ/s)	13.1±0.4	11.8±0.5*	11.3±0.4*
LVET (s)	0.043±0.001	0.050±0.002*	0.051±0.002*
HR (beats/min)	715±13	637±23*	643±7*
EF (%)	0.917±0.003	0.917±0.004	0.912±0.005

Data is presented as mean±SE. *$p<0.05$ vs. *db/+* control mice. Septal wall thickness(SWT), posterior wall thickness(PWT), left ventricular internal dimension diastole(LVIDd), left ventricular internal dimension systole(LVIDs), left ventricular mass(Lvmass), fractional shortening(FS), circumferential shortening(Vcf), left ventricular ejection time(LVET), heart rate(HR), ejection fraction(EF).

Table 4.3.2.2 – Echocardiographic assessment of 12 week old *db/+* and *db/db* mice or 12 week old *db/db* mice fed sildenafil (500 mg/kg body weight daily) for 6 weeks. Mice were analyzed under fed conditions and were conscious.

	***db/+*(13)**	***db/db*(14)**	***db/db* + Sildenafil(13)**
SWT (mm)	0.78±0.01	0.75±0.01	0.71±0.01*
LVPWT (mm)	0.78±0.01	0.75±0.01	0.71±0.01*
LVIDd (mm)	3.56±0.06	3.37±0.04	3.47±0.07
LVIDs (mm)	1.55±0.04	1.57±0.06	1.55±0.06
LVmass	89.4±6.8	84.1±3.2	83.1±4.2
FS (%)	56.4±0.8	53.4±1.5	55.4±1.3
Vcf (circs/s)	12.5±0.3	10.8±0.2*	11.2±0.4*
LVET (s)	0.045±0.003	0.050±0.001	0.050±0.002
HR (beats/min)	704±6	645±11*	655±11*
EF (%)	0.910±0.005	0.886±0.010	0.901±0.009

Data is presented as mean±SE. *$p<0.05$ vs. *db/+* control mice. Septal wall thickness(SWT), posterior wall thickness(PWT), left ventricular internal dimension diastole(LVIDd), left ventricular internal dimension systole(LVIDs), left ventricular mass(Lvmass), fractional shortening(FS), circumferential shortening(Vcf), left ventricular ejection time(LVET), heart rate(HR), ejection fraction(EF).

4.3.3 Isolated Heart Perfusions

At the end of the 6 wk treatment period animals were sacrificed and their hearts removed for *ex vivo* working heart perfusions. Cardiac contractile function was monitored and the ability to oxidize glucose was determined. Both aortic flow and cardiac output of hearts isolated from diabetic animals were reduced when compared to hearts isolated from nondiabetic control mice(Figure 4.3.3.1), confirming previous observations of contractile dysfunction in perfused *db/db* hearts(Chapter 3). Treatment of *db/db* mice for 6 wks with sildenafil did not influence contractile function of isolated hearts from diabetic mice. On the other hand, sildenafil treatment did increase the ability of isolated *db/db* hearts to oxidize glucose(Figure 4.3.3.2). Glucose oxidation of isolated hearts from *db/db* mice was reduced, as was demonstrated in Chapter 3. Diabetic mice

treated with sildenafil for 6 wks displayed a similar rate of glucose oxidation to nondiabetic mice.

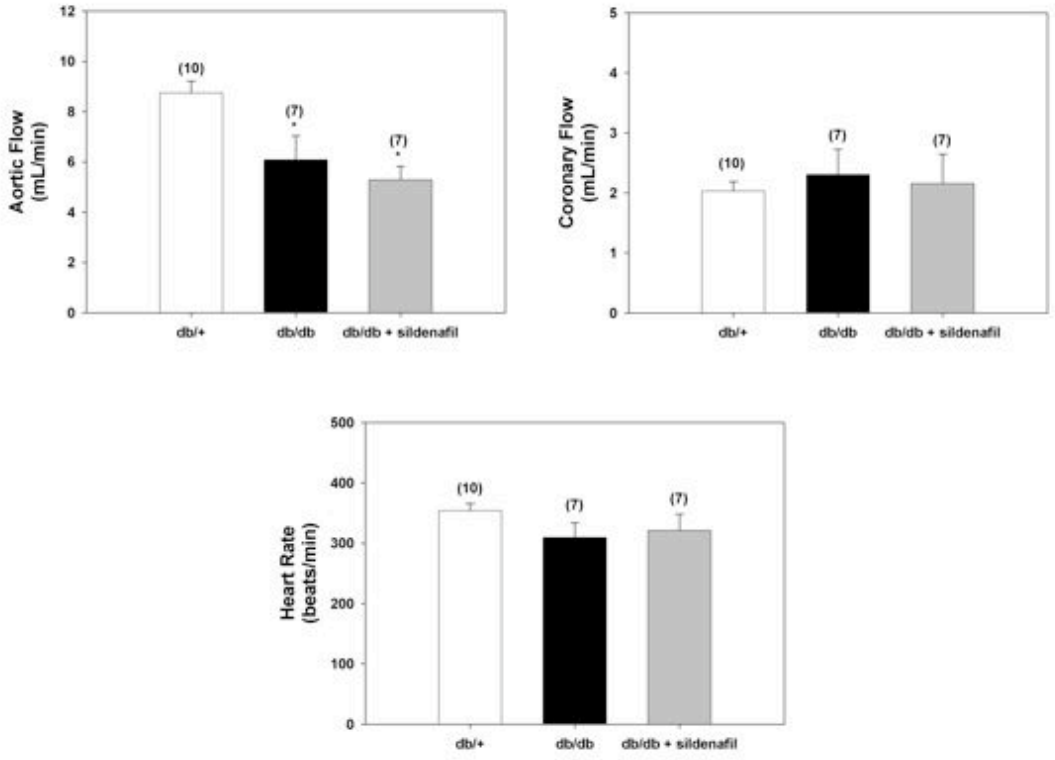

Fig. 4.3.3.1 – Cardiac function of isolated hearts from *db/+* and *db/db* mice and *db/db* mice fed sildenafil for 6 wks (500 mg/kg body weight/day). Hearts were perfused for 60 min with 11 mM [U-^{14}C]glucose and 0.7 mM palmitate. Data are presented as mean±S.E.(n). *$p<0.05$ vs. *db/+* hearts.

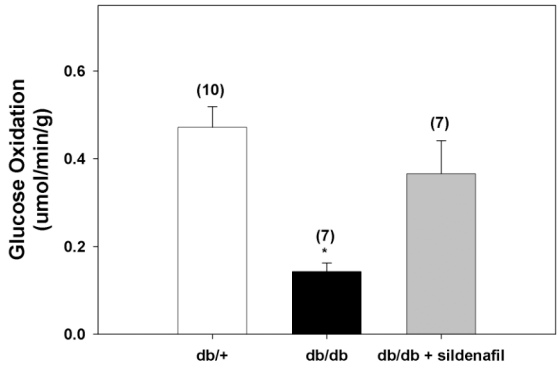

Fig. 4.3.3.2 – Exogenous glucose oxidation of isolated-perfused hearts from *db/+*, *db/db* and *db/db* mice treated with sildenafil for 6 wks (500 mg/kg body weight/day). Hearts were perfused for 60 min with 11 mM [U-^{14}C]-glucose and 0.7 mM palmitate. *p<0.05 vs. *db/+*.

Following the initial perfusion for 60 min, hearts were subjected to a brief period of global ischemia followed by 60 min of reperfusion. Rates of glucose oxidation during reperfusion were similar to those found during perfusion prior to the bout of ischemia (Fig. 4.3.3.3). Despite the normalization of glucose oxidation in isolated hearts from sildenafil-treated *db/db* mice (pre-ischemic and post-ischemic), there was no improvement in contractile recovery following ischemia (Fig. 4.3.3.3).

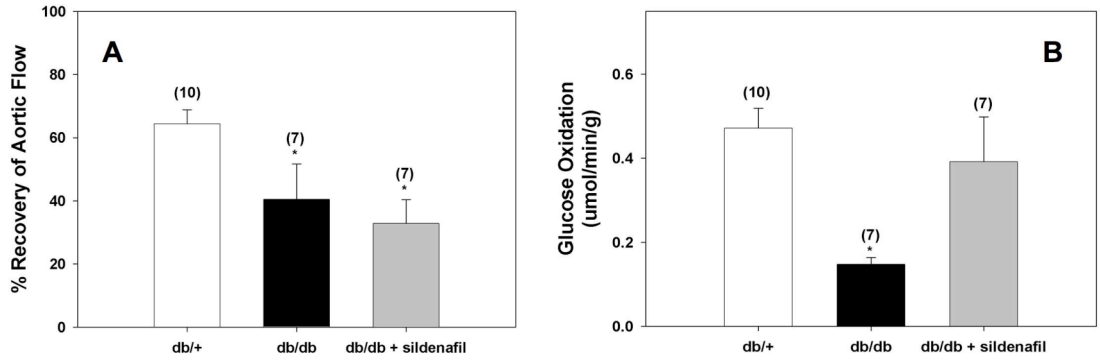

Fig. 4.3.3.3 – Functional and metabolic response to ischemia-reperfusion by isolated hearts from *db/+*, *db/db* and *db/db* mice treated with sildenafil for 6 wks. Percent recovery of aortic flow vs. preischemic contractile function (A), and average rates of glucose oxidation (B), over the reperfusion period. *p<0.05 vs. *db/+*.

To assess any direct affects that sildenafil may have on hearts from *db/db* mice, hearts were perfused with or without sildenafil (50 nM) in the perfusate for 100 min and glucose oxidation and cardiac function were measured. Despite the long duration of perfusion, function was stable over time (Fig. 4.3.3.4). There was no effect of sildenafil on aortic flow(Fig. 4.3.3.4, A) or heart rate (Fig 4.3.3.4, C). The addition of sildenafil to the perfusate also did not lead to a significant increase in the coronary flow (Fig. 4.3.3.4, B), although there was a trend towards an increase as the duration of perfusion increased. The oxidation of exogenous glucose was not different between hearts perfused with or without sildenafil (Fig. 4.3.3.5).

At the end of the perfusion period, hearts were frozen and the ventricles were weighed to determine both ventricular wet and dry weights. Despite the echocardiographic findings indicating some alterations in ventricular wall thickness,

ventricular wet and dry weights of the hearts from sildenafil-treated *db/db* mice were not different from *db/db* mice or *db/+* mice fed regular chow(Table 4.3.1.1).

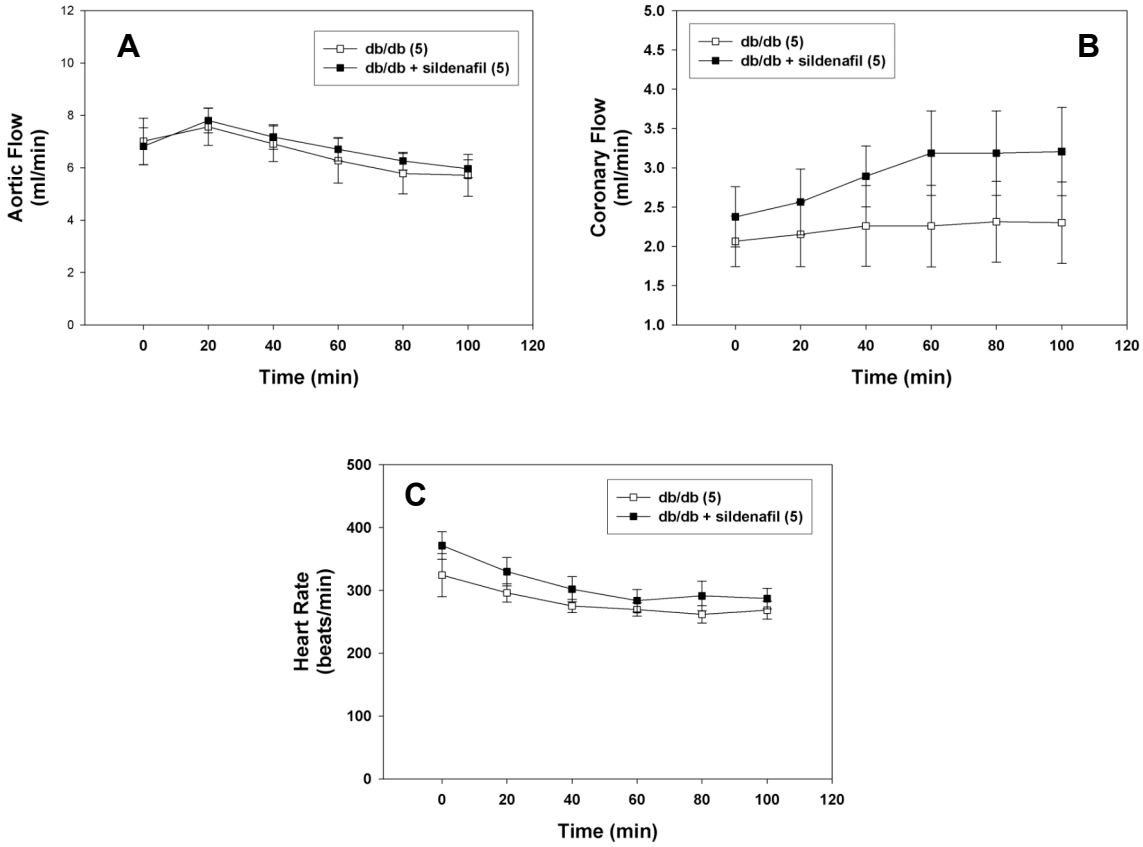

Fig. 4.3.3.4 – Contractile function of isolated hearts from *db/db* mice with or without sildenafil included in the perfusate at a concentration of 50 nM. Hearts were perfused for 100 min. Hearts were perfused with the same KHB as used previously.

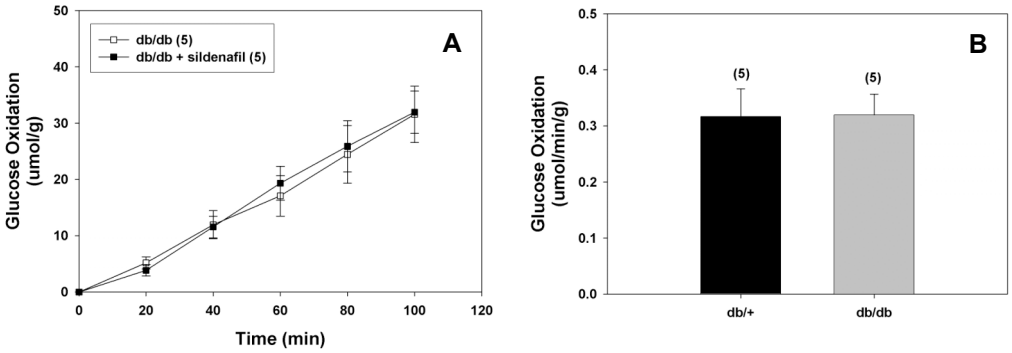

Fig. 4.3.3.5 – Cumulative (A) and average rates (B) of glucose oxidation over the 100 min perfusion of isolated hearts from *db/db* mice, perfused with or without sildenafil. Hearts were perfused with 11 mM ^{14}C-glucose and 0.7 mM palmitate.

4.4 Discussion

Treatment of type 2 diabetic *db/db* mice with sildenafil for 6 wks led to a reduction in fed blood glucose concentrations and increased the ability of isolated *db/db* hearts to oxidize exogenous glucose. The ability of sildenafil to alter cardiac metabolism does not appear to be a direct effect as the addition of sildenafil to the perfusate of isolated hearts had no effect on cardiac metabolism. Despite the alterations in cardiac metabolism, sildenafil was unable to improve basal cardiac contractile function or the recovery of contractile function after ischemia-reperfusion. Sildenafil treatment was associated with some reduction in both septal and posterior wall dimensions, but not changes in heart dry weight.

Under basal conditions in non-diseased hearts, PDE5 inhibitors have not demonstrated an ability to alter cardiac function[224]. Challenge of hearts with β-adrenergic agonists in the presence of PDE5 inhibition blunts the increase in contractility in response to these inotropic agonists, mirroring some of the observations common in the failing heart in which chronic downregulation of PDE5A activity may be responsible for the reduced inotropic response to β-adrenergic stimulation[222]. Conversely, the chronic inhibition of PDE5A in the presence of hypertrophic stimuli, such as aortic banding, prevents or restores normal cardiac function and blocks the hypertrophic alterations of the myocardium[224]. In this study, the administration of sildenafil had no effect on cardiac function in the diabetic state after either chronic or acute administration. This supports the contention that the cardiomyopathy of the diabetic heart is not hypertrophic.

Previously Takimoto et al.[224] documented that sildenafil was able to block as well as reverse the hypertrophic response to aortic banding in mice, with no effect of sildenafil on the hearts of sham-operated mice however. This result is in contrast with the current study in which there was a decrease in wall dimensions of the left ventricle in hearts from sildenafil-treated *db/db* mice. This difference may be due to the higher dosage of sildenafil that was used in our current investigation. Takimoto et al.[224] utilized a dosage regimen of 100 mg/kg body weight/day whereas we used a higher dosage of 500 mg/kg body weight/day. The cardiac-specific overexpression of guanylyl cyclase prevented the hypertrophic response to isoproternol and aortic banding in mouse hearts, but was without effect on heart weight or left ventricular wall dimensions in the absence of a hypertrophic stimuli[225], supporting the results of Takimoto et al.[224]. However, the

cardiac-specific knockdown of guanylyl cyclase activity by Holtwick et al.[226] led to a pressure-independent pathological hypertrophy, suggesting that cGMP can influence cardiac geometry independent of external stimuli.

The ability of sildenafil to reduce the hyperglycemia of *db/db* mice could be due to a number of mechanisms. Sildenafil could increase the secretion of insulin by the pancreas, increase the metabolism of glucose by muscle, reduce the production of glucose by the liver, or increase the storage capacity/insulin sensitivity of adipose tissue similar to the actions of thiazolidinediones. The expression of PDE5 in the pancreas is very low, however. Inhibition of phosphodiesterases have been shown to increase insulin secretion, however this has largely been attributed to increases in cAMP levels[230-232]. Some recent work has suggested that cGMP may potentiate the secretion of insulin for a given stimulus[238, 239], however this effect has to be examined further. PDE5 is expressed in the liver, and inhibitors of cAMP-specific phosphodiesterases have been associated with increases in hepatic glucose production. However this was not the case with sildenafil which had either no effect or an ability to reduce hepatic glucose production under specific liver perfusion conditions[240]. Anti-diabetic actions of sildenafil on adipose tissue have not been demonstrated.

There is an accumulating body of evidence suggesting that NO potentiates glucose uptake in skeletal muscle. In humans, glucose uptake by exercising muscle is decreased by NO synthase (NOS) inhibition[241, 242], a response that is independent of the ability of NO to augment blood flow. Interestingly the effect of NOS inhibition on glucose uptake was greater in diabetic humans than in nondiabetics[241]. The inhibition of NOS also reduced the ability of skeletal muscle to take up glucose following stimulation

of AMPK[243]. In isolated rat muscle strips the PDE5 inhibitor zapranist led to increased glucose metabolism[244]. Obese/diabetic rats were resistant to the effects of zapranist. Conversely, another PDE inhibitor T-1032 reduced the ability of insulin to increase glucose uptake in rat hindlimb *in vivo*[245].

In the heart, the role of NO/cGMP in regulating glucose utilization is less clear. A number of studies have shown in the beating heart that NOS inhibition increased rather than decreased glucose utilization[246, 247]. The difficulty in interpreting some of these studies, especially those completed *in vivo* or *in situ*, is that NOS inhibition leads to an increase in cardiac work due to systemic effects on vascular resistance[246]. Separating these effects from direct effects on cardiovascular metabolism has proven to be difficult. In isolated/perfused hearts, inhibition of NOS has also been shown to increase glucose utilization in some studies[248] while having limited consequence in others[249]. It would seem that there is a significant workload component related to the ability of cGMP to alter cardiac metabolism. This was demonstrated by Depre et al.[250] who found that cGMP had no effect on cardiac metabolism at low workload while at high workload cGMP reduced the ability of the heart to increase glucose metabolism. This would seem to be in opposition to what has been found in skeletal muscle where increases in contraction appear to increase the ability of cGMP to potentiate glucose utilization. In a recent paper with isolated heart tissue, it was shown that AMPK and cGMP may share a common signaling pathway to some degree in the heart[251]. NOS inhibition was actually associated with a reduction in glucose utilization and cGMP potentiated glucose utilization via AMPK. In a similar study the administration of a NOS inhibitor during an *in vivo* clamp study decreased the uptake of 2-deoxyglucose by the hearts of Zucker fatty

rats[252]. The data presented here was not able to show an effect of sildenafil on glucose metabolism when given acutely in the perfusate. It may be that the diabetic *db/db* heart is resistant to the effects of sildenafil in a similar manner to skeletal muscle from the obese Zucker rat[244]. Functional studies have shown that in the absence of a stimulus known to increase cGMP levels, PDE5 inhibition alone is not able to augment cGMP levels[223, 224]. The direct effects of cGMP on the heart need to be examined further as they relate to cardiac metabolism. In the current study, alterations in cardiac metabolism appear to be caused by indirect actions of sildenafil. This result is similar to previous work with PPAR agonists[136, 138] and the data presented in Chapter 3, in which improvements in the diabetic status of the *db/db* mouse were associated with alterations in the cardiac metabolism.

Interestingly, including sildenafil in the pefusate failed to result in a significant increase in coronary flow. This may be due to the fact that coronary flow is already substantial increased in isolated hearts perfused with crystalline buffer and there is a significant reduction in coronary flow reserve[186,311]. The elevations in coronary flow in crystalline buffer perfused hearts appear to be related to the reduced oxygen carrying capacity of the buffer. Inclusion of erythrocytes in the perfusate, or the use of blood as a perfusion buffer, leads to a substantial reduction in rates of coronary flow in isolated hearts and an increase in coronary flow reserve[311].

The intriguing aspect of this study in relation to what was observed in Chapter 3 relates to the correction of metabolism that was observed despite only a modest reduction in hyperglycemia. The treatment of *db/db* mice with COOH led to substantial corrections in the blood glucose levels of *db/db* mice such that *db/db* mice treated with COOH were

no longer hyperglycemic. In this study, only a modest reduction in blood glucose levels occurred in response to sildenafil. Despite only the partial correction in glycemic status, cardiac glucose oxidation was increased. In Chapter 3 one can be reasonably certain that the effect on cardiac metabolism is indirect, while that cannot be concluded in this case.

CHAPTER 5 – POTENTIAL MECHANISMS RESPONSIBLE FOR ALTERED METABOLISM BY THE DIABETIC HEART

Chapter 3 indicates that the oxidation of FA by isolated perfused working hearts form *db/db* mice is increased. Switching the FA source from Alb-FA to CM-TG results in a similar phenotype of increased FA oxidation[137], suggesting that the utilization of FA for the generation of ATP is increased regardless of the source. As PPARα is a key regulator of cardiac FA oxidation many have suggested that it is central in the alterations occurring in the diabetic heart[160, 253]. This was supported by the similarities in both metabolism and function of the cardiac-specific PPARα overexpressing mouse[159] in relation to the *db/db* mouse. However, the mRNA expression of PPARα is not consistently increased when measured in hearts from *db/db* mice[45, 159]. Downstream targets of PPARα do show elevations in mRNA expression in the *db/db* mouse heart[45, 159], however the activity of these targets has not been extensively studied in order to quantify the significance of changes in mRNA expression levels.

The administration of a PPARγ agonist in Chapter 3 led to a reduction in plasma glucose and FA levels. It could be possible that the reduction in plasma FA led to a reduction in cardiac FA oxidation through a reduced PPARα activation. The control of FA utilization in the *db/db* heart may be more complex than that. Aasum et al. demonstrated that the use of a PPARα agonist to correct cardiac metabolism in the *db/db* mouse heart had no effect on the mRNA expression of PPARα targets ACO or mCPT-1 despite a reduction in both circulating plasma glucose and FA concentrations[136]. Similarly, a recent report on the effects of rosiglitazone, a thiazolidinedione PPARγ

agonist, on FAT/CD36 and FATP protein expression and activity in Zucker fatty rats following 16 d of treatment demonstrated that only the protein expression in adipose tissue of putative FA transporters were altered[208]. In adipose tissue FAT/CD36 was increased in concert with an increased FA uptake capacity. In the heart treatment failed to alter FAT/CD36 expression and no change in the ability to uptake FA was evident, ie. giant vesicles generated from the hearts of treated and untreated Zucker fatty rats retained the ability to accumulate intramyocardial lipid. The reduction in myocardial TG measured in this study appeared to be due to a reduction in circulating metabolites and not an alteration in the ability of hearts to accumulate lipid. FAT/CD36 is another PPARα target and theroretically should be reduced following treatment as there is a reduction in the presumed physiologic ligand of PPARα *in vivo*, ie. FA.

The mechanisms responsible for the changes in cardiac metabolism in diabetic hearts remain somewhat of a mystery. The following chapter will attempt to understand better the capacity of mitochondria to oxidize FA and the influence of diabetes.

5.1 Fatty Acid Utilization by Isolated Mitochondria

5.1.1 Carnitine Palmitoyl Transferase-1 (CPT-1)

The rate-limiting step in the oxidation of fatty acids is suggested to be the transport of fatty acids across the outer mitochondrial membrane (Fig. 5.1.2.1). This process is dependent on the enzyme CPT-1. As was discussed in Chapter 1 (Section 1.2), the activity of CPT-1 is regulated by malonyl CoA. Unpublished data from our laboratory suggests that the concentration of malonyl CoA in the *db/db* mouse heart was elevated, suggesting that the activity of CPT-1 should be reduced. This is contradicts the

observed increased rates of fatty acid oxidation observed in *db/db* hearts observed in Chapters 3 and in previous publications[39-41, 136, 138]. Therefore, clearly reduced malonyl CoA inhibition of CPT-1 is not a contributory mechanism for the observed enhanced FA oxidation by *db/db* hearts.

The liver isoform of CPT-1 is able to alter its sensitivity to inhibition by malonyl CoA during starvation or STZ-induced diabetes in rodents[254, 255]. The heart expresses primarily the muscle isoform of CPT-1 or mCPT-1. It has been argued that mCPT-1 is unable to alter its sensitivity to malonyl CoA[93, 98, 256], however experimental evidence for this has only been presented in starvation. It has been suggested that the relationship between malonyl CoA and mCPT-1 may be more complex than has been suggested. In skeletal muscle, the sensitivity to malonyl CoA was inversely correlated to the ability to oxidize fatty acids and the existence of malonyl CoA insensitive pools of CPT have been proposed[257]. Malonyl CoA sensitivity was also increased in skeletal muscle from trained humans[258]. Differences in malonyl CoA have also been presented between mitochondrial populations[259]. The heart does not express only mCPT-1, but rather expresses both muscle and liver isoforms[256]. Pathological conditions such as STZ-induced diabetes may increase in the relative expression of the liver isoform of CPT-1[256]. The liver isoform of CPT-1 is not only able to alter its sensitivity to malonyl CoA[254, 255], but is also substantially less sensitive to malonyl CoA under basal conditions than the muscle isoform[94, 95, 98].

Changes in the sensitivity of CPT-1 to malonyl CoA could represent a mechanism whereby FA oxidation could be increased in the *db/db* mouse, especially in the face of elevated malonyl CoA levels within the myocardium. Therefore the function of CPT-1

and its sensitivity to malonyl CoA in mitochondria from *db/+* and *db/db* mice was assessed.

5.1.2 Uncoupling protein-3 (UCP3)

A recent candidate has emerged that may influence the ability of mitochondria to maintain high rates of fatty acid oxidation. Uncoupling protein (UCP) 3 has gained recent interest as a potential regulator of fatty acid oxidation. UCP3 is a member of the uncoupling proteins (for recent reviews of the uncoupling proteins see[260, 261]). UCP1 is the best-characterized member of the uncoupling proteins and is important in reactions leading to non-shivering thermogenesis in brown adipose tissue[262]. UCP3 shares ~58% of its amino acid sequence with UCP1[260]. The physiologic role of UCP3 has not yet been determined. Under basal conditions UCP3 does not uncouple oxidative metabolism[263, 264]. Conditions that generally favour increased fatty acid oxidation are associated with increased UCP3 expression in skeletal muscle. Elevations in the plasma fatty acid concentration, such as that which occurs during fasting, are associated with increases in mRNA expression of UCP3[265-267]. Overexpression of the UCP3 protein in L6 myotubes leads to increases in the oxidation of palmitate[268]. The increase in palmitate oxidation is not merely a consequence of increased uncoupling as the oxidation of glucose is not increased. Mitochondria isolated from skeletal muscle of transgenic mice overexpressing UCP3 showed increased CPT-1 activity and reduced accumulation of intramuscular triglycerides[269].

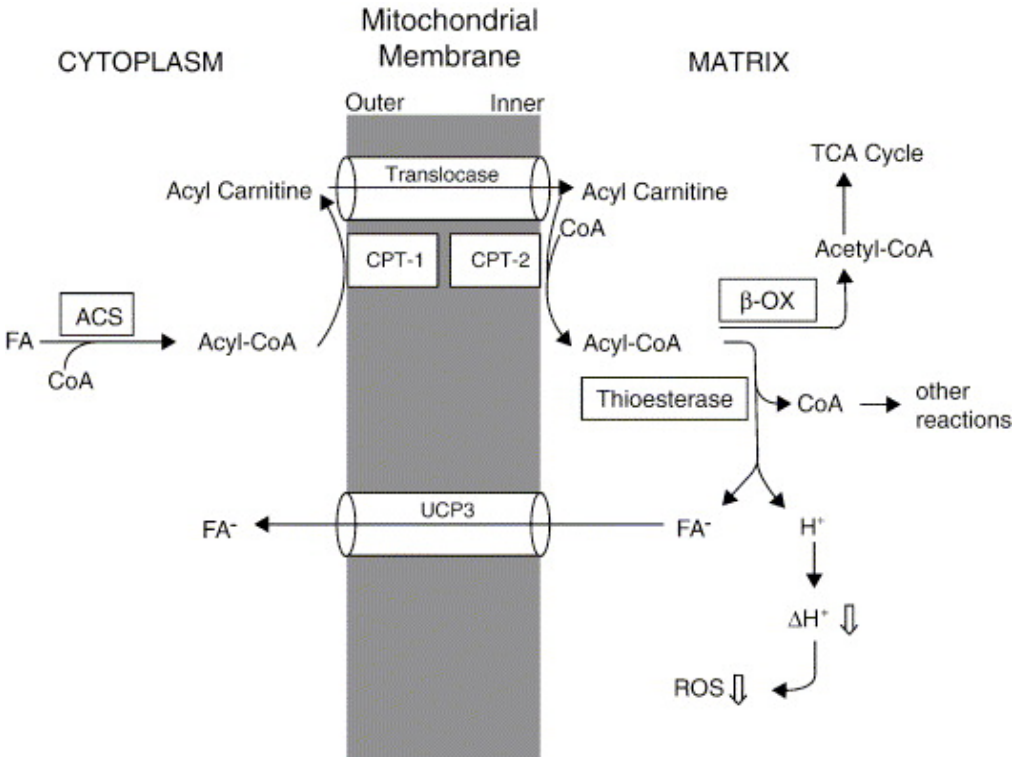

Fig. 5.1.2.1 - Function of cardiac UCP3 in relation to FA metabolism. UCP3 is hypothesized[270, 271] to transport fatty acid anion (FA^-), generated from thioesterase activity, out of mitochondria. As a consequence, high rates of FA oxidation (β-Ox) can be sustained. The thioesterase reaction produces CoA necessary for a number of mitochondrial reactions, plus H+. The resulting reduction in proton-motive force (ΔH+) will result in the decreased production of reactive oxygen species (ROS).

An accepted function of the UCPs is the transport of FA anions[272, 273]. In truth, UCPs are the only proteins that have definitively been shown to act as FA transporters despite the assertion by many that FAT/CD36 and FATP act as FA transporters across the plasma membrane[274]. A number of models have been formulated which incorporate the correlations between UCP3 expression levels and rates of FA oxidation with the known function of UCPs as FA transporters[275, 276]. Fig. 5.1.2.1 depicts the model first developed

by Drs. Himms-Hagen and Harper[271], which suggests that UCP3 facilitates high rates of FA oxidation when faced with FA oversupply. This model argues that when FA are the predominant substrate being used acyl CoA levels may accumulate in the mitochondria. At this point the concentration of free CoA becomes limiting. Mitochondrial thioesterases, which show similar expression patterns to UCP3, hydrolyze acyl CoA to FA anion and free CoA. Thioesterase-generated CoA can then replenish the free CoA pool. The FA anion generated is essentially trapped in the mitochondria. UCP3 transports the toxic FA anion out of the mitochondria and in doing so serves to reduce the proton gradient across the inner mitochondrial membrane. Both the generation of free CoA and the reduction in the proton gradient would help maintain FA oxidation in the face of oversupply. As the proton gradient increases flux through the electron transport chain would be slowed, therefore this modest "uncoupling" could help to maintain flow and the futile cycle responsible for the generation of free CoA would help supply CoA for processes beyond mitochondrial uptake of FA.

The expression of UCP3 in heart has been investigated largely at the mRNA level. The mRNA expression of UCP3 is sensitive to the plasma fatty acid concentration[277]. Activation of PPARα, a major controller of fatty acid utilization in the heart, also leads to increases in UCP3 mRNA[278], while the absence of PPARα in the PPARα (-/-) mouse removes the ability of cardiac UCP3 to respond to its environment[278]. Type 1 diabetic models have demonstrated increased expression of UCP3 mRNA[278-280], while type 2 diabetic/obesity models have demonstrated slightly increased or similar expression of UCP3[53, 279, 281, 282]. UCP2 is another member of the uncoupling proteins that has putative functions in the control of fatty acid metabolism[275, 276]. UCP2 mRNA expression in heart

however, was not responsive to changes in the plasma fatty acid concentration, PPARα activators, or diabetes/obesity[283,284]. As well, despite the relatively high mRNA expression of UPC2 in the heart relative to UCP3, significant protein expression of UCP2 is not evident[310]. The protein expression of UCP3 in cardiac tissue has only been documented in a few instances and the protein expression in diabetic animals has only recently been reported[285, 286].

Chapters 3 clearly demonstrated that *db/db* hearts show increased rates of FA oxidation that is reduced following PPARγ treatment. UCP3 upregulation could be a mechanism whereby FA oxidation is unregulated. Therefore the protein expression of UCP3 in cardiac mitochondria from *db/db* mice was assessed, along with the protein expression in other models of varying capacity to oxidize FA. The effect of UCP3 loss was also determined as it could influence cardiac metabolism in the diabetic state.

5.2 Methods

5.2.1 Experimental animals

Male mice were used in all experiments. C57Bl/KsJ-lepr^{db}/ lepr^{db} (*db/db*) and their lean, nondiabetic heterozygote littermates (*db/+*) were purchased from Harlan Laboratories (Indianapolis, IN). Mice arrived at 5-6 wk of age and were sacrificed at 12 wks of age. Transgenic mice overexpressing the human form of the insulin regulatable glucose transporter (hGLUT-4) in mice (homozygous for the hGLUT-4 transgene) were from the breeding colony maintained at the University of Calgary. The phenotype of the hGLUT-4 *db/db* mouse has been described previously[39]. CD-1 mice were purchased from Charles River (Quebec). CD-1 mice arrived at 9 weeks of age and were allowed to

acclimatize for 1 wk before experimentation. UCP3(-/-) and wild type C57Bl/6J mice were from the breeding colony at the University of Ottawa and experiments were carried out at the specified ages. A separate control group of C57Bl/6J mice was ordered from Jackson Laboratories. Mice arrived at 10-12 wk of age and experiments commenced at 18 wk of age. All mice were maintained on a 12-12 light/dark cycle with *ad libitum* access to food and water.

5.2.2 Induction of Insulin-Deficient Diabetes

The induction of diabetes using the selective β-cell toxin streptozotocin(STZ) was carried in an identical manner with all mouse models. The protocol was adapted from Neitzel et al.[137]. Briefly STZ was prepared in 100 mM citrate buffer (pH 4.5) at a concentration of 30 mg/ml. STZ or vehicle was injected via intraperitoneal (i.p.) injection over a 3 day period. Mice received 85, 75, and 55 mg/kg body weight STZ on days 1, 2, and 3 respectively. Control mice received equal volumes of citrate buffer. Isolation of mitochondria for protein measurements and working heart perfusions were then preformed 2 wk after the 3 d injection protocol.

5.2.3 Mitochondrial Isolation

Mitochondria were isolated as reported previously with some modifications [287, 288]. All procedures were carried out at 0-4^{o}C. Buffers used were as follows: Buffer 1: 140 mM KCl, 20 mM HEPES, 5 mM $MgCl_2$, 2 mM EGTA, 1 mM ATP, 1% BSA; Buffer 2: 140 mM KCl, 20 mM HEPES, 5 mM $MgCl_2$, 1 mM EGTA, 1% BSA. Hearts were removed from fed animals following i.p. injection of 10 mg sodium pentobarbital

and washed in ice-cold Buffer 1. Hearts were minced on ice and suspended in 10 volumes of Buffer 1 supplemented with 4 Units of nagarse/g of tissue. Nagarse was used to maximize mitochondrial yield and provide a mixed population of both subsarcolemmal and intramyofibril mitochondria. The suspension was homogenized in a Potter-Elvehjem homogenizer with a loose fitting Teflon pestle. The suspension was then diluted 10 fold in Buffer 1 without nagarse. Following homogenization with a tight fitting Teflon pestle the suspension was filtered through 250 μm nytex mesh and centrifuged at 9681 g_{max}. The pellet was resuspended in 25 ml of Buffer 2 and centrifuged at 482 g_{max}. The supernatant was centrifuged at 4303g_{max}. The pellet was resuspended in 25 mL of Buffer 2 without BSA and centrifuged at 4303g_{max}. The final mitochondrial pellet was resuspended in ~ 200 μL of Buffer 2 without BSA. The protein concentration was determined by the Lowry method.

In Western blotting experiments for UCP3 content, hearts were flash-frozen and stored at -80^{o}C until isolation of mitochondria.

5.2.4 CPT1 Assay

Following mitochondrial isolation, 10 uL aliquots of mitochondria in Buffer 2 without BSA were assayed for CPT1 activity. 10 uL of mitochondrial suspension was added to 90 uL of reaction buffer. Final concentrations in the reaction were as follows: 117 mM Tris·HCl (pH 7.4), 0.28 mM reduced glutathione, 4.4 mM ATP, 4.4 mM $MgCl_2$, 16.7 mM KCl, 2.2 mM KCN, 40 mg/L rotenone, 0.5% BSA, 300 uM palmitoyl-CoA, 5 mM L-carnitine with 0.5 uCi L-[^{3}H]carnitine, and (0-2 uM) malonyl CoA. The reaction was carried out at 37^{o}C and started by the addition of 10 uL of mitochondrial suspension.

After 7 min the reaction was ended by the addition of 60 uL of ice-cold 1 M HCl. The formation of palmitoyl-[^{3}H]carnitine was used as an indication of CPT activity. Palmitoyl-[^{3}H]carnitine was removed by the addition of 400 uL of water-saturated butanol, followed by three washes with distilled water. At the end of the washing process, 100 uL was removed and counted. The sensitivity of CPT-1 to malonyl CoA was calculated as a percentage of the maximal activity in the absence of malonyl CoA.

5.2.5 Mitochondrial FA Oxidation

The oxidation of palmitate was assessed in isolated mitochondria from *db/+* and *db/db* hearts. 40 uL of mitochondrial suspension was added to 160 uL of reaction buffer. The final concentrations in the reaction mixture were as follows: 100 mM sucrose, 10 mM Tris·HCl, 10 mM KH_2PO_4, 100 mM KCl, 1 mM $MgCl_2$, 1 mM L-carnitine, 0.1 mM malate, 2 mM ATP, 0.05 mM CoA, 1 mM DTT, 0.3 % BSA, and 0.150 mM [1-^{14}C]palmitate (0.5 uCi/mL). The reaction was carried out in a 96 well plate, with a rubber stopper connected to a scintillation vial inserted into the well such that it was sealed. An opening in the stopper allowed for the $^{14}CO_2$ released by the reaction to be trapped by filter paper soaked with 200 uL of 2 M NaOH. The reaction was started by the addition of mitochondrial suspension (40 uL) and carried out at 37^{o}C for 30 min. The reaction was stopped by the injection of 100 uL of ice-cold 4N H_2SO_4. Scintillant was added to the scintillation vial for radioactivity determination.

5.2.6 Citrate Synthase Activity

The ratio of citrate synthase activity in mitochondrial suspensions before and after repeated freeze-thaw cycles to disrupt mitochondrial membranes was used as an index of the intactness of the mitochondrial preparation. Citrate synthase activity was measured as previously reported[258, 289]. Briefly, mitochondria were diluted 20 fold with Buffer 2 without BSA (see mitochondrial isolation) with or without 0.04% Triton X-100. Citrate synthase activity in diluted mitochondria without Triton X-100 was defined as the activity present in intact mitochondrial samples. Mitochondria with Triton X-100 underwent 3 freeze-thaw cycles and citrate synthase activity was measured, with this value representing the activity present when all mitochondrial membranes had been broken.

5.2.7 Western Blotting

70 μg of mitochondrial protein was loaded into each sample lane on a 12% polyacrylamide minigel. Following transfer onto nitrocellulose, membranes were blocked in 2% casein. Membranes were then incubated overnight at 4^{o}C with primary antibody diluted 1:1000 (UCP3, AB-3046; Chemicon, Temecula, CA) in blocking buffer. The secondary antibody was incubated at 1:10000 dilution for 1 hour at room temperature in blocking buffer. Recombinant UCP3 (prepared in the laboratory of Dr. Harper, University of Ottawa) was used as a positive control. Blots were visualized using ECL kits (Amersham Pharmacia). Blot intensity was normalized to the amount of total protein loaded and the intensity of the positive control for that particular blot.

5.2.8 Isolated Working Heart Perfusions

Hearts were isolated and perfused in working made[135] as described in Chapters 2 and 3. The heart was perfused with buffer containing 0.7 mM [9,10-^{3}H]palmitate bound to 3% BSA with 11 mM glucose. Cardiac function and FA oxidation was measured.

5.2.9 Incorporation of [9,10-^{3}H]palmitate into TG

A subset of *db/+* and *db/db* hearts were perfused for 90 min as described in 5.2.8. At the end of the 90 min perfusion protocol hearts were flash-frozen and stored at -80^{o}C. A portion of the frozen tissue was used to determine the rate of incorporation of radiolabel into tissue TGs. The portion of tissue was homogenized in ice-cold chloroform:methanol (2:1 v/v). The homogenized tissue was diluted with 1 mL of water and vortexed. Following centrifugation at 3500 rpm for 10 min the lower phase was evaporated to dryness and resuspended in 1mL of chloroform. 50 uL of sample was run on a thin layer chromatography plate along with standards for TG, MG, DG, FFA, and PL. The plate was run in 100 mL of solvent (heptane:diethyl ether:acetic acid, 25:75:1) for ~1 hr. After separation of tissue lipids, the bands were visualized and scraped into scintillation vials. The amount of radioactivity present in each lane was related to the specific activity of the buffer substrate. Esterfication into TG represented 72.7% of radioactivity incorporated into all lipid classes.

Statistical Analysis

Data are expressed as means±standard error. Differences in means were determined by ANOVA with the Student-Neuman-Keuls test for pairwise comparison. Means were considered to be statistically significant when the p value was less than 0.05.

5.3 Results

5.3.1 CPT-1 Sensitivity to Malonyl CoA

The presence of nagarse in the isolation medium did not significantly affect the isolation procedure, as the majority of mitochondria isolated were intact (86.6±2.1%). As well, sensitivity to malonyl CoA was retained.

The sensitivity of isolated mitochondria from *db/+* and *db/db* hearts displayed similar responses to inhibition by malonyl CoA (Fig. 5.3.1.1). The tissue concentration of malonyl CoA is believed to be 0.7 uM, therefore the concentrations chosen were physiologically relevant. The concentration of malonyl CoA in the *db/db* heart is closer to 2 uM, however (unpublished data).

The ability of mitochondria from *db/+* and *db/db* hearts to oxidize fatty acids was also examined (Fig. 5.3.1.2). The ability to oxidize exogenous palmitate was not different between *db/+* and *db/db* cardiac mitochondria. The FA oxidation rates presented provide an indication of the metabolism of palmitate from the activation of FA by acyl CoA synthase to the generation of $^{14}CO_2$ during flux through the TCA cycle (Chapter 1, Fig. 1.2.1.1).

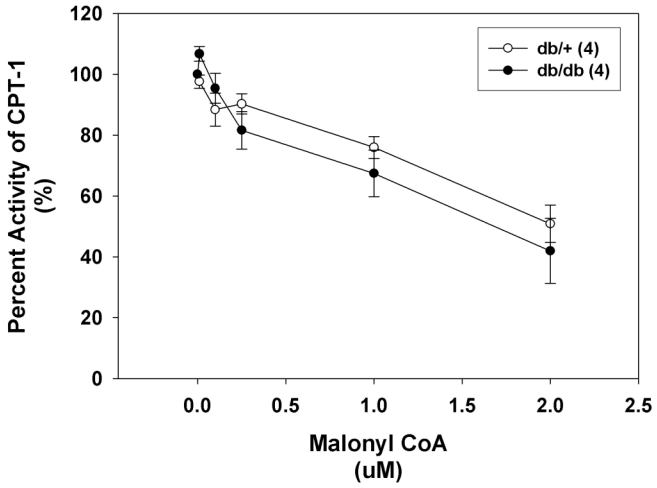

Fig. 5.3.1.1 – The sensitivity of CPT-1 activity in isolated mitochondria from *db/+* and *db/db* mice to inhibition by malonyl CoA. Experiments were performed on freshly isolated mitochondria. Data are presented as mean±S.E., n number in parentheses.

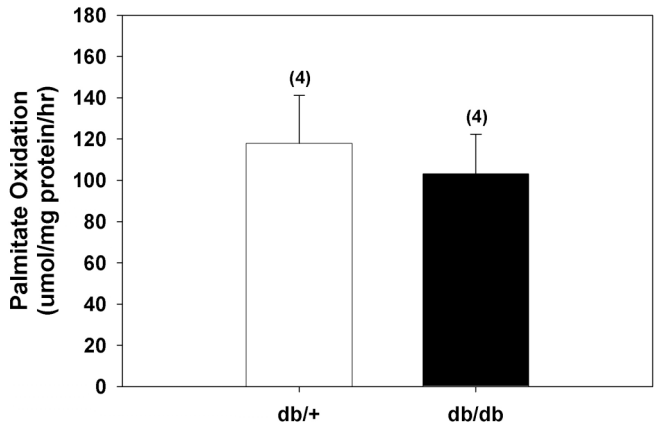

Fig. 5.3.1.2 – Mitochondrial oxidation of palmitate. Freshly isolated mitochondria from *db/+* and *db/db* hearts were incubated with 0.15 mM [14C]-palmitate for 30 min as described in the methods section (4.2.1). Data are presented as mean±SE with n value indicated in parantheses.

5.3.2 Cardiac Protein Expression of UCP3 in Type 2 Diabetic Mouse Hearts

To access what role UCP3 may have in the upregulation of cardiac fatty acid oxidation in type 2 diabetic hearts, the cardiac expression of UCP3 was determined in hearts from the *db/+* mouse, the diabetic *db/db* mouse in which there is significant upregulation of fatty acid oxidation, and the hGLUT- mouse in which there is a normalization of FA oxidation[39]. All mice were 12 wk old at the time of sacrifice. It has been shown previously that the *db/db* mouse is obese and hyperglycemic[39, 40]. The hGLUT4- mouse is obese, hyperglycemic under fed conditions, and hyperlipidemic despite normalization of both cardiac metabolism and cardiac function[39]. A representative Western blot detailing the protein expression of UCP3 in cardiac tissue can be seen in Fig. 5.3.2.1. Protein expression was clearly evident in hearts from *db/+* mice (lane 2). UCP3 was not expressed in skeletal muscle from UCP3(-/-) mice (lane 5) while the recombinant protein was clearly labeled by the UCP3 antibody (lane 1). Skeletal muscle from UCP3(-/-) mice was used as a negative control, as the expression of UCP3 in cardiac tissue is not yet widely accepted. There was also no UCP3 in cardiac mitochondria from UCP3(-/-) mice (data not shown).

The diabetic status did not significantly affect UCP3 protein levels in *db/db* hearts (Fig. 5.3.2.1). Importantly, there was no upregulation of UCP3 in hearts from *db/db* mice compared to hearts from *db/+* mice. Protein expression was significantly upregulated in the hGLUT4-*db/db* mouse heart; hearts from hGLUT4-*db/db* mice displayed an approximate 3 fold increase in the protein expression levels of UCP3 over hearts from *db/+* and *db/db* mice (Fig. 5.3.2.1).

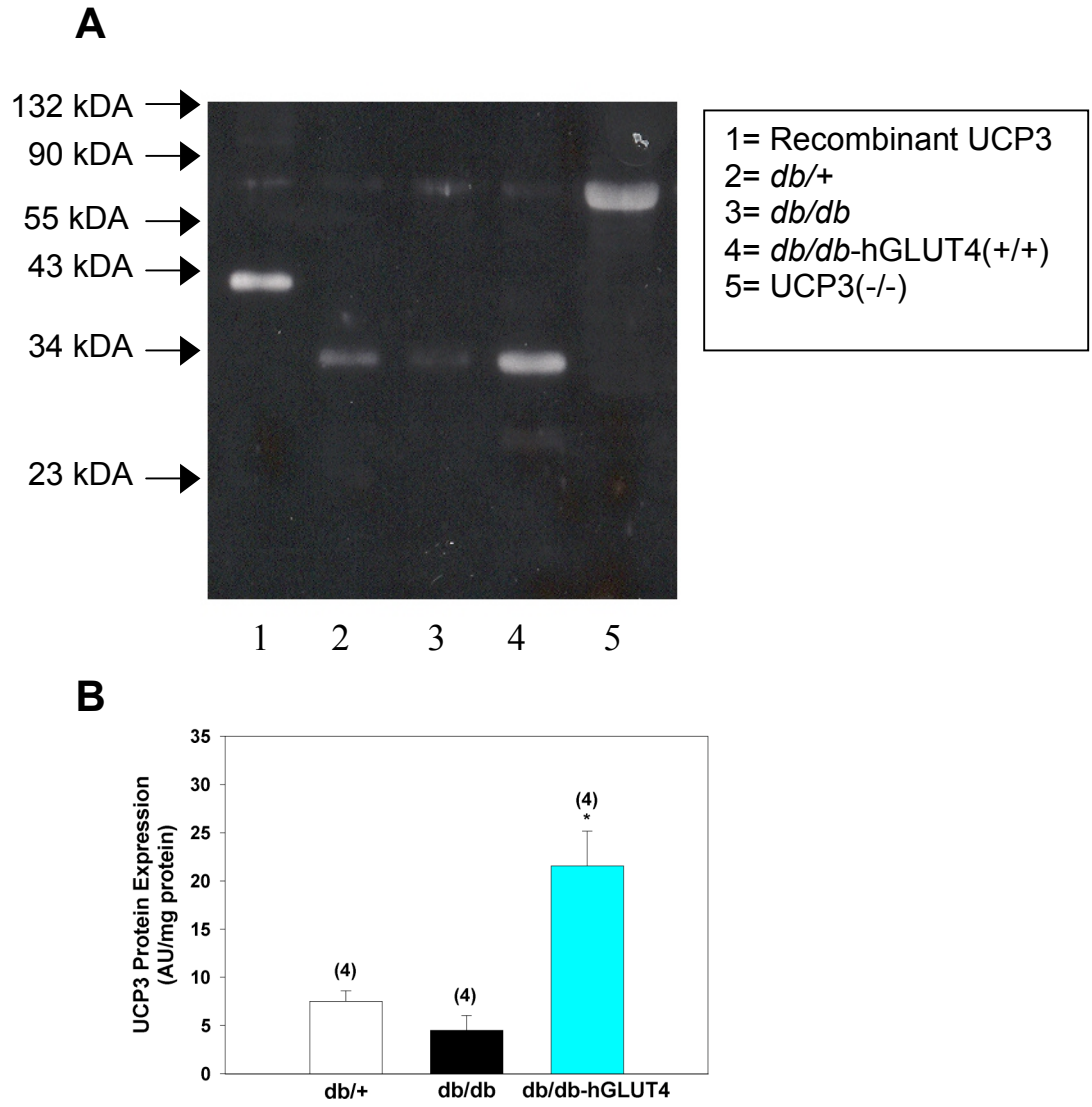

Fig. 5.3.2.1 - UCP3 protein expression in cardiac mitochondria from *db/+*, *db/db*, and hGLUT4- mice (fed conditions). A: Representative blot of cardiac UCP3 protein expression. 70 μg of mitochondrial protein was loaded into each lane. B: Average of the UCP3 protein levels measured from 4 different gels. n values provided were appropriate. Data is presented as mean±SE. *$p<0.05$ vs. control *db/+* hearts.

5.3.3 Oxidation vs. Esterification in Type 2 Diabetic Mouse Hearts

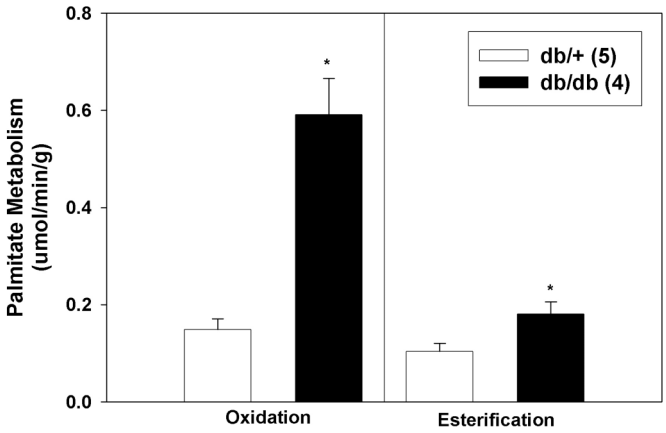

Fig. 5.3.3.1 – Comparison of the rates of oxidation and esterification of [9,10-3H]palmitate into TG by hearts from control *db/+* and diabetic *db/db* mice over 90 min of isolated working heart perfusion. *p<0.05 vs. *db/+*.

Past attempts to directly measure the uptake of FA by mouse hearts through the use of giant vesicles generated from cardiac tissue[80, 290], a measure independent of the effects of metabolism, have proven difficult due to the small size of the heart. As an indirect measure of the supply of FA to the isolated *db/db* mouse heart, the oxidation and esterification of exogenous palmitate was compared in *db/+* and *db/db* hearts. Hearts from diabetic *db/db* mice show increases in not only cardiac FA oxidation, as has been demonstrated in previous chapters, but also show increased rates of esterification of exogenous palmitate over 90 min of perfusion with 11 mM glucose and 0.7 mM [9,10-^{3}H]palmitate. Therefore despite the absence of an increase in UCP3 protein expression in hearts from *db/db* mice, over-utilization of FA is evident in isolated *db/db* hearts.

5.3.4 Cardiac Protein Expression of UCP3 in Type 1 Diabetic Mice

STZ injection induces an insulin-deficient state through selective destruction of the β-cells. Mice were 12 wk old at the time of sacrifice. CD-1 mice receiving STZ (2 wks duration) became diabetic, as determined by their glycemic status (9.0±0.8 mM in control mice vs. 31.8±2.9 in diabetic mice), and weighed significantly less than their non-diabetic counterparts (38.8±0.6 g in control mice vs. 35.8±0.7 g in diabetic mice). Although our laboratory has previously presented data showing elevated cardiac fatty acid oxidation rates in *db/+* and Swiss Webster hearts following STZ[137], the effect of insulin-deficient diabetes on cardiac fatty acid oxidation rates in STZ-CD-1 hearts has not been previously documented. Fig. 5.3.4.1 shows a significant increase in palmitate oxidation in diabetic hearts. UCP3 protein levels in hearts from diabetic mice are also shown in Fig. 5.3.4.1. The induction of diabetes tended to increase cardiac UCP3 protein expression, however this increase did not reach significance. No differences in cardiac function were seen (data not shown).

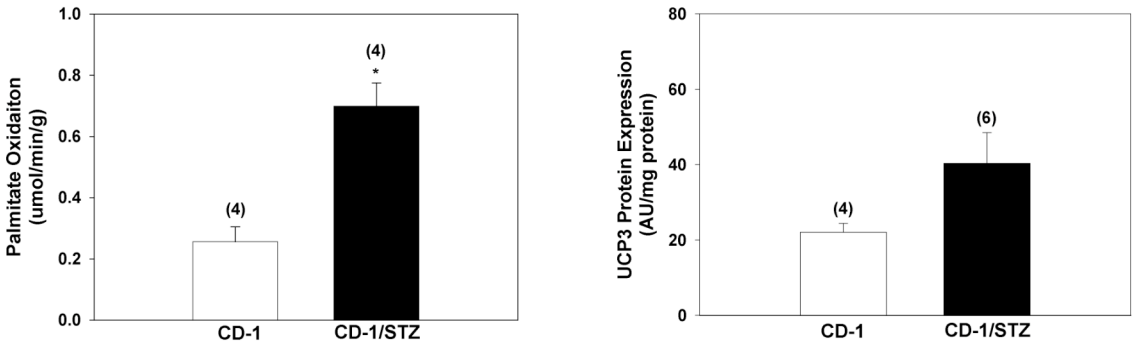

Fig. 5.3.4.1 – Palmitate oxidation of isolated hearts, and UCP3 protein expression in hearts from CD-1 control mice and CD-1 mice injected with STZ to induce insulin-deficient diabetes.

5.3.5 Streptozotocin-Induced Diabetes and the UCP3(-/-) mouse

The protein expression of UCP3 in diabetic hearts did not correlate with the observed rates of fatty acid oxidation (Figs. 5.3.2.1 and 5.3.4.1). Therefore diabetes was induced in the UCP3(-/-) mouse to better understand the role of UCP3 in alterations in FA oxidation previously documented STZ-induced diabetes. STZ treatment led to hyperglycemia in both UCP3(-/-) mice and control mice (Table 5.3.5.1). Diabetic mice weighed significantly less than non-diabetic animals upon sacrifice; the lower sacrifice weight resulted from an inability to gain body weight during the 2 weeks following induction of diabetes. The ages of mice used can also be found in Table 5.3.5.1. The loss of UCP3 in the knockout animals did not appear to alter the response to STZ based on the similar degrees of hyperglycemia and weight loss.

Table 5.3.5.1 - Physical characteristics of UCP3(-/-) mice and C57Bl/6 control mice before and after treatment with vehicle (citrate buffer) or 215 mg/kg body weight STZ

	Age (weeks)	Initial Body Weight (g)	Final Body Weight (g)	Blood Glucose (mM)	Ventricular Wet Weight (mg)	Ventricular Dry Weight (mg)
Wild type	24±3(5)	31.4±2.1(5)	33±1.9(5)	7.7±0.4(5)	154±8(4)	29±2(4)
UCP3(-/-)	15±0(5)	29.4±1.3(5)	30.8±1.0(5)	9.1±0.9(5)	169±5(4)	29±3(4)
Wild type STZ	24±3(6)	31.6±0.4(6)	27±0.4(6)*	22.5±2.9(6)*	145±10(5)	24±1(5)
UCP3(-/-) STZ	17±2(6)	28.4±0.9(6)	27±0.7(5)*	22.8±1.6(5)*	153±6(5)	27±1(5)

Data are presented as mean±SE followed by n values in brackets. *$p<0.05$ vs. control vehicle treated mice. Data are from fed mice.

The cardiac function of isolated perfused working hearts can be seen in Fig. 5.3.5.1. There was no effect of either diabetes or loss of UCP3 on cardiac function, as measured by aortic flow and cardiac output. Rates of exogenous palmitate oxidation can be seen in Fig. 5.3.5.2. Contrary to previous reports[137], and the response seen in CD-1 diabetic hearts (Fig. 5.3.2.1), there was no effect of diabetes alone on cardiac palmitate oxidation (0.35±0.05 μmol/min/g in control C57Bl/6J vs. 0.22±0.03 μmol/min/g in C57Bl/6J diabetic mice)(Fig. 5.3.5.2). In non-diabetic hearts, the absence of UCP3 did not impact the rate of palmitate oxidation (0.35±0.05 μmol/min/g in control C57Bl/6J vs. 0.26±0.1 μmol/min/g in UCP3(-/-) mice). However, the combination of both diabetes and UCP3 absence produced a reduction in rates of palmitate oxidation (Fig. 5.3.5.2), below the level of fatty acid oxidation in nondiabetic control hearts (0.35±0.05 μmol/min/g in control C57Bl/6J vs. 0.08±0.03 μmol/min/g in UCP3(-/-) diabetic mice). If the rate of palmitate oxidation was normalized to cardiac function, the combined effect of diabetes and absence of UCP3 produced an even more striking result.

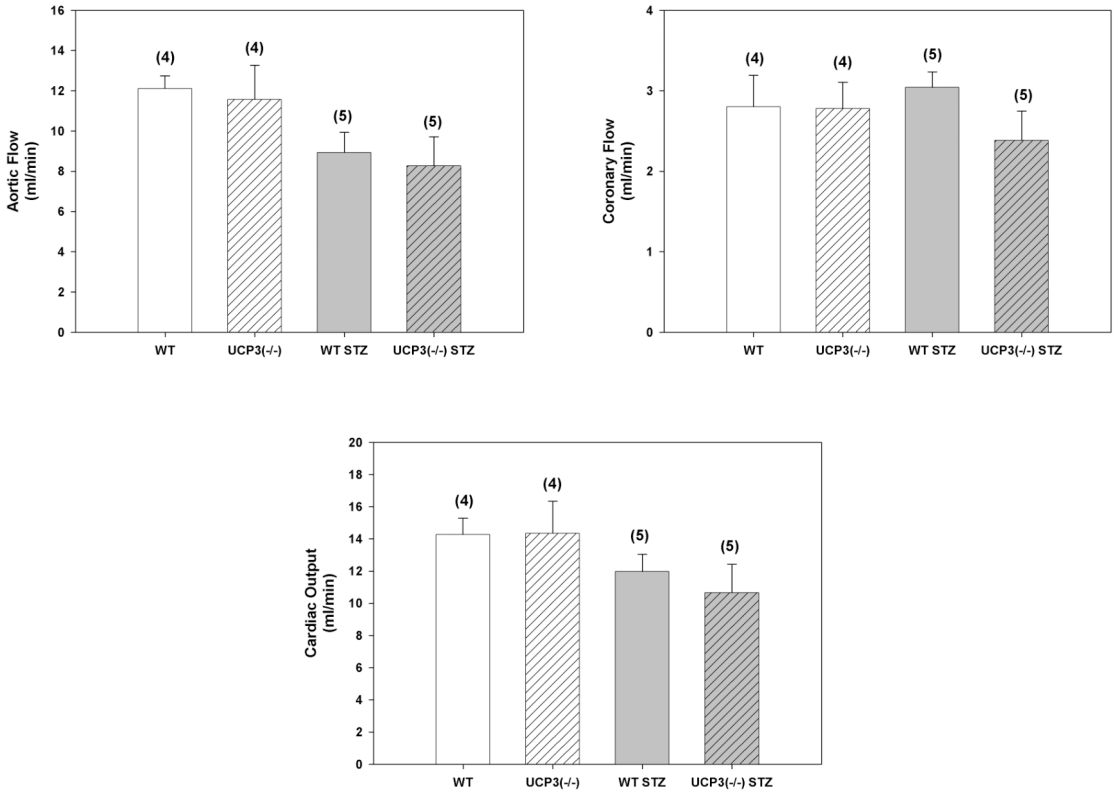

Fig. 5.3.5.1 – Cardiac function of isolated hearts from wild-type C57Bl/6 mice and UCP3 knockout mice maintained on the C57Bl/6 background. Average rates of aortic and coronary flows as well as cardiac output for the 60 min of perfusion are presented.

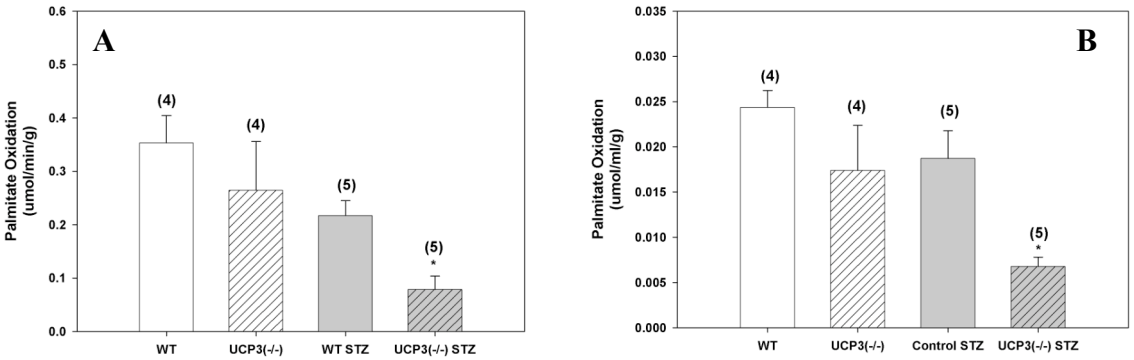

Fig. 5.3.5.2 – Palmitate oxidation of isolated hearts perfused with 11 mM glucose and 0.7 mM 3H-palmitate. The oxidation of palmitate was measured in hearts from wild-type C57Bl/6 mice and UCP3 knockout mice maintained on the C57Bl/6 background. STZ-induced diabetes was induced as described. A, average rates of palmitate oxidation normalized to ventricular dry weight. B, average rates of palmitate oxidation normalized to both ventricular dry weight and cardiac output. *$p<0.05$ vs. control mice.

The lack of an effect of STZ-induced diabetes on palmitate oxidation rates in C57Bl/6 (Fig. 5.3.5.2) is in opposition to previous work by our laboratory[137] and others [191, 291]. The mice used in the UCP3(-/-) study were older than our laboratory had used previously. The working heart perfusions were also performed at the University of Ottawa rather than the University of Calgary. Mice of a similar age and the same background as those used in the UCP3(-/-) study were ordered from Jackson Laboratories. Following the induction of diabetes, hearts were perfused and the palmitate oxidation of isolated hearts was studied in both control mice and insulin-deficient mice. Similar results were encountered with age-matched C57Bl/6 mice to those used in Ottawa; palmitate oxidation was not significantly elevated in perfused hearts after the induction of STZ-induced diabetes in C57Bl/6 mice.

5.4 Discussion

No differences were observed in the ability of mitochondria from *db/+* and *db/db* hearts to metabolize FA under the assay conditions studied. The oxidation of FA by isolated mitochondria was not altered in diabetic mice and the activity of CPT-1 was equally sensitive to inhibition by malonyl CoA. The measurement of CO_2 production in isolated mitochondria from mouse hearts provides an indication of ACS activity, CPT-1 function and flux through β-oxidation and the TCA cycle. Mitochondria from diabetic *db/db* mice did not display an inherent increased ability to oxidize FA. This is in contrast to data from isolated hearts when they are perfused with both glucose and FA (Chapter 3). This also contrasts with some reports on the mRNA expression of mitochondrial enzymes involved FA oxidation in *db/db* hearts. CPT-1 mRNA expression was increased in *db/db* hearts along with PGC-1[159]. However these results are very similar to those garnered from freshly isolated cardiomyocytes[117].

UCP3 protein was expressed in control heart mitochondria at measurable levels in the absence of any underlying pathophysiology. The cardiac expression of UCP3 was not altered significantly in diabetic hearts from either type 2 *db/db* or insulin-deficient diabetic mice, even when this was associated with increased rates of cardiac fatty acid oxidation (*db/db* hearts, STZ-treated CD-1 hearts). The overexpression of hGLUT4 resulted in a significant upregulation of UCP3 protein in hearts from transgenic mice despite the fact that this model shows normalization of the elevated cardiac FA oxidation in hearts. Even though there was no correlation between the protein expression of UCP3 and cardiac fatty acid oxidation rates in diabetic hearts, the absence of UCP3 in combination with diabetes led to a reduction in fatty acid oxidation. The absence of

UCP3 alone did not lead to an alteration in cardiac fatty acid oxidation under control conditions.

A recent report by Murray et al.[285] suggested that UCP3 protein expression is increased in hearts from both STZ-induced diabetic mice and *db/db* mice. This report did not use either positive or negative controls and quantity of mitochondria loaded into each lane was also not provided. The upregulation of protein expression between control and diabetic mice was modest in both the *db/db* and STZ-induced models. The lack of an increase in UCP3 protein in *db/db* hearts as measured in this Chapter is in agreement with observations from other chronic diabetes/obesity models, the *ob/ob* mouse and the Zucker fatty rat[53, 279, 281]. Abel's group has examined both the mRNA and protein expression of UCP3 in hearts from wild type *ob/+* and obese insulin-resistant *ob/ob* mice[45, 286]. Although there was as much as a 2 fold increase in mRNA expression[45] there was no difference in protein expression[286]. This group has also looked at mRNA expression of UCP3 in hearts from *db/db* mice[45] and showed a similar fold increase in *db/db* hearts to *ob/ob* hearts. The use of the UCP3 knockout mouse as a negative control for protein expression was also utilized in these studies[286]. Cardiac mRNA expression of UCP3 was not increased in Zucker fatty rat hearts, despite substantial increases in plasma FA and TG levels[27928153]. Also, the correlation between cardiac fatty acid oxidation and UCP3 mRNA levels was not strong in hearts from the Zucker fatty rat and lean controls[28153]. UCP3 mRNA expression is increased in obesity models induced by high fat feeding[282278]. Monogenic models of obesity/diabetes tend to have a more severe phenotype. What this observation means in the scope of such differences is unclear.

The absence of any differences in UCP3 expression in the mouse hearts is not surprising if one considers the differences in fatty acid oxidation in the beating heart versus isolated cellular systems and the data presented here concerning mitochondrial FA oxidation in *db/db* hearts. Carroll et al. found that in isolated cardiomyocytes there was no difference in fatty acid oxidation from *db/+* and *db/db* hearts[117]. Even at the level of the mitochondria, Kuo et al. did not find an increased oxidative capacity for fatty acid substrates in the mouse heart[292], results that are consistent with our measurements of palmitate oxidation by isolated mitochondria from *db/db* hearts; this contrasts with data from beating perfused *db/db* hearts which show an elevation in fatty acid oxidation (Chapter 3), as described previously[39, 40, 136]. If there was an increase in UCP3 expression in the mouse heart, based on its proposed role in fatty acid oxidation (Fig. 5.1.2.1), it would seem likely that at the cellular or mitochondrial level there would be an elevation in fatty acid oxidation, similar to the observations from skeletal muscle of Bezaire et al. and Maclellan et al.[268, 269].

Overexpression of GLUT4 has been associated with increased mRNA expression of UCP3 in skeletal muscle[293]. The degree of overexpression of GLUT4 was directly related to the increase in UCP3 mRNA. One would expect that if UCP3 plays a substantial role in the regulation of cardiac fatty acid oxidation, then UCP3 should not be elevated in hGLUT4-*db/db* hearts, as there is no elevation in cardiac fatty acid oxidation[39]. This conclusion is complicated by the global overexpression of hGLUT4, resulting in generalized systemic changes in the transgenic mouse. The uptake of glucose is significantly increased in this model[117, 294]. The overexpression of GLUT4 may be overriding any input from UCP3 on the oxidation of substrates. In non-diabetic hearts in

which there has been a global overexpression of GLUT4, there is still an increase in plasma lipid levels[295, 296], most likely due to reduced fatty acid utilization in the face of increased glucose metabolism. One could argue that in the absence of exogenous glucose, that UCP3 could then take on more of a regulatory role. In freshly isolated cardiomyocytes, however, there is no difference in the fatty acid oxidation rates in the absence of exogenous glucose versus the fatty acid oxidation rates following the addition of exogenous glucose with or without insulin (R. Carroll, unpublished observations).

Another proposed model of UCP3 action is to control the concentration of fatty acids in the mitochondria during periods in which cellular fatty acid levels increase. Although the hGLUT4-*db/db* mouse exhibits corrections in both its cardiac function and cardiac metabolism, its diabetic status in terms of the plasma concentrations of glucose and lipids is not improved[39] except under fasting conditions. The concentration of fatty acids in the circulation is an important determinant of their uptake, through both creating a diffusion gradient and activating signaling pathways involving various putative fatty acid transporters[77, 79, 297]. Fatty acid uptake could remain elevated in the hGLUT4-*db/db* mouse and thereby lead to elevations in cellular lipid content. UCP3 has been proposed to export fatty acids out of the mitochondria. Schrauwren and Hesslink have proposed a model by which increases in fatty acid uptake in excess of the oxidative capacity of muscle will increase the entrance of non-acylated fatty acids into the mitochondria [163]. As there are no acyl-CoA synthases in the mitochondria, these fatty acids are unmetabolizable and prone to peroxidation. UCP3 could be acting to export these fatty acids out of the mitochondria, thereby helping to maintain mitochondrial function. UCP3 in the hGLUT4-*db/db* mouse may be functioning to maintain mitochondrial function

through controlling intramitochondrial fatty acid concentrations. Data on the uptake of fatty acids into the heart and fatty acid concentrations within the myocardium could help to increase our understanding of this model. Conversely, the db/db mouse heart appears to also exhibit symptoms of FA oversupply relative to its oxidative capacity. Rates of esterfication are elevated despite the increases in oxidation, suggesting that FA uptake is in excess of the capacity to utilize FA. At 12 wks of age there is significant accumulation of myocardial TG (Chapter 3).

Brand and colleagues have proposed a model whereby UCP3 acts to control production of reactive oxygen species (ROS) rather than controlling the rate of fatty acid oxidation[275, 276]. Oxidative stress is increased in diabetes. The lack of an increase in UCP3 in the *db/db* heart may contribute to the cardiac dysfunction seen in this model. The increase in UCP3 expression in the hGLUT4-*db/db* heart may also contribute to the correction in cardiac function that has been previously documented, by reducing ROS production.

In contrast to models of chronic diabetes, in models of more acute diabetes the mRNA expression of UCP3 has been found to be increased[279, 282]. STZ-induced diabetes or high fat feeding have been associated with elevations in UCP3 mRNA expression. In the current study, a significant increase in UCP3 protein in hearts from insulin-deficient mice was not seen, despite a strong trend in that direction. The correlation between elevations in UCP3 mRNA and UCP3 protein expression in hearts has not been determined. Hoeks et al. found that increases in cardiac mRNA expression did not lead to statistically significant increases in UCP3 protein levels following dietary manipulation[298].

Contrary to the CD-1/STZ diabetic mouse, an increase in fatty acid oxidation in perfused hearts from insulin-deficient C57Bl/6J mice was not observed. This may be an effect of the different mouse backgrounds. Early work by Coleman and colleagues found the Bl/6J mouse was more resistant to the loss of leptin signaling than was the C57Bl/KsJ mouse[299]. The mouse on the Bl/6J strain was substantially less hyperglycemic than the mouse on the KsJ background. Conversely, the Bl/6J mouse was found to be more susceptible to metabolic derangements induced by high fat feeding than the CD-1 mouse[300]. The Bl/6J mouse also showed some variation in its response to high fat feeding[300]. Rossmeisl et al. found that responses to diet-induced obesity were different between the Bl/6 mouse and the AKR mouse[301]. Diabetes was induced in the Bl/6 mice used for this study as they were clearly hyperglycemic and failed to gain weight. The differences in the rates of fatty acid oxidation cannot be attributed to a failure to induce diabetes.

UCP3 absence alone failed to affect fatty acid oxidation in control hearts, in agreement with previous observations indicating a mild phenotype in the UCP3(-/-) under control conditions[302, 303]. The loss of UCP3 had a dramatic effect on the fatty acid oxidation rates in STZ-induced diabetic hearts; the rate of fatty acid oxidation was reduced below that in control hearts expressing UCP3. STZ-induced diabetes has been associated with increased cardiac lipid accumulation. In the absence of UCP3, the induction of diabetes could reduce the ability of the heart to compensate for increased fatty acid uptake into the myocardium. Information on the ability of these hearts to oxidize substrates other than palmitate would be useful in making the distinction as to whether the loss of UCP3 signaling in diabetes represents a reduction in mitochondrial

function or an alteration in substrate utilization. Some degree of signaling by UCP3 is necessary during the induction of diabetes to maintain normal rates of cardiac fatty acid oxidation. The functional correlates of such signaling pathways need to be assessed further. In a recent study, McLeod et al.[304] measured a significant increase in ROS production during anoxia-reoxygenation in UCP3-deficient H_9C_2 cardiomyoblasts. UCP3 expression was knocked down through the use of RNA interference. This study argues for a significant role of uncoupling proteins in the preconditioning response and cardiac ROS buffering. Clarke and colleagues have argued that UCP3 could be potentially detrimental for the heart due to potential inefficiencies following uncoupling[305], however in the *ob/ob* mouse although mitochondrial uncoupling is evident this must be due to some other mechanism[286]. Support for this hypothesis was drawn from an increase in uncoupling protein expression in patients undergoing cardiac surgery that was proportional to their fatty acid levels in the circulation. A better understanding of cardiac function in the UCP3(-/-) mouse is important not only for the diabetic heart, but other pathophysiologic conditions with cardiac consequences.

In summary, the oxidation of palmitate was not increased in the cardiac mitochondria of type diabetic mice and there were no differences in the sensitivity of isolated mitochondria to malonyl CoA inhibition. UCP3 protein expression does not have to be upregulated to allow for the increase in cardiac fatty acid oxidation that is seen in the intact heart of the type 2 diabetic mouse. Nor is there significant increase in UCP3 protein expression in insulin-deficient models that show increased cardiac fatty acid oxidation. The loss of UCP3, however, reduced the ability of diabetic hearts to oxidize fatty acids. A greater understanding of the factors leading to this reduction in fatty acid

oxidation will provide greater insight into the role of UCP3 in the diabetic heart. This study is the first to show that UCP3 is important in the oxidation of fatty acids in diabetes but it questions the view that UCP3 expression is a contributor to the elevated rates of fatty acid oxidation in diabetic hearts.

6. GENERAL DISCUSSION AND FUTURE DIRECTIONS

Hearts from *db/db* mice displayed an altered substrate utilization pattern and impaired contractile function. Two separate studies demonstrated that corrections in cardiac metabolism were without effects on cardiac function. The increase in FA oxidation could not be tied to alterations in mitochondrial function nor the protein expression of UCP3.

The absence of any correlation between cardiac function and cardiac metabolism in the *db/db* heart is an unexpected result. A significant body of data has been put forward suggesting a link between cardiac metabolism and the development of a diabetic cardiomyopathy[39, 61, 62]. Two possibilities may be responsible for the results encountered in this body of work – the interventions occurred at a time at which the cardiac dysfunction was irreversible, or the relationship between cardiac metabolism and cardiac function is merely correlative.

The question of prevention versus reversibility is one that has been examined in insulin-deficient models[189, 306], but not one that has been studied in type 2 diabetic models. In a study of the chronic effects of insulin-deficient diabetes on cardiac morphology in rodents, some of the ventricular remodeling could be corrected, whereas other aspects were resistant to treatment if intervention occurred too late[307]. 12 wks of diabetes led to decreased myocyte cross-sectional area, decreased mitochondrial and myofibrillar volume densities, and an increase in the extracellular matrix. All of these changes were prevented if insulin treatment began 3 d after induction of diabetes. If insulin treatment was delayed until 6 wks after induction of diabetes, most of the remodeling was corrected by 26 wks however the extracellular matrix remained increased

compared to nondiabetic hearts. Insulin treatment of STZ rats for 4 wks, following 6 wks of STZ-induced diabetes both prevented and restored cardiac function in isolated hearts[189]. Conversely, PKCβ expression and intramyocardial DAG concentrations were not corrected by periods of euglycemia following brief period of diabetes[180]. Insulin treatment for 4 wks, 4 wks after the induction of diabetes[306], or the inhibition of myocardial FA oxidation with etomoxir for 8 d[308], 5 wks after induction of diabetes, failed to reduce DAG levels. PKCβ expression was also unaffected by transplantation of islets into rats, diabetic for 2 wks at the time of transplantation and followed for 3 wks post-transplantation[180]. Therefore some evidence exists suggesting that some of the proposed metabolic pathways may be chronically altered despite attaining euglycemia. The development of environmentally-induced obesity/diabetes models may be integral in examining these questions further. The *db/db* mouse begins to show signs of insulin resistance (whole body) at 10 d following birth with increases in the circulating insulin levels[299]. Therefore intervention even at 6 wks of age may be too late. The earliest study to date describing the phenotype of the *db/db* mouse suggests that alterations in the metabolic profile of the heart may occur as early as 4 wks of age[45], in advance of alterations in circulating metabolites and contractile dysfunction. The study of Belke et al.[39], which was able to relate corrections in cardiac metabolism to improvements in cardiac function in the *db/db* mouse represented a transgenic approach, and essentially equates to an inborn correction. It is important that diet-induced obesity models are developed that allow both the prevention and reversal of cardiac function/metabolism to be examined more clearly in type 2 diabetes. Or conversely monogenic models that develop diabetes at a more advanced age and maintain some degree of "normal"

physiology prior to the development of diabetes. Models such as these would allow intervention to occur in advance of diet manipulation, or at different time points after the development of diabetes and obesity. The first study to show a beneficial effect of troglitazone on cardiac function in the ZDF rat involved treatment of 14 wks[51]. To date this is the only work this group has presented using this model and the lack of a shorter treatment period is somewhat conspicuous by its absence.

The other important questions raised in this work concern the mechanism responsible for the alterations in cardiac metabolism. Clearly when perfused with glucose and FA, *db/db* hearts display increased oxidation of FA and reduced oxidation of glucose. No differences in mitochondrial FA oxidation were identified however, similar to results from isolated cells incubated with FA and glucose[117]. In unstimulated cardiac myocytes no differences in the oxidation of FA are seen[114, 117], and the ability of insulin and glucose to suppress FA oxidation is maintained[117], suggesting that the glucose/FA cycle is intact. This is somewhat contradictory to the hypothesis that suggests that activation of PPARα signaling cascade is involved in the upregulation of FA oxidation[133, 253]. Rather it appears that the differences between cardiac metabolism in diabetic hearts and nondiabetic hearts are only visible when energy demand is increased. Diabetic hearts show an inability to upregulate glucose metabolism when energy demand is increased, therefore the only alternative is to increase FA metabolism. This certainly suggests a demand component and a mechanism surrounding energy sensing in the heart. However, AMPK does not appear to be involved[117, 121]. A greater understanding of the relationship between cardiac workload and the differences in cardiac metabolism between control and

diabetic hearts is needed. Caution does need to be taken when relating *ex vivo* studies to the *in vivo* situation, however.

The *db/db* mouse exhibits a severe form of hyperglycemia as evident in the preceeding work. Even though there is a reduction in GLUT4 expression in the *db/db* heart[309], the elevated plasma glucose concentrations *in vivo* may be able to overcome the defect through mass action. With the development of more sophisticated tools to measure cardiac metabolism *in vivo* in small models such as the mouse[216], the ability to gain a better understanding of cardiac metabolism *in vivo* is possible. The work presented here suggests that the *db/db* mouse is over-reliant on FA for the generation of ATP. However a recent study completed *in vivo* suggests that the elevations in plasma glucose are sufficient to overcome this over-reliance on FA *in vivo* in the hearts of *db/db* mice[216]. Although it can be considered an advantage of the isolated working heart apparatus to be able to control the concentration of all substrates provided to the heart, it also represents a limitation and a potential source of bias. Perfusing isolated db/db hearts with 11 mM glucose and 0.7 mM palmitate provides an indication of myocardial metabolism specifically under those condtions. The development of more sophisticated clamp protocols, such as the one mentioned above, will allow for a clearer understanding of cardiac metabolism of the diabetic heart.

Diabetes remains a significant concern and although this present body of work does not support a linkage between cardiac metabolism of isolated hearts and cardiac function, there certainly remains a substantial body of work in support of such a linkage. Through both the development of animal models more similar to the development and progression of diabetes in humans, and the miniturization of techniques to more easily

examine cardiac metabolism and function *in vivo*, a better understanding of diabetic cardiomyopathy will emerge.

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