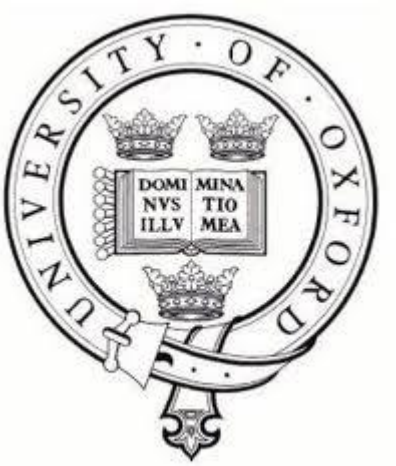
Effect of Hypoxia on Cardiac Metabolism and Function in the Type 2 Diabetic Heart



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ABSTRACT

The effect of hypoxia on cardiac metabolism and function in the type 2 diabetic heart

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Type 2 diabetic patients have impaired cardiac ischaemia-reperfusion recovery and higher rates of mortality following a myocardial infarction. Hypoxia is a key component of ischaemia and therefore, this thesis is aimed to investigate the effect of hypoxia on metabolism and contractile function of the type 2 diabetic heart. In combination with high fat feeding, different doses of streptozotocin (STZ) (15, 20, 25 and 30 mg/kg) were used to determine the optimal dose needed for induction of diabetes in male Wistar rats. A novel type 2 diabetic model was developed and characterised by hyperinsulinaemia, hyperglycaemia and dyslipidaemia. The effects of chronic hypoxia were investigated by housing diabetic rats in a hypoxic chamber (11% O_2) for 3 weeks. Results showed that the HIF signalling pathway was not impaired in diabetic hearts. PPARα targets (MCAD, UCP3 and PDK4) were downregulated by chronic hypoxia in control hearts but not in diabetic hearts, suggesting PPARα overactivation in diabetic hearts. Acute hypoxic perfusions (16 minutes normoxia, 36 minutes hypoxia and 20 minutes reoxygenation) were performed to investigate the effect of acute hypoxia on metabolism and cardiac function. Diabetic hearts had impaired metabolic response to acute hypoxia, associated with decreased cardiac function during acute hypoxia and reoxygenation. In the final study, sulfo-N-succinimidyl oleate (SSO), a FAT/CD36 inhibitor was administered prior to acute hypoxia to modulate metabolism in diabetic hearts. The previously seen maladaptation of diabetic hearts to acute hypoxia was improved by SSO. In diabetic hearts, SSO increased glycolysis during acute hypoxia, and normalised fatty acid oxidation and decreased triglyceride deposition upon reoxygenation, associated with improved cardiac function at the end of experiment compared to untreated diabetic hearts. In conclusion, the elevated lipid metabolism contributed to metabolic inflexibility in diabetic hearts, which is associated with the impaired response to hypoxia, and the inhibition of lipid metabolism was associated with improved cardiac function in diabetic hearts following hypoxia.

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ABBREVIATIONS

ACC	acetyl-CoA carboxylase
ADP	adenosine diphosphate
AMPK	AMP-activated protein kinase
ANOVA	analysis of variance
ANT	adenine nucleotide translocator
ATP	adenosine triphosphate
β-OHB	β-hydroxybutyrate
BSA	bovine serum albumin
CAT	carnitine acyl-transferase
CoA	coenzyme A
CPT	carnitine palmitoyltransferase
CS	citrate synthase
DFO	desferrioxamine
EDP	end-diastolic pressure
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycoltetracetic acid
FABPpm	fatty acid binding protein
FAD(H_2)	flavin adenine dinucleotide (reduced)
FAT/CD36	fatty acid translocase/ cluster of differentiation 36
FIH	factor inhibiting HIF
GLUT	glucose transporter
GTP	guanosine triphosphate
GWW	gram wet weight
HIF	hypoxia inducible factor

IFM	interfibrillar mitochondria
KH	Krebs-Henseleit
LDH	lactate dehydrogenase
LVDP	left ventricular developed pressure
MCAD	medium chain acyl-CoA dehydrogenase
MCT1	monocarboxylate transporter 1
NAD(H)	nicotinamide adenine dinucleotide (reduced)
NEFA	non-esterified fatty acids
NMR	nuclear magnetic resonance
p-VHL	von Hippel Lindau protein
PDH	pyruvate dehydrogenase
PDK	pyruvate dehydrogenase kinase
PFK	phosphofructokinase
PHD	prolyl-4-hydroxylase
PPAR	peroxisome proliferator-activated receptor
RXR	retinoid X receptor
SSM	subsarcolemmal mitochondria
SSO	sulfo N-succinimidyl oleate
TAG	triglycerides
TCA	tricarboxylic acid
UCP3	uncoupling protein 3
VEGF	vascular endothelial growth factor
VLDL	very low-density lipoprotein

CHAPTER 1

GENERAL INTRODUCTION

1.1 TYPE 2 DIABETES

Diabetes is a worldwide epidemic responsible for 1.5 million deaths in 2012 worldwide[1] and type 2 diabetes is becoming more prevalent especially in developed countries, where individuals are becoming more obese while adopting an increasingly sedentary lifestyle. In 2011, the estimated diabetes prevalence worldwide was 366 million compared to 153 million in 1980, and it is predicted to affect 552 million people by 2030[2, 3]. Diabetic patients are at risk of multiple complications, categorised as macrovascular complications (coronary artery disease, peripheral arterial disease, and stroke) and microvascular complications (diabetic nephropathy, neuropathy, and retinopathy)[4]. In addition, diabetes is also becoming a significant financial burden to countries with high prevalence of the disease. Assuming there is no inflation, the cost of care for diabetes and treatment of its complications in the UK will rise from £9.8 billion in 2010 to £16.9 billion in 2035. Diabetes UK outlined the criteria for diagnosis of type 2 diabetes include random venous plasma glucose concentration ≥ 11.1 mmol/l; or

a fasting plasma glucose concentration ≥ 7.0 mmol/l (whole blood ≥ 6.1 mmol/l); or

two hour plasma glucose concentration ≥ 11.1 mmol/l two hours after 75g anhydrous glucose in an oral glucose tolerance test[5].

Up to 95% of diabetic cases are type 2 diabetes and insulin resistance is one of the hallmarks of type 2 diabetes, defined by the inability of the cell or organ to respond to insulin stimulation[6]. Insulin is secreted by pancreatic β-cells in response to elevated glucose concentrations and is one of the key regulators of metabolism. Type 2 diabetes is a progressive disease, where insulin resistance and impaired glucose tolerance develop usually 9 to 12 years before the disease is diagnosed[7]. The earliest detectable peripheral insulin resistance in type 2 diabetes historically was observed in muscle[8].

There have been many proposed mechanism that may contribute to the development of insulin resistance. Proposed mechanisms include the defect of the insulin signalling pathway itself[9], the defect of glucose transporters[10] and lipotoxicity, following the increased fatty acid metabolism observed in type 2 diabetes[11]. Figure 1.1 shows the proposed mechanism of fatty acid-induced insulin resistance. Different factors contributing to the increase in visceral fat, causing lipotoxicity, include overeating, smoking, alcohol, sedentary lifestyle, genetics and aging[12].

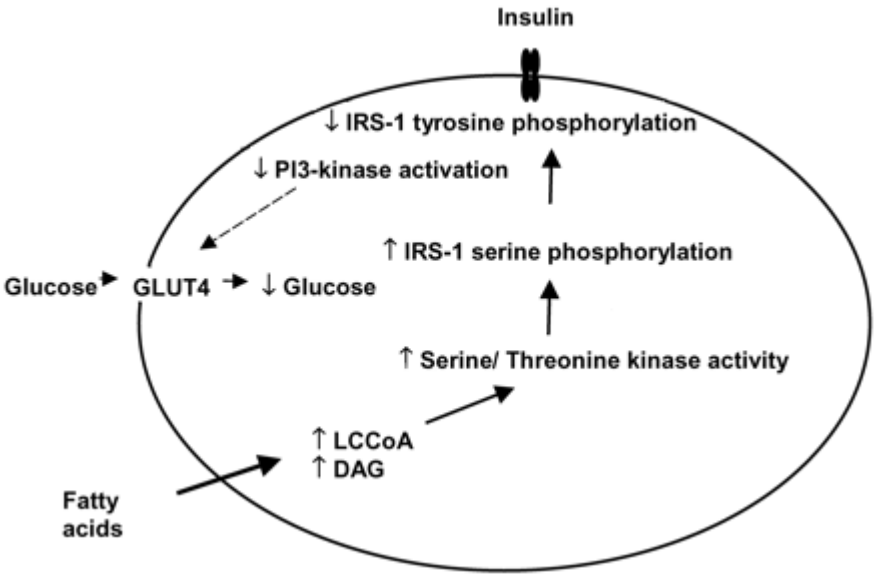

Figure 1.1: Fatty acid-induced insulin resistance. LCCoA; long-chain acyl CoA, DAG; diacylglycerol. Adapted from Savage *et al.*[13].

The insulin resistance of peripheral organs, including liver, fat and muscle, leads to glucose intolerance, which is initially compensated by increased pancreatic β-cell insulin secretion. This hyperinsulinemia may be sustained to a certain extent depending on β-cell plasticity, or it may cause β-cell apoptosis as the β-cells are unable to meet the high demands for insulin by peripheral organs, leading to hypoinsulinaemia, hyperglycaemia and insulin-dependent diabetes[14, 15]. In fact, it was shown that patients with impaired glucose tolerance had 40% decreased β-cell mass and type 2 diabetic patients had 63% decreased β-cell mass compared with control patients[16]. In addition to

make the matter worse, the remaining β-cells in type 2 diabetic patients were shown to have even lower sensitivity to glucose, further impairing the ability to secrete insulin via glucose-stimulated insulin secretion[17]. Late stage type 2 diabetes is characterised by peripheral insulin resistance, systemic hypoinsulinaemia, hyperglycaemia and dyslipidaemia[18, 19]. Figure 1.2 summarises the developmental stages of type 2 diabetes.

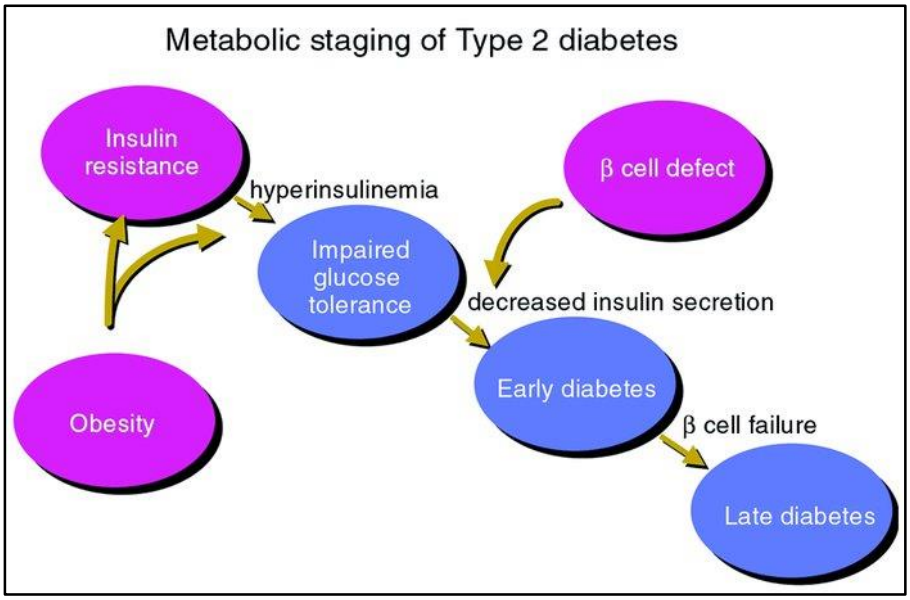

Figure 1.2: Summary of the development of type 2 diabetes. Adapted from Salteil[20].

Cardiovascular disease remains the major cause of mortality amongst type 2 diabetic patients[18, 21]. Diabetic patients have impaired cardiac ischaemiareperfusion recovery and have higher rates of mortality following a myocardial infarction[22]. Cardiovascular diseases associated with diabetes include angina, myocardial infarction, stroke, peripheral artery disease and congestive heart failure[23]. A cohort study in 1.9 million people in England indicated that heart failure and peripheral arterial disease are the most common initial manifestations of cardiovascular disease in type 2 diabetes[24]. Following a myocardial infarction, diabetic patients have almost double the rate of mortality and 3 times the rate of progression to congestive heart failure than non-diabetics, and this is independent of other risk factors such as smoking, alcohol

consumption and age[25]. In addition, type 2 diabetes can also affect cardiac structure and function independent of hypertension and coronary artery disease, and this was termed diabetic cardiomyopathy in 1972[26]. Now the term generally describes diastolic dysfunction which may become more apparent with the presence of hypertension or myocardial ischaemia[27]. The pathogenesis of diabetic cardiomyopathy is multifactorial and the proposed mechanisms include activation of the renin-angiotensin system[28], impaired cardiac calcium homeostasis[29], mitochondrial dysfunction that leads to increased oxidative stress[30, 31] and altered cardiac substrate metabolism[32, 33]. The main interest of this thesis is the altered cardiac substrate metabolism in diabetic hearts and its role in the adaptation to hypoxia.

1.2 CARDIAC ENERGY METABOLISM IN THE HEALTHY HEART

The healthy heart produces and consumes 3.5 - 5 kg of ATP every day, to sustain continuous myocardial contraction[34]. In order to cater for this need for energy, the heart metabolises primarily long-chain fatty acids which contribute 60-70% to energy production, while glucose and lactate represent 20% and 10% of the production, respectively. The healthy heart preferentially uses fatty acid as a fuel as not only can palmitate be oxidised to produce more ATP than glucose per carbon atom, it also consists of more carbon atoms per molecule, thus producing almost 3 times the ATP per molecule of substrate compared with glucose (Table 1.1) [34]. However, palmitate yields the least ATP per oxygen atom consumed, making it a less preferred substrate under hypoxic conditions. The flux through metabolic pathways is very dynamic and substrates selection can vary depending on availability of substrates, oxygen, hormones, and workload[35-37]. Regardless of the substrate chosen by the heart, more than 95% of ATP production is derived from mitochondrial oxidative phosphorylation, with the rest being derived from glycolysis and GTP formation in the tricarboxylic acid (TCA)

cycle[38]. The importance of ATP is especially emphasised during stressful conditions such as ischaemia or hypoxia as the heart will need to maintain the production of ATP for continuous contraction despite the lack of substrates or oxygen. Given that glucose is a more oxygen-efficient substrate for ATP production, it is natural that a healthy heart will switch substrate metabolism from predominantly fatty acids towards glucose. However, the glycolytic pathway is uncoupled from the mitochondrial oxidative phosphorylation as the heart increase ATP via the oxygen-independent glycolytic pathway and decrease oxygen consumption by the mitochondria[39]. In addition, the decrease in ATP levels has been shown to activate the ATP-sensitive potassium (K_{ATP}) channels, leading to action potential duration shortening and limiting Ca^{2+} influx to protect the heart from post-ischaemic damage[40, 41].

Table 1.1: ATP generation from different substrates per molecule, carbon and oxygen atom.

Substrate	**ATP yield per molecule**	**ATP yield per carbon atom**	**ATP yield per oxygen atom consumed**
Glucose	32	5.2	2.58
Lactate	15	4.9	2.46
Pyruvate	12	4.1	2.50
Palmitate	105	6.7	2.33

1.2.1 Glucose uptake and metabolism

Glucose metabolism can be divided into cytosolic glycolysis and mitochondrial oxidation. Most mammalian cells import glucose by a process of facilitative diffusion mediated by members of the glucose transporter family (GLUT). Although there are fourteen GLUT members expressed in humans, only GLUT1 to 4 have been shown to specifically regulate glucose homeostasis[42]. The main isoforms expressed in the heart

are GLUT1 and GLUT4, with GLUT1 more ubiquitously distributed and proposed to be a constitutive transport protein, with GLUT4 being responsible for the insulin-stimulated glucose uptake as it can translocate from cytosolic vesicles to the sarcolemma[43, 44].

Upon entering the cardiomyocytes, the glucose molecule is phosphorylated by hexokinase to produce glucose-6-phosphate, which can enter either glycolysis, pentose phosphate shunt or glycogen synthesis[34] (Figure 1.3). The heart stores glycogen following insulin stimulation[45], suggesting that glycogen may be an important on site fuel source that can be rapidly broken down into glucose for oxidation and ATP production. This has been shown in rat hearts, which increased glycogen breakdown during a sudden increase in work[46]. Following the phosphorylation of glucose, glucose-6-phosphate is isomerised to fructose-6-phosphate by phosphoglucose isomerase. The next step involves phosphofructokinase-1 (PFK-1) phosphorylating fructose-6-phosphate to fructose-1,6-biphosphate, and PFK-1 is one of the key regulatory enzymes in the glycolytic pathway. PFK-1 is allosterically inhibited by downstream phosphoenolpyruvate, ATP, and citrate, while activated by high concentrations of AMP[47]. The end product of glycolysis, pyruvate, can either enter the mitochondria for oxidation or be reduced to lactate in the cytosol. In normoxia, pyruvate is decarboxylated by pyruvate dehydrogenase (PDH) to form acetyl CoA in the matrix of mitochondria, which can enter TCA cycle for oxidation. PDH is a key enzyme in substrate metabolism and is regulated by pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphatase (PDP)[48]. PDK regulates PDH by reversibly phosphorylating PDH and inhibiting its activity[34]. The PDK isozymes mostly found in the heart are PDK1 and PDK4, which can be activated by the acetyl-CoA, NADH and ATP[49, 50]. On the other hand, PDP activates PDH by dephosphorylating it, and PDP is

activated by insulin and Ca^{2+}, by distinct and separate mechanisms with additive effects[51].

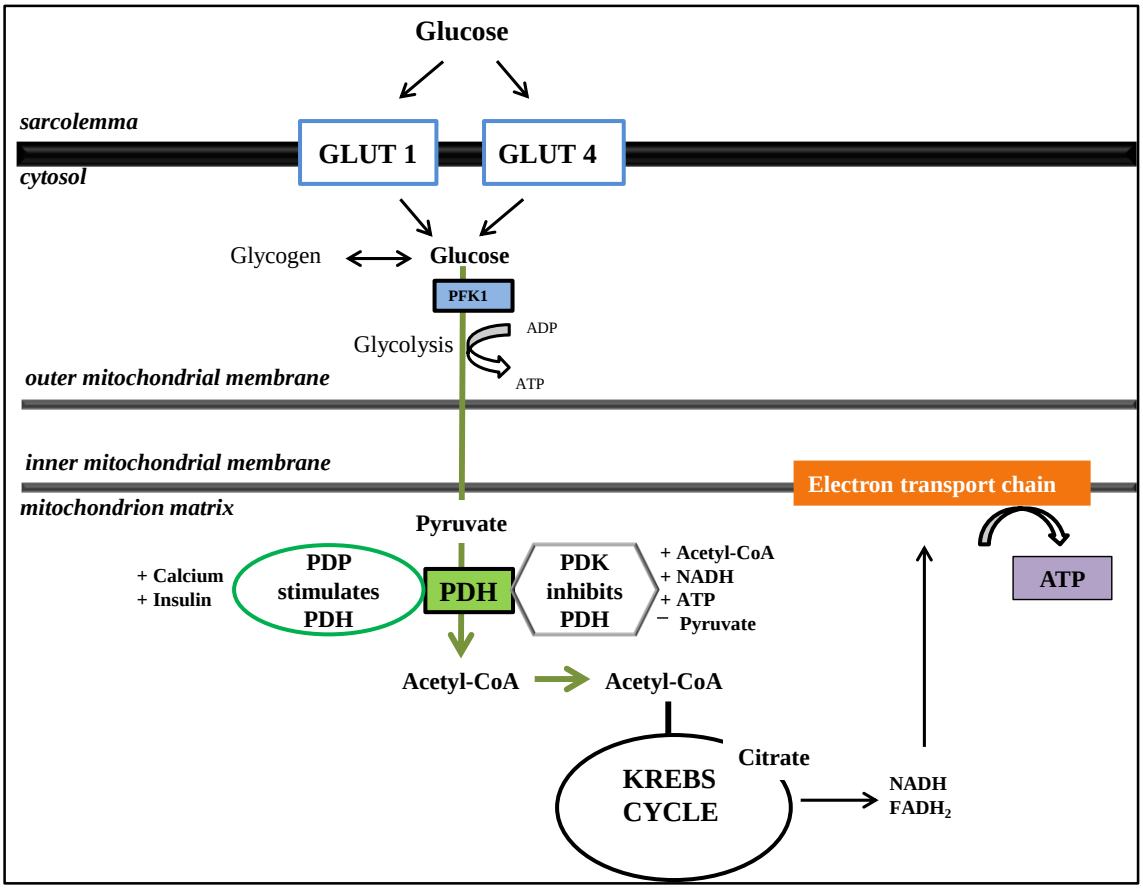

Figure 1.3: Glucose uptake and metabolic pathway. Glucose enters the cytosol via facilitative diffusion via GLUT1 and GLUT4, and is then converted to pyruvate via glycolysis. Pyruvate is transported into the mitochondria and decarboxylated to acetyl-CoA by PDH before entering the TCA cycle to produce NADH and $FADH_2$. PDH is a key regulator of substrate metabolism and is inhibited by PDK and stimulated by PDP. The electrons liberated by the oxidation of NADH and $FADH_2$ are passed along the electron transport chain to produce ATP. GLUT; glucose transporters, PFK1; phosphofructokinase 1, PDH; pyruvate dehydrogenase, PDP; pyruvate dehydrogenase phosphatase, PDK; pyruvate dehydrogenase kinase.

1.2.2 Fatty acid uptake and metabolism

In a healthy heart, circulating free fatty acid concentrations range between 0.2 and 0.6 mM[52] but the concentrations can vary depending on circumstances or disease states,

such as fasting, diabetes and myocardial infarction[53-55]. Fatty acids are transported to the heart in forms of non-esterified fatty acids (NEFA) bound to albumin or esterified to triacylglycerides contained in chylomicrons or very low-density lipoproteins (VLDL)[34, 38]. Although it has previously been demonstrated that NEFA are the primary fuel preferred by the heart[56], Hauton *et al.* showed that in isolated working heart, NEFA and triglycerides from chylomicrons or VLDL are equally capable of supporting heart function, as cardiac mechanical function was maintained regardless of lipid substrate supplied[57]. Saddik and Lopaschuk proposed that a proportion of NEFA that enters the heart gets esterified into endogenous myocardial triglycerides before being lipolysed into fatty acids again to be oxidised[56], suggesting there is a dynamic pool of lipids with turnover rates depending on hormonal and metabolic signals. Therefore, the concentration of exogenous fatty acids plays an important role in the regulation of triglyceride lipolysis[58] as increased concentrations of exogenous fatty acids inhibit endogenous triglyceride lipolysis in isolated perfused hearts and stimulate triglyceride synthesis[59].

Fatty acids are transported into the cardiomyocytes by passive diffusion or via a protein carrier-mediated pathway (Figure 1.4). These proteins include fatty acid translocase (FAT/CD36), plasma membrane fatty acid binding protein (FABPpm) and fatty acid transport proteins (FATP1 and 6). FAT/CD36 has been proposed to play a major role in the transport of fatty acid across the sarcolemmal membrane of cardiomyocytes, as the inhibition of FAT/CD36 in cardiomyocytes resulted in 50% decrease in fatty acid uptake and oxidation[60]. Cytosolic fatty acids are esterified to long chain fatty acyl coenzyme A (acyl-CoA) by acyl-CoA synthetase on the mitochondrial outer membrane. Carnitine palmitoyltransferase-1 (CPT1) in the intermembrane space replaces the CoA group on the long chain acyl-CoA with carnitine and carnitine acyltranslocase (CAT)

shuttles the fatty acyl-carnitine across the inner mitochondrial membrane in exchange for a free carnitine. In the inner membrane, CPTII converts the fatty acyl-carnitine back to long chain fatty acyl-CoA in the mitochondrial matrix[34]. The long chain fatty acyl-CoA undergoes β-oxidation in the mitochondrial matrix, resulting in the production of acetyl CoA and reduced cofactors NADH and $FADH_2$, which enter the electron transport chain to produce ATP.

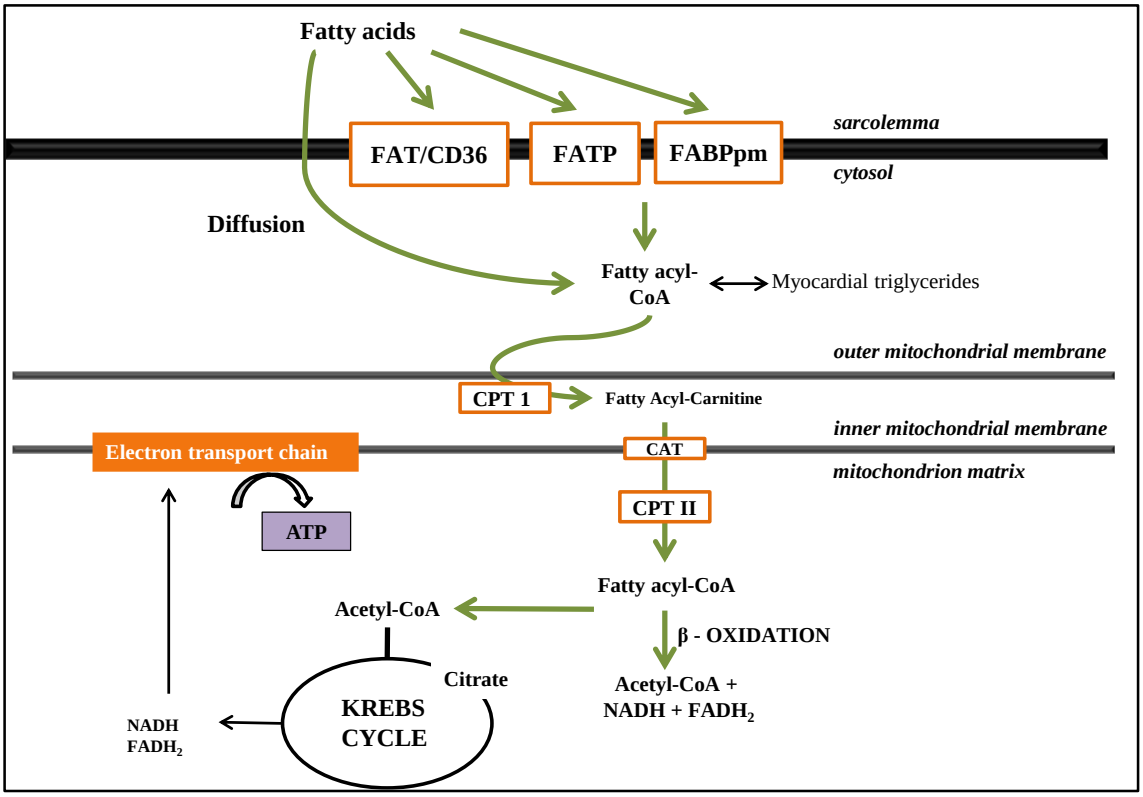

Figure 1.4: Fatty acid uptake and metabolic pathway. Fatty acids enter the cell via passive diffusion or protein carrier-mediated pathway and are converted to fatty acyl-CoA. CPT1 replaces the CoA group with carnitine in the space between outer and inner membranes. Then CAT shuttles the acyl-carnitine across the inner mitochondrial membrane where CPTII converts the molecule back to fatty acyl-CoA for β-oxidation. FAT/CD36; fatty acid translocase/cluster of differentiation 36, FATP; fatty acid transport protein, FABPpm; plasma membrane fatty acid-binding protein, CPT1; carnitine palmitoyltransferase-1, CAT; carnitine acyltranslocase, CPTII; carnitine palmitoyltransferase-II.

Glucose and lipid metabolism pathways converge at acetyl-CoA which can enter the mitochondrial TCA cycle, producing NADH and $FADH_2$ from dehydrogenase reactions. These proton carriers are oxidized to NAD^+ and FAD by Complex I (NADH–ubiquinone reductase) and from succinate dehydrogenase reaction of the electron transport chain (Figure 1.5). The electrons from the previous oxidation move via ubiquinone to Complex III (ubiquinone–cytochrome c reductase) and via cytochrome c to Complex IV (cytochrome oxidase), where they are then transferred to the terminal electron acceptor, oxygen, which is reduced to water. The protons generated from the oxidation of NADH and $FADH_2$ are pumped into the intermembrane space through complex I, III, and IV. These protons increase the electrochemical gradient across the membrane and become the driving force for ATP synthase to produce ATP from ADP. Adenine nucleotide translocase (ANT) is responsible for shuttling ATP and ADP across the mitochondrial membrane. However, uncoupling proteins (UCP) may leak the protons from the intermembrane back into the matrix, reducing the electrochemical gradient and generate heat instead. This is called mitochondrial uncoupling and it may decrease cardiac efficiency and affect cardiac function. UCP3 is one of the isoforms expressed in the heart and Cole *et al.* showed an increase in UCP3 expression in high fat fed rat hearts[61], suggesting that UCP3 may be affected by altered metabolism.

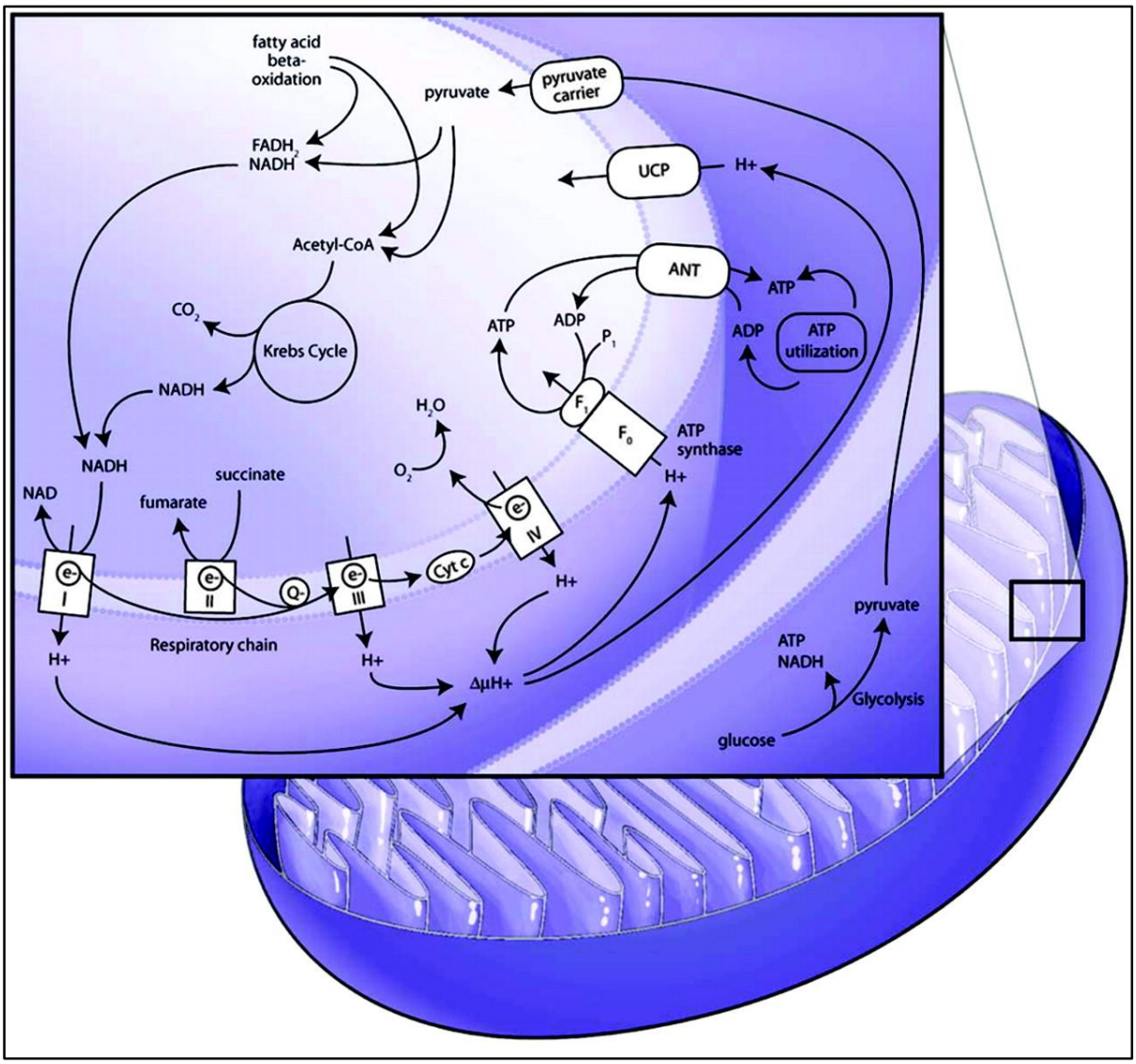

Figure 1.5: Mitochondrial electron transport chain. ANT; adenine nucleotide translocase, UCP; uncoupling protein, Q; coenzyme Q, Cyt c; cytochrome c. Adapted from Kim *et al*.[62].

1.3 THE RANDLE CYCLE AND METABOLIC FLEXIBILITY

In 1963, Randle *et al.* described the glucose-fatty acid cycle as the relationship between glucose, fatty acid flux and fuel selection in mammalian organs[63]. The authors illustrated the competition between glucose and fatty acids for oxidation in muscle and adipose tissue, and demonstrated that the utilization of one nutrient inhibited the use of the other directly without hormonal involvement. This ensures that multiple fuels are not simultaneously metabolised and that fuels are not wasted.

It has been demonstrated that fatty acids metabolism inhibits glucose utilisation in the heart[63], and that glucose uptake, hexokinase, PFK-1 and PDH are the key steps in glucose metabolism that are inhibited[64, 65]. An increase in fatty acid β-oxidation increases acetyl-CoA and NADH which inhibits PDH via PDK activation[66]. The increase in acetyle-CoA that enters the TCA cycle increases citrate concentrations, which inhibits PFK-1[67]. Finally the inhibition of PFK-1 increases in glucose-6-phosphate, which inhibits hexokinase[68]. The degree of inhibition of glucose metabolism by fatty acid oxidation varies between the key steps involved, with the least impact on glucose uptake, followed by PFK-1 flux and then PDH, which could undergo almost complete inhibition[68, 69]. In addition, if the heart is exposed to a prolonged increase in fatty acid metabolism, fatty acids can bind to the transcription factor, peroxisome proliferator-activated receptor α (PPARα) to stimulate a cascade of transcriptional events that increase expression of genes involved in fatty acid uptake and in mitochondrial and peroxisomal β-oxidation of fatty acids[70-72]. However, PPARα does not only increase fatty acid metabolism but it also upregulates PDK4 mRNA and protein levels in the heart[73, 74], suppressing the glucose metabolic pathway as well.

As the increase in fatty acid metabolism inhibits glucose metabolism, the vice versa is also true that an increase in glucose metabolism inhibits fatty acid oxidation[63]. An increase in glycolysis produces more mitochondrial acetyl-CoA, which can be indirectlt exported into the cytosol[67]. Cytosolic acetyl-CoA can be carboxylated by acetyl-CoA carboxylase (ACC) to produce malonyl-CoA, which is a potent inhibitor of CPT1, blocking fatty acid entry into the mitochondria for oxidation. Citrate and glutamate are allosteric activators of ACC[75, 76] while long and medium chain fatty acids act as negative feedback inhibitors of ACC[77].

Insulin also plays a role in ACC regulation by stimulating its dephosphorylation and activation[78]. Insulin is a hormone, which is a major regulator of metabolism and it plays an important role in shifting substrate metabolism towards glucose utilisation by stimulating glucose uptake and utilisation[79] while suppressing fatty acid oxidation[80].

The term ‘cardiac metabolic flexibility’ is used to describe the capacity of the heart to match substrate selection and oxidation with substrate availability[81]. This flexibility also facilitates utilisation of the most efficient substrate for a given environment, and is achieved through metabolic signals that regulate key steps of metabolic pathways[82]. Therefore, the Randle cycle, in combination with hormonal regulation, is vital for the heart so that it is able to switch fuel selections depending on different environments such as fasted state, insulin-stimulated post-prandial state or hypoxia, to ensure continuous supply of ATP.

1.4 ABNORMAL CARDIAC ENERGY METABOLISM IN THE TYPE 2 DIABETIC HEART

The type 2 diabetic heart is metabolically abnormal and its metabolism is shifted away from glucose metabolism and the dependence of the heart on fatty acid metabolism is increased[33]. Insulin-stimulated glucose uptake in the heart is primarily mediated by GLUT4 and studies on animal models have shown that diabetic hearts have decreased GLUT4 concentrations[83, 84], suggesting that the decrease in glucose metabolism is occuring from the very first step in the pathway. Diabetic patients have increased plasma free fatty acids and Kim *et al.* have found that elevated free fatty acids may be responsible for decreasing whole body glucose uptake by 15% and glycolysis by 30-50%[85]. Further down the glucose metabolism pathway, there is a significant decrease in PDH flux via increase in PDK4 levels in diabetic rat hearts, reaffirming that glucose metabolism is downregulated at multiple key steps in type 2 diabetes[86]. In addition, the downregulation of glucose also means that there is insufficient acetyl-CoA produce from the glucose metabolism pathway to be converted to malonyl-CoA by ACC, which is needed to inhibit CPT1 in order to limit fatty acid oxidation.

Elevated plasma free fatty acid concentrations are common in type 2 diabetic patients[87]. This in turn facilitates increased fatty acid uptake into cardiomyocytes and subsequently increases fatty acid oxidation[38]. Increased fatty acid metabolism in the diabetic heart can also be seen via increased expression of PPARα and its downstream targets, which are responsible for transcriptional upregulation of fatty acid uptake and oxidation genes[88]. One of the downstream products of PPARα is UCP3, which is expressed on the inner mitochondrial membrane and has been shown to uncouple mitochondrial respiration from ATP synthesis[89]. The expression of UCP3 was shown to be increased in diabetic rat hearts[90] and in high fat fed mouse hearts[61]. In addition, increased long

chain fatty acid supply to skeletal and cardiac muscles upregulates UCP3 mRNA and protein levels, with greatest effect being seen in cardiac muscle[91]. Medium chain acyl-coenzyme A dehydrogenase (MCAD), another PPARα target, is an enzyme responsible for the oxidation of fatty acids with chain lengths between 6 and 12 carbons in β-oxidation. It was demonstrated that MCAD activity was elevated in high fat fed rat hearts[61]. Interestingly, diabetic hearts are exposed to both hyperglycaemia and hyperlipidaemia but yet choose to upregulate fatty acid metabolism and decrease glucose metabolism, which is likely due to the insulin resistance in the diabetic heart[63, 92]. This increase in lipid availability and metabolism also increases the chance of lipid peroxidation by reactive oxygen species produced in the mitochondria and consequently damage the mitochondria, decreasing mitochondrial oxidative capacity[93].

This inability of the diabetic heart to upregulate glucose metabolism despite the hyperglycaemic state indicates an inflexibility of metabolism to a given stimulus. Cardiac metabolic inflexibility was seen in diabetic hearts as they maintained elevated lipid metabolism regardless of any stimuli[94]. Cardiac metabolic flexibility is especially important during events such as myocardial infarction when the heart needs to switch its dependency from fatty acid metabolism to the more anaerobic glycolysis. However, in the type 2 diabetic heart, insulin resistance and glucose metabolism inhibition by elevated lipid metabolism via the Randle cycle may impair ‘metabolic flexibility’ and prevent the natural ability of the heart to switch fuel utilisation under different circumstances.

1.5 HYPOXIA, CARDIOVASCULAR DISEASE AND METABOLISM

Hypoxia results from an imbalance between oxygen demand and supply and, in a tissue, is caused by inadequate blood flow, known as ischaemia, and/or a reduction in arterial blood oxygen partial pressure[95]. During a myocardial infarction, the heart undergoes an ischemic assault, causing a shortage of substrates and oxygen needed for cellular metabolism, and the accumulation of waste products such as lactate in the cells. Hypoxia is therefore a component of ischaemia. Hypoxia activates the hypoxia-inducible factor (HIF), which is a central transcription factor that activates a large number of genes responsible for oxygen delivery, angiogenesis, cell proliferation, cell differentiation, and metabolism[96, 97]. HIF is a heterodimer composed of an alpha subunit, such as HIF-1α, and a beta subunit, which is constitutively expressed and stable irrespective of the oxygen level[98]. HIF-1 was discovered by the identification of a hypoxia response element (HRE) in the 3′ enhancer of the gene for erythropoietin, responsible for erythrocyte proliferation in hypoxia[99]. Following the cloning of HIF-1α, HIF-2α was identified and cloned, revealing that it shares 48% amino acid sequence identity with HIF-1α[100]. HIF-3α, which was discovered later, is also expressed in a variety of tissues, although HIF-3α is not as extensively studied compared to the other 2 family members[101]. However, HIF-1 is the more relevant family member of HIF in the heart and hence will be focused on in this thesis.

The HIF-1α subunit is virtually undetectable under normoxic conditions, since it is rapidly degraded by the ubiquitin–proteasome pathway. In normoxia, HIF-1α is hydroxylated on proline residues 402 and 564 by the prolyl hydroxylase domain (PHD) family of enzymes, which require oxygen and 2-oxoglutarate, as co-substrates, and iron (Fe^{2+}) and ascorbate, as co-factors. The involvement of iron as co-factor has been well documented in studies using hypoxia-mimetic such as iron antagonists and chelators,

such as desferrioxamine (DFO) and cobalt chloride[102]. Upon hydroxylation, HIF-1α is recognised by the von Hippel–Lindau protein (VHL), which is part of an ubiquitin ligase complex, known as the E3 ligase complex, which targets HIF-1α for polyubiquitination and subsequent proteasomal degradation[98].

The asparagine 803 residue of HIF-1α is also hydroxylated under normoxic conditions by a specific asparagine hydroxylase named Factor Inhibiting HIF (FIH), which impairs the interaction of the transcriptional co-activators p300/CREB binding protein with the HIF-1α c-terminal transactivation domain. This leads to further repression of the transcriptional activity of HIF[98, 103].

In hypoxia, the proline and asparagine residues of HIF-1α are no longer hydroxylated as there is no oxygen available for the PHD and FIH enzymes, and therefore HIF-1α escapes degradation. HIF-1α can then translocate to the nucleus to dimerise with HIF-1β, and initiate transcription via hypoxia response elements (HREs) within the target genes[104, 105]. Consequently, HIF-1 activates the expression of genes that ensure cell survival at low oxygen levels. Summary of the HIF pathway regulation can be seen in Figure 1.6.

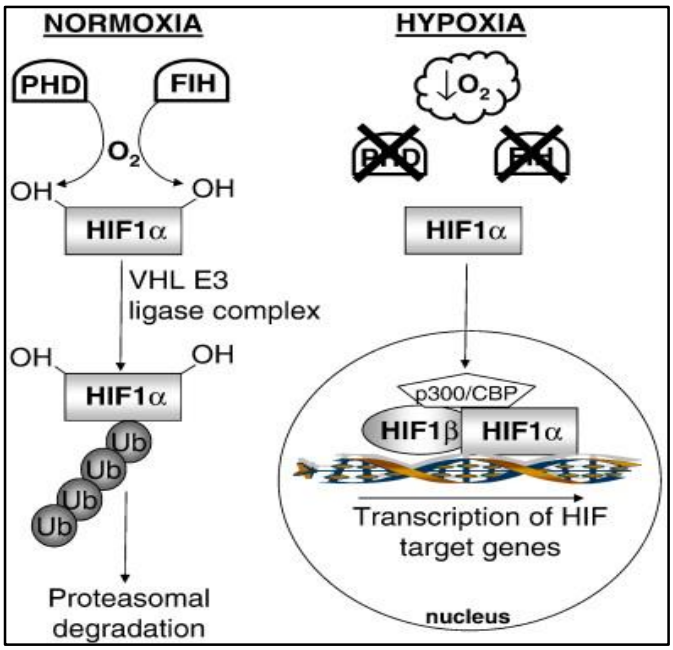

Figure 1.6: Regulation of the HIF pathway in normoxia and hypoxia. PHD; prolyl hydroxylase, FIH; factor inhibiting HIF, VHL; Von Hippel-Lindau factor, Ub; ubiquitin, CBP; CREB-binding protein. Adapted from Heather and Clarke[15].

1.5.1 Cardiac metabolism in hypoxia

In hypoxia, HIF-1 promotes cell survival under hypoxic conditions by mediating a switch from oxidative to glycolytic metabolism. HIF does this by upregulating glucose uptake, via GLUT1, and glycolysis to optimise oxygen consumption efficiency via anaerobic ATP production[106-108]. In rat hearts, there was increased hexokinase protein levels following 3 weeks of chronic hypoxia[109] and similarly, HL-1 cardiomyocytes were shown to have increased glycolytic capacity following hypoxia[110]. In addition, HIF also activates PDK1, which inhibits PDH, uncoupling glycolysis and the TCA cycle to decrease mitochondrial glucose oxidation and oxygen consumption[111]. HIF is also shown to activate VEGF in tumour cells in order to increase angiogenesis[112], resulting in increase in blood flow and oxygen delivery to the tumour cells. In a healthy heart, this could be essential in chronic hypoxia as a coping mechanism to increase blood flow and oxygen delivery to the heart.

Due to the fact that fatty acids produces less ATP per mole of oxygen consumed compared with glucose, there is a decrease fatty acid oxidation in conjunction with an increase in glycolysis to decrease oxygen consumption. Bass *et al*. showed that in rat hearts, a decrease in fatty acid utilisation after prolonged exposure to high altitude hypoxic conditions[113]. There was decreased enzyme activity of β-hydroxyacyl-CoA dehydrogenase, a fatty acid β-oxidation enzyme, and CPT1 in rat hearts following chronic hypoxia[114, 115]. The decrease in fatty acid metabolism in hypoxia is likely due in part to the decrease in PPARα activation but there were contrasting findings as to whether the decrease in PPARα is a HIF-dependent or independent process[116-118].

Hypoxia and HIF-1α also directly target the mitochondria, by downregulating enzymes involved in the TCA cycle and electron transport chain, to decrease oxidative phosphorylation. Heather *et al.* demonstrated that the activity of aconitase, a TCA cycle enzyme was decreased in hypoxia[119]. In hypoxia, HIF-1 upregulates proteins associated with optimising Complex IV activity in hypoxia[98]. In addition, chronic hypoxia has been shown to decrease activities of complex I, II and IV[119].

1.5.2 Hypoxia and diabetes

Type 2 diabetic patients have higher rates of mortality following myocardial infarction[25], and hypoxia is an important component of ischaemia. One of the proposed causes of the decreased survivability of diabetic patients following ischaemia is that diabetic hearts have abnormal adaptation to hypoxia, which would increases the damage from ischaemia-reperfusion injury.

Marfella *et al.* demonstrated that there was an increase in infarct size in type 1 diabetic rat hearts due to hyperglycaemia, associated with reduced HIF-1α expression[120] and later showed that human type 2 diabetic hearts had reduced expression of HIF-1α and

vascular endothelial cell growth factor (VEGF) in ischaemic regions[121]. VEGF is a HIF target gene and plays a vital role as an endothelial-specific mitogen in angiogenesis[122], which is crucial in ischaemia or hypoxia to ensure adequate oxygen is delivered to the cells for survival. The decrease in VEGF in diabetes is supported by data from studies that showed that there is decrease in myocardial capillary density in diabetic rats[123, 124], which may be detrimental in chronic hypoxia as the capillaries are unable to maintain oxygen delivery to the myocardium.

From a metabolic perspective, hypoxia and diabetes have opposing effects on substrate selection and utilization, with hypoxia promoting glycolysis and suppressing lipid metabolism, while diabetes promotes lipid metabolism and suppresses glycolysis. Coupled with the cardiac metabolic inflexibility reported, the diabetic heart may be less able to shift its metabolism in response to hypoxia, resulting in the maladaptation of diabetic hearts in hypoxia. This may contribute to the deleterious effects following myocardial infarction in the type 2 diabetic heart

1.6 CURRENT THERAPIES OF TYPE 2 METABOLISM THAT MODULATE METABOLISM

Sulphonylureas such as glyburide, glipizide, and glimepiride act on pancreatic β-cells to stimulate insulin secretion, similar cascade of events as glucose-stimulated insulin secretion[125]. However the most concerning side effect of this class of drug is hypoglycaemia. There are also nonsulfonylurea agents that stimulate short-lived burst of insulin secretion such as Repaglinide and nateglinide with the same side effect of causing hypoglycaemia in addition to possible weight gain[126]. Metformin is a biguanide widely used to treat type 2 diabetic patients, which act as an insulin-sensitising agent

instead of stimulating insulin secretion. Although the mechanism of action of metformin is not fully understood, it has been demonstrated that metformin targets AMPK[127] and it decreases hepatic gluconeogenesis, thus improving fasting hyperglycemia. Metformin is associated with significant decrease in macrovascular complications, myocardial infarction, and stroke in the UKPDS[128]. Thiazolidinediones such rosiglitazone and pioglitazone, is another class of insulin-sensitising agent, which binds to PPARγ and affect transcriptional activity related to lipid metabolism in adipocytes. Thiazolidinediones has been shown to decrease serum free fatty acid concentrations in diabetic patients and consequently increased insulin sensitivity in muscle tissue[129, 130], implicating the role of free fatty acid in insulin resistance development. Another class of drugs inhibit glucose absorption by competitively inhibiting α-glucosidase in the gastrointestinal tract, preventing breakdown of oligo- and disccharides into monosaccharides[131]. Currently available α-glucosidase inhibitors are acarbose, miglitol, and vaglibose. The most common side effects include bloating, abdominal discomfort, diarrhea, and flatulence[132]. Fibrates such as gemfibrozil and fenofibrate act on PPARα to decrease triglyceride synthesis and promote high density lipoprotein production[133]. While orlistat is not a treatment for type 2 diabetes per se, it is used to treat obesity and acts as a gastric and pancreatic lipase inhibitor that decreases free fatty acid and cholesterol absorption[134].

1.7 THESIS AIMS

The main objective of this thesis was to investigate the effect of hypoxia on cardiac metabolism and function in the type 2 diabetic heart. The main hypothesis of this thesis is that type 2 diabetic hearts have abnormal metabolism and defects in the HIF signalling pathway. The studies of this thesis were conducted to examine how the type 2 diabetic hearts changes its metabolism in response to both chronic and acute hypoxia, and the associated changes in cardiac function. Others suggest that the HIF signalling pathway is impaired in the type 1 diabetic heart[120, 121, 135], hence the HIF signalling pathway in type 2 diabetic heart was hypothesised to be impaired as well. At the end of this thesis, the aim was to determine whether the abnormal response in diabetic hearts to hypoxia could be improved using a metabolic modulator.

The first aim of the thesis was to establish a type 2 diabetic rat model which closely resembles human type 2 diabetes pathophysiology including insulin resistance, obesity, hyperglycaemia, and dyslipidaemia. A new rat model was needed as most of the studied type 2 diabetic models are mouse models, which were unsuitable for our studies due to technical limitations of heart size. In addition, rat models that were available were lean, took a long time to develop or had phenotypes that are too severe, resembling type 1 diabetes. Therefore this chapter aimed to develop a type 2 diabetic rat model that was inexpensive and easy to induce compared to other existing rat models, using combination of low dose STZ and high fat diet. The cardiac characterisation of the new model was conducted to determine the severity of the model as well as resemblance to human type 2 diabetes.

The second aim was to investigate the effect of chronic hypoxia on cardiac metabolism and contractile function in diabetic rats housed in the normobaric hypoxic chamber.

This was aimed to test if the type 2 diabetic heart had impaired metabolic adaptation to chronic hypoxia and if there were any changes in the HIF signalling pathway. In addition, metabolic flexibility in the diabetic heart in chronic hypoxia was investigated.

The third aim was to investigate the adaptation of the diabetic heart to acute hypoxia and its effect on cardiac metabolism and contractile function. Since diabetic hearts have bigger infarct size following ischaemia[120], acute hypoxia, as a component of myocardial ischaemia was used to determine if the type 2 diabetic heart had an impaired functional adaptation to acute hypoxia. In addition, the metabolic changes in the diabetic hearts during acute hypoxia and upon reoxygenation were studied.

Lastly, Chapter 6 aimed to use sulfo-N-succinimidyl oleate (SSO), a FAT/CD36 inhibitor, to inhibit fatty acid uptake and oxidation, as a metabolic modulator to improve cardiac metabolism and contractile function of the diabetic heart in acute hypoxia. This would determine if fatty acid metabolism indeed plays a key role in the maladaptation to acute hypoxia in the diabetic heart.

CHAPTER 2

MATERIALS AND METHODS

2.1 ANIMAL HANDLING AND GENERATION OF TYPE 2 DIABETIC MODEL

All animal studies conducted conformed to the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act of 1986 and to University of Oxford institutional guidelines. Healthy male Wistar rats, purchased from Harlan, UK, were housed on a 12hr light/dark cycle and kept under controlled conditions of temperature, light and humidity, with *ad libitum* access to high fat diet or chow (see Appendix 1 for composition) and water. The high fat diet contains 60% kcal from fat, 35% kcal from protein and 5% kcal from carbohydrate (see Appendix 2 for composition). Rats weighing ~250 – 350g were used for the generation of the diabetic model. Control and diabetic rat groups were weight-matched at the start of the study. For induction of type 2 diabetes, rats were fed high fat diet for 2 weeks followed by an intraperitoneal injection of streptozotocin (STZ) (Sigma, UK) after fasting overnight. STZ was dissolved in 300 µl citrate buffer (30mM citric acid and 20mM sodium citrate, pH 4) immediately prior to injection. Please refer to Chapter 3 for the different doses of STZ initially tested, to define the optimal dose of 25 mg/kg, which was used in all other subsequent chapters. The diabetic rats were monitored closely and weighed daily for a week after the injection. The diabetic rats were maintained with high fat diet and control rats with chow for a further 4 weeks after the injection for all subsequent studies except the model development study (Chapter 3) where rats were only maintained for 1 more week after the injection[136] (Figure 2.1).

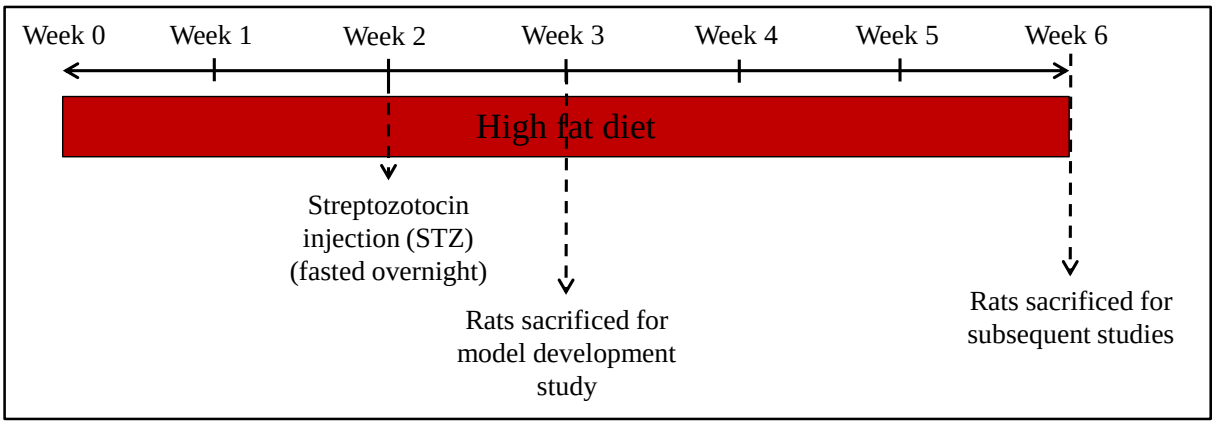

Figure 2.1: Timeline of type 2 diabetic rat model generation using high fat diet in combination with low dose STZ.

2.2 MEASUREMENT OF BLOOD PLASMA METABOLITES AND HAEMATOCRITS

Fasting blood plasma was obtained via the saphenous vein while rats were anaesthetized with 1.5% isoflurane in oxygen. Terminal blood was collected following rat heart excisions. The glucose meter, Accu-Chek Compact Plus Diabetes Monitoring Kit was used to determine blood glucose from a small volume of blood. The glucose meter contains disposable strips with dehydrated glucose oxidase, glucose dehydrogenase or hexokinase and rehydrates the enzyme upon contact with blood sample[137]. The meter also incorporated enzyme into a biosensor that generates an electron that is detected by the meter. Glucose concentrations from the glucose meter were compared with plasma glucose concentrations from plasma analyses and found to be consistent. Plasma was separated via centrifugation at 16000 g for 5 mins at 4˚C and stored at -80 ^{o}C. Blood was also collected to measure haematocrit using a microhaematocrit reader (Hawksley, UK). Plasma was analysed on ABX Pentra 400 (Horiba ABX Diagnostics) for glucose, lactate, triglycerides (TAG), non-esterified fatty acids (NEFA), cholesterol and β-hydroxybutyrate (β-OHB) concentrations. Insulin concentrations were measured using a rat insulin ELISA kit (Mercodia).

2.3 ISOLATED HEART PERFUSIONS

2.3.1 Preparation of Krebs-Henseleit buffer

The Krebs-Henseleit (KH) buffer containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.97 mM $CaCl_2$, 0.5 mM EDTA, 25 mM $NaHCO_3$, 11 mM glucose and 1.2 mM K_2HPO_4 was prepared and filtered. Fatty acid-free bovine serum albumin (BSA) (Lampire, USA) (1.5% w:v) and 0.4 mM palmitate were added to the KH buffer (please see Appendix 3 for details). This solution was placed in dialysis tubing and left to dialyse overnight against BSA- and palmitate-free KH buffer, to allow chelation of free calcium by albumin. Immediately prior to use, the free calcium concentration was measured using a Radiometer blood gas analyser and adjusted if necessary, to a final concentration of 1.35 mM.

2.3.2 Ex vivo Langendorff heart perfusion

Rats were anaesthetised using intraperitoneal pentobarbitone and hearts were rapidly excised, arrested in ice-cold KH buffer and weighed. Hearts were cannulated via the aorta, and perfused in Langendorff mode with KH buffer (gassed 95% O_2, 5% CO_2) at 37^{o}C, at a constant perfusion pressure of 100 mmHg. The left atrium was opened and a fluid-filled, PVC balloon was inserted into the left ventricle (Figure 2.2) and inflated to an end-diastolic pressure of 4-8 mmHg. This was connected by polyethylene tubing to a pressure transducer, and the signal acquired via a bridge amplifier connected to a PowerLab data acquisition system (ADInstruments, Oxfordshire, UK) (Figure 2.3). This system measures pressure development within the left ventricle, with the peak and nadir of the wave representing the end-systolic and end-diastolic pressure, respectively. Heart rates were determined by trigger points on the ascending limb of the pressure waveform. Left ventricular developed pressure (LVDP) was determined as end-systolic

pressure minus end diastolic pressure (EDP) (Figure 2.4). Rate pressure product (RPP) was calculated as the product of LVDP and heart rate. A healthy rat heart should be achieving an RPP of 35000 mmHg/min as published by previous studies in the group[138, 139] and any control hearts with RPP less than 15000 mmHg/min were excluded as the decrease may have been caused by technical difficulties. There are also other challenges and limitations with this perfusion setup. Firstly, the hearts were perfused under superoxia with pO_2 of 500 mmHg due to the fact that KH buffer does not contain haemoglobin as oxygen carrier and if the pO_2 was lower, the hearts would undergo hypoxia and affect cardiac function and metabolism. This also posed a limitation in perfusing chronically hypoxic hearts in Chapter 4 as it may alter cardiac metabolism when perfused under superoxic environment. However this is a limitation that has been acknowledged and efforts have been put into improving the protocol for future studies. Another limitation is that the perfusion setup used a constant pressure setting which posed a limitation for coronary flow measurements in Chapter 5 when hearts were perfused in acute hypoxia. In acute hypoxia, it was predicted that coronary flow will decrease following the decrease of cardiac function but because of the constant pressure in the perfusion rig, the buffer was forced down into the heart regardless of the demand of the heart, therefore not fully representing a physiological coronary flow in acute hypoxia.

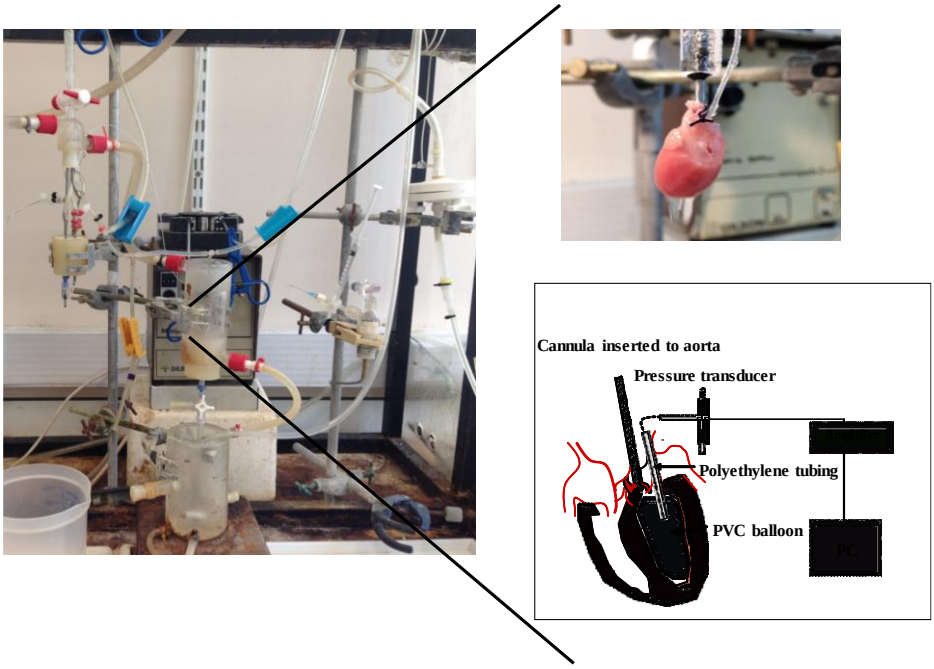

Figure 2.2: A PVC, fluid filled balloon was inserted into the left ventricle, and connected to a pressure transducer and data acquisition system, allowing for measurement of cardiac function

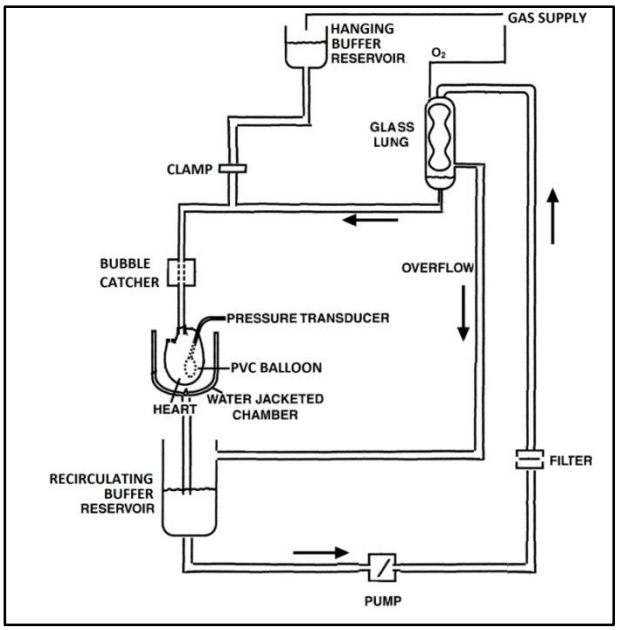

Figure 2.3: Schematic diagram of the Langendorff isolated heart perfusion set up adapted from Schenkman 2001[140]. Isolated heart is cannulated via the aorta and kept in a water-jacketed chamber to maintain a constant temperature of 37°C. Perfusion buffer was filtered through 5.0 μm filters (Millipore, UK) and oxygenated using a glass lung located above the heart, at a height equivalent to a perfusion pressure of 100 mmHg.

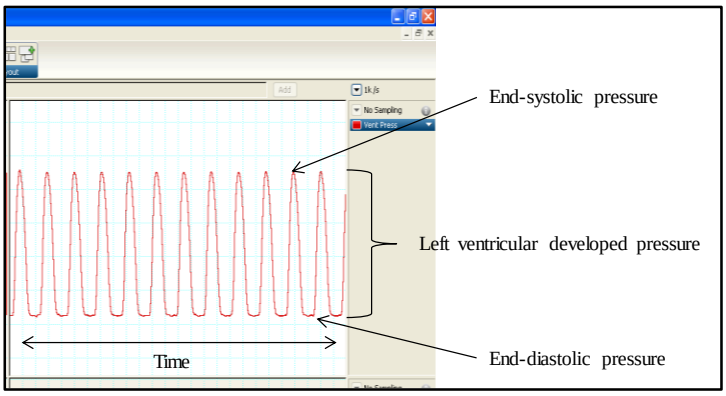

Figure 2.4: Example of a PowerLab file, showing left ventricular developed pressure obtained by deducting end-diastolic pressure from end-systolic pressure

2.3.3 Determination of glycolytic rates

Hearts were perfused with 200 ml recirculating KH buffer containing 11 mM glucose and 0.4 mM palmitate bound to 1.5% fatty acid-free bovine serum albumin, and 0.16 µCi/ml of D[5-^{3}H]-glucose (Amersham, UK). Following a 10 min stabilisation period, basal glycolytic rates were determined by collecting 1 ml aliquots of recirculating buffer every 4 mins for 20 mins. Insulin-stimulated glycolytic rates were determined after addition of 0.3 U/l insulin to the recirculating buffer, and 1 ml aliquots were again collected every 4 mins for a further 32 mins. Glycolytic rates were determined in perfusion aliquots, as the conversion of ^{3}H-glucose to $^{3}H_2O$ via enolase which converts 2-phosphoglycerate to phosphoenolpyruvate and releases H_2O as a byproduct. The aliquots of perfusate contain both $^{3}H_2O$ and ^{3}H-glucose. Therefore, the $^{3}H_2O$ was separated from ^{3}H-glucose using a Dowex 1 x 4 chloride form, 100-200 mesh (Sigma, UK) anion exchange column. Preparation of the Dowex was made by adding 250 g of Dowex resin into a solution containing 1.25 M NaOH and 1.61 M boric acid. The mixture was then mixed gently and the beads were repeatedly washed with distilled H_2O until the mixture reached pH<7.5. The exchange columns were prepared by using glass Pasteur pipettes. The bases of the pipettes were filled with glass wool and while

the pipettes were held with a rack, Dowex resin was added up to the neck of the pipette. The columns were washed through once with distilled H_2O and allowed to drain. Scintillation vials were placed underneath the columns to collect samples. Then 200 µl of perfusate was added to the column and incubated for 15 minutes, allowing the ^3H-glucose to bind to the column and ^3H_2O to be eluted into the vials. A volume of 2 ml of distilled H_2O was added into the column to wash down any residual samples. The scintillation vials were collected after the columns were fully drained and 10 ml of Ecolite liquid scintillation cocktail (MP Biomedicals, US) was added before the radioactivity (cpm) was measured by the scintillation counter.

In addition, a duplicate of 0.2 ml perfusate at time 0 was added directly into scintillation vials and had its radioactivity counted to determine the specific activity of the buffer. For each aliquot sample, glycolytic rates were calculated using the equations:-

Total glycolytic flux per heart (µmol/heart):

$$\frac{[\text{sample cpm} - \text{sample time 0 cpm}] \times \text{recirculating buffer volume (ml)}}{[\text{specific activity.ml}^{-1} - \text{sample time 0 cpm}] \times \text{glucose (mM)}}$$

Total glycolytic flux per gram wet weight (µmol/gww):

$$\frac{\text{Total glycolytic flux (µmol.heart}^{-1})}{\text{Heart weight (g)}}$$

The glycolytic rates per gram wet weight (µmol/gww/min) were calculated by plotting the total glycolytic flux for all samples against time. A minimum of 5 data points were fitted with linear regression and the gradient was equal to the glycolytic rate.

2.3.4 Determination of palmitate oxidation rates

A separate set of hearts were perfused with 200 ml recirculating KH buffer containing 11 mM glucose and 0.4 mM palmitate bound to 1.5% fatty acid-free bovine serum

albumin (Sigma, UK) and containing 0.16 µCi/ml [9,10-^{3}H]-palmitate (Amersham, Bucks, UK). Following a 10 min stabilisation period, aliquots of recirculating buffer were collected every 4 minutes during the perfusion protocol. Palmitate oxidation rates were determined by the production of $^{3}H_2O$ from the electron transport chain as described by Barr *et al.*[141]. The aliquots of perfusate contain both $^{3}H_2O$ and ^{3}H-palmitate, so the $^{3}H_2O$ was separated via Folch extraction. A volume of 1.88 ml of chloroform: methanol (1:2 v/v) solution, 625 µl chloroform and 625 µl KCl-HCl solution (2 M KCl, 0.4 M HCl) were added to 0.5 ml of perfusate sample. The mixture was rotated on a laboratory Stuart rotator SB3 at 40 rpm for 1 hour before removing the top aqueous layer into another tube, while the organic layer was discarded. To the extracted aqueous solution, 1 ml chloroform was added, 1 ml methanol and 0.9 ml KCl-HCl solution before it was mixed again by rotation for 1 hour at 40 rpm. The top aqueous layer was once again removed and retained, and 0.5 ml (in duplicate) of this phase was added into a scintillation vial containing 10 ml of Ecolite liquid scintillation cocktail (MP Biomedicals, US) and the radioactivity (cpm) of the sample was counted. In addition, a duplicate of 0.5 ml perfusate at time 0 was added directly into scintillation vials and had its radioactivity counted to determine the specific activity of the buffer.

For each aliquot sample, palmitate oxidation rates were calculated using the equations:-

Total palmitate oxidation per heart (µmol/heart):

$$\frac{[\text{sample cpm} - \text{sample time 0 cpm}] \times 8.26 \times 2 \times \text{recirculating buffer volume}}{[\text{specific activity.ml}^{-1} - \text{sample time 0 cpm}] \times 2 / \text{palmitate (mM)}}$$

Total palmitate oxidation per gram wet weight (µmol/gww):

$$\frac{\text{Total palmitate oxidation (µmol.heart}^{-1})}{\text{Heart weight (g)}}$$

The dilution factor of 8.26 was due to the dilution of the buffer aliquot during the extraction protocol. As 0.5 ml aliquots were diluted in scintillation fluid and counted for radioactivity, to normalise to counts per ml values were multiplied by 2. The rates of palmitate oxidation per gram wet weight (μmol/gww/min) were calculated by plotting total palmitate oxidation for all samples against time. A minimum of 5 data points were fitted with linear regression and the gradient was equal to the palmitate oxidation rate.

2.3.5 Determination of lactate efflux

Lactate assay buffer (0.2 M Tris base, 10 mM EDTA, 1.55 M hydrazine hydrate and 0.15 mM nicotinamide adenine dinucleotide (NAD)) was prepared. A final volume of 2 ml of solution in a cuvette was prepared with either perfusate or lactate standards (from a 2 mM lactate stock solution), and 1 ml of the lactate assay buffer (Table 2.1). An absorbance at 340 nm was determined for all standards and samples before 10 μl lactate dehydrogenase (LDH) was added into each cuvette. Following incubation for 20 minutes at room temperature, the absorbance was again determined, to measure the increase in reduced NAD (NADH) concentrations. Lactate concentrations (mM) were calculated from the differences in absorbance, using the equation of the linear regression line fitting the standard curve. Lactate efflux rate (μmol/gww/min) was determined by plotting the amount of lactate in perfusate against time.

The reaction involved in this assay is as follow:

Table 2.1: Concentrations and volumes of assay buffer, H_2O, lactate stock solutions and samples in lactate assay.

Final lactate concentration (mM)	Lactate assay buffer (µl)	Distilled H_2O (µl)	Lactate stock solution or sample (µl)
0	1000	1000	0
0.00625	1000	993.75	6.25
0.0125	1000	987.5	12.5
0.025	1000	975	25
0.05	1000	950	50
0.075	1000	925	75
X	1000	900	100

2.4 HEPATIC AND CARDIAC TRIGLYCERIDE ASSAY

Frozen liver and heart tissue were crushed using a pestle and mortar under liquid nitrogen and 25-50 mg of tissue were weighed out and added to 10 ml glass test tubes containing 5 ml Folch solution (2:1 chloroform:methanol v/v). An additional 3ml Folch solution was added and the mixture was rotated on a Stuart rotator SB3 at 40 rpm for 1 hour. Then 2 ml water was added and mixed for another hour. Samples were left overnight to separate. The upper phase was removed and discarded leaving the lower organic phase, which was dried under air. Next, 1 ml ethanol was added and mixed thoroughly, and a duplicate of 100 µl sample was taken and left overnight to evaporate. The final sample was prepared by resuspension in 20 µl ethanol and the TAG concentration was measured using a commercial kit (Randox).

2.5 HEPATIC AND CARDIAC GLYCOGEN ASSAY

Approximately 50 mg freeze-clamped tissue was weighed out, and added to 100 µl of a solution of 30% (w/v) potassium hydroxide to destroy endogenous protein. Samples

were heated at 105°C for 1 hour and 50 µl of a solution of 2% sodium sulphate and 600 µl ethanol was added. Samples were incubated at 4°C overnight and centrifuged at 16000 g for 5 mins at 4˚C to pellet the glycogen, before being resuspended in 500 µl of 80% ethanol by sonication. After drying at 40°C overnight, 375 µl water was added and glycogen was converted to glucose by addition of 125 µl amyloglucosidase enzyme buffer (1M sodium acetate solution was made by mixing 0.82 g of sodium acetate with 10 ml of 1M acetic acid, and added to 5 mg of amyloglycosidase (Sigma, UK) to make up 10 ml of amyloglycosidase enzyme buffer). After incubation at 37°C for 1 hour, 100 µl water was added and the final solution centrifuged, before being analysed for glucose concentrations on ABX Pentra 400 autoanalyser (Horiba ABX Diagnostics). Results were expressed as µmol glucosyl units per gram wet weight (µmol/gww).

2.6 CARDIAC PYRUVATE DEHYDROGENASE (PDH) AND CITRATE SYNTHASE (CS) ACTIVITY ASSAY

Approximately 50 mg tissue was weighed out in duplicate, and homogenised with a Polytron homogeniser for 30 seconds at 5680 g in an extraction buffer containing 25 mM HEPES (pH 7), 25 mM KH_2PO_4, 25 mM KF, 1 mM dichloroacetate, 3 mM EDTA, 1 mM ADP, 1 mM dithiothreitol, 0.05 mM leupeptin and 1% Triton. Samples were freeze-thawed 3 times and then centrifuged at 4930 g for 5 mins at 4˚C to remove debris. The assay buffer for PDH activity assay containing 50 mM HEPES (pH 7.2), 1 mM $MgCl_2$, 80 µM EGTA, 1 mM dithiothreitol, 4 µM rotenone, 1.67 mM NAD, 0.1 mM co-enzyme A, 0.2 mM thiamine pyrophosphate and 16.7 mM lactate and 2 U/ml LDH, and was incubated at 30°C for 5 minutes prior to the addition of each sample[142, 143]. Following addition of 25 µl of each sample to 975 µl of PDH assay buffer, the reaction was followed spectrophotometrically at 340 nm over a 2 minute time period. The initial rate of NADH production was determined by the gradient over the first 60

seconds from which PDH activity was calculated. The 5 minute incubation period was to produce pyruvate from lactate and then the addition of the tissue lysates will start the pyruvate to acetyl-CoA conversion by PDH (Figure 2.5),

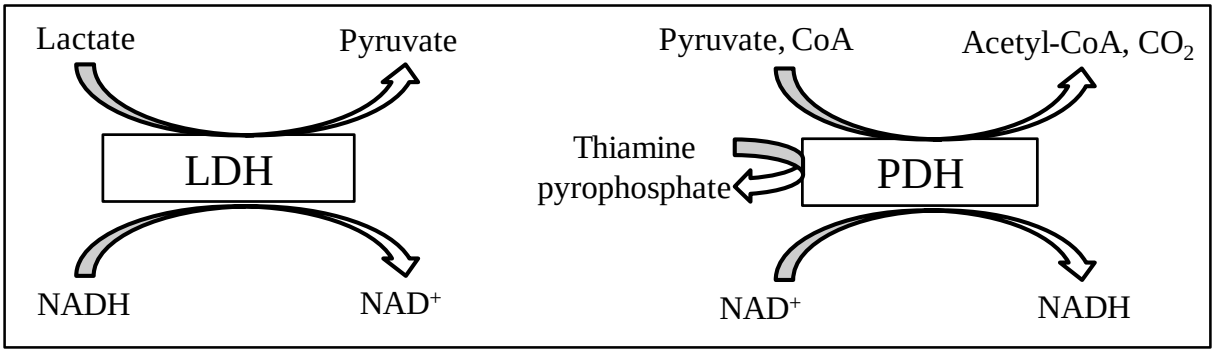

Figure 2.5: The reactions driven by LDH in the PDH assay buffer during the 5 minute incubation and then upon the introduction of tissue lysates after the incubation, PDH activity was measured as the rate of NADH production.

On the same extracts, a citrate synthase activity assay was conducted concomitantly. A volume of 50 µl of extracted sample was added to a cuvette containing 100 µl CS assay buffer (1mM dinitrobenzoic acid and 1 M Tris HCl (pH 8.1), 50 µl acetyl CoA and 750 µl water). The mixture was incubated at 37ºC for 5 minutes before adding 50 µl of 10mM oxaloacetate and immediately reading at 412 nm over a 2 minute time period. The rate of enzyme activity, represented by thionitrobenzoic acid (TNB) production, was measured by the gradient over the first 60 seconds[144].

The reaction catalysed by citrate synthase is as follow:

$$\text{Acetyl-CoA} + \text{Oxaloacetate} + H_2O \longrightarrow \text{Citrate} + \text{CoA-SH} + H^+$$

The reaction which is measured is as follow:

$$\text{CoA-SH} + \text{Dinitrobenzoic acid} \longrightarrow \text{Thionitrobenzoic acid} + \text{CoA-S-S-TNB}$$

2.7 CARDIAC MEDIUM-CHAIN ACYL-COA DEHYDROGENASE (MCAD) ACTIVITY ASSAY

Approximately 20 mg of crushed tissue was added to a homogenisation buffer of 50 mM KH_2PO_4, 1 mM EDTA and 2 mM $MgCl_2$ to make a 5% homogenate, and kept on ice. A volume of 900 µl of reaction mixture (100 mM KH_2PO_4, 1 mM EDTA, 0.5mM $Na_2S_4O_6$ and 0.2 mM ferrocenium hexafluorophophate ($FcPF_6$) (pH 7.4)) was added to a warmed (37°C) cuvette with 20 µl sample, and then 50 µM octanoyl-CoA was added. Absorbance was measured at 300 nm for 3 mins at 37°C and MCAD activity calculated. This method is as described by Lehman *et al.*[145].

2.8 WESTERN BLOTTING

All western blotting experiments were carried out with the assistance of Dr. Lisa Heather. All western blots were optimised by different tissues and fragments of the hearts to identify the band of interest. For example, tissues that express GLUT4 such as cardiac tissue were blotted together with liver tissue, which normally does not express GLUT4, therefore allowed us to identify the band of interest with confidence. However, the limitation of this experiment is that it cannot measure the translocation of proteins to the sarcolemma and measure the total protein in the tissue instead. This is more relevant especially in the acute settings where translational changes to switch substrate metabolism could not occur in the short period of time and most changes were attributable to protein translocations to increase or decrease substrate uptake.

2.8.1 Lysate preparation

Approximately 50 mg of crushed heart tissue was added to 500 µl of lysis buffer (Table 2.2), containing protease inhibitor, and homogenized using a Polytron homogenizer for 30 seconds at 5680 g. The lysates were boiled for 5 min and centrifuged at 16000 g for

10 min at 4˚C, the supernatant was removed and aliquots taken for determination of protein concentration (Bicinchoninic acid protein assay kit, Perbio, UK). To the remaining supernatant, 5% β-mercaptoethanol (v/v) was added, followed by boiling for 5 min and storing at -80 ºC.

2.8.2 Polyacrylamide gel electrophoresis and protein transfer

Equal concentrations of protein were diluted in Laemmli loading buffer (Table 2.2) and loaded onto 12.5% polyacrylamide gels. Gels were run at 120 V for approximately 2 hours. A piece of Immobilon-P membrane (Millipore, UK) and two pieces of chromatography paper (BioRad, UK) were cut to the same size as the gel. These were soaked with the gel for 30 min in sodium dodecyl sulphate (SDS) transfer buffer (Table 2.2), layered onto the transfer apparatus (Biorad, UK) and transferred at 0.18 A per gel for 1 hour. The membranes were stained with Ponceau S stain (Sigma, UK) to determine successful protein transfer and even protein loading. Blots were washed in Tris-buffered saline (TBS)-Tween (Table 2.2) and blocked in 5% (w/v) dried milk in TBS-Tween (25 ml per blot) for at least 1 hour.

2.8.3 Immunoblotting, detection and band quantification

The membrane was washed for 1 hour in TBS-Tween, with the solution changed every 15 min. The specific primary antibody (see Table 2.3 for list of antibodies) was diluted in 5% (w/v) dried milk in TBS-Tween (12 ml per blot), and incubated on the membrane overnight. The membranes were then washed for 1 hours in TBS-Tween, with the solution changed every 15 min. The appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz, USA) was diluted in 5% (w/v) dried milk in TBS-Tween (12 ml per blot), and incubated on the membrane. Membranes were washed for a further hour in TBS-Tween, with the solution changed every 15 min. The membrane was covered in enhanced chemiluminescence (ECL) detection solution (Amersham,

UK) and sandwiched between sheets of acetate. Membranes were exposed to X-ray film and developed using a Compact X4 automatic X-ray film processor (X-ograph Imaging systems, UK). Protein bands were quantified using Un-Scan-It, Version 6.1 (Silk Scientific, USA)[146].

Table 2.2: Composition of buffers used in western blotting experiments.

Lysis buffer	**Laemmli loading buffer**
75 mM tris-HCl pH 6.8 3.8% SDS (w/v) 4 M urea 20% glycerol (v/v)	187.5 mM tris-HCl 6% SDS (w/v) 30% glycerol (v/v) 0.003% bromophenol blue (w/v) pH 6.8
SDS transfer buffer	**TBS-Tween**
25 mM tris 192 mM glycine 20% methanol (v/v)	0.9% NaCl (w/v) 10 mM tris-base 0.05% Tween (v/v) pH 7.4

Table 2.3: Primary antibodies used in western blotting experiments.

Primary antibody	Primary antibody supplier	Dilution	Secondary antibody	Dilution
GLUT4	Donation of Prof. Geoff Holman, University of Bath, UK	1:12500	Goat anti-rabbit	1:2500
GLUT1	Abcam, UK	1:1000	Goat anti-rabbit	1:2500
FAT/CD36	Donation of Dr Narendra Tandon, Otsuka Maryland Medicinal Laboratories, USA	1:50000	Goat anti-mouse	1:2500
FABPpm	Donation of Dr Jorge Calles-Escandon, Wake Forest University School of Medicine, USA	1:50000	Goat anti-rabbit	1:3571
FATP1	Santa Cruz, CA, USA	1:200	Donkey anti-goat	1:1786
MCT1	Santa Cruz, CA, USA			
PDK4	Donation of Prof. Mary Sugden, Queen Mary's, University of London, UK	1:50000	Goat anti-rabbit	1:3846
UCP3	Abcam, UK	1:2500	Goat anti-rabbit	1:2500
PDK1	Novus Biologicals	1:3000	Goat anti-rabbit	1:2500
HIF1α	Cell Signalling	1:500	Goat anti-rabbit	1:2500

2.9 STATISTICAL ANALYSIS

Results are expressed as means ± standard error of the mean (SEM). Data were analysed using one way or two way analysis of variance (ANOVA) with post-hoc t-testing. $p < 0.05$ was taken as the level of statistical significance. Relevant statistical analysis is described in detail in specific chapters. Softwares SPSS (IBM) and Prism (GraphPad) were used for the statistical analyses.

CHAPTER 3

TYPE 2 DIABETIC RAT MODEL DEVELOPMENT

3.1 ABSTRACT

Type 2 diabetes is characterised by hyperinsulinaemia, hyperglycaemia and dyslipidaemia, accompanied by abnormalities in cardiac metabolism including increased lipid and decreased carbohydrate metabolism. The aim of this study was to develop a new rat model of type 2 diabetes and investigate the cardiac and systemic metabolic phenotypes. Male Wistar rats were fed a high fat diet for two weeks, followed by a single intraperitoneal injection of streptozotocin (STZ), and sacrificed a week after the injection. Different doses of STZ (15, 20, 25, 30 mg/kg body weight) were tested, to determine the optimal dose to induce type 2 diabetes. There was a dose-dependent increase in plasma glucose (from 12.7 mM in controls to 14.7 – 25.1 mM in diabetes) and a dose-dependent decrease in plasma insulin levels with increasing concentration of STZ (from 3.03 ug/L in controls down to 1.04 ug/L with the highest dose of 30 mg/kg STZ). There was an increase in cardiac and hepatic triglycerides in diabetic rats compared with controls, in a dose-independent manner. Cardiac UCP3 protein levels were significantly increased, whereas cardiac GLUT4 protein levels were significantly decreased in diabetic hearts compared with controls. Cardiac PDH activity displayed a dose-dependent relationship between enzyme activity and STZ concentration. Cardiac glycolytic rates were measured in 15 mg/kg STZ high-fat fed diabetic rats and there was no significant difference compared with control hearts, suggesting 15 mg/kg was insufficient to impair cardiac glucose metabolism as seen in type 2 diabetes. Consequently, the dose of 25 mg/kg of STZ was chosen for future studies, as it most closely resembled type 2 diabetes without causing overt pancreatic dysfunction. Here we have established a novel type 2 diabetic rat model using combination of low dose STZ and high fat feeding that is inexpensive, easy to induce and exhibits human type 2 diabetes pathophysiology.

3.2 INTRODUCTION

As the prevalence of type 2 diabetes grows on a global scale, a great understanding of the disease and the development of better therapeutics is needed. To achieve this, thoroughly characterised and clinically relevant animal models of type 2 diabetes are required. Animal models should resemble the human type 2 diabetes pathology, displaying insulin resistance, obesity, hyperglycaemia, and dyslipidaemia. There are currently a few existing rodent models that have been commonly used and characterised[147,148]. The ob/ob and db/db mouse models have been used to demonstrate cardiac metabolism and efficiency in type 2 diabetes[149, 150] but these models have the tendency to develop cardiac hypertrophy which may not be ideal to investigate cardiac metabolism in diabetic hearts independent of diabetic cardiomyopathy. Two other genetic mouse models which are commonly used are Akita diabetic mouse and OVE26 mouse, but these are type 1 diabetic models rather than type 2 diabetes. From a technical perspective, a mouse model is not the most suitable for experiments involving isolating mitochondria, as the techniques require larger amounts of heart tissue than a mouse heart can provide.

Of the existing rat models, the Goto-Kakizaki rat model has been extensively studied but unfortunately it is lean and obesity is one of the key characteristics of human type 2 diabetes[151]. The Zucker fatty rats are obese, and have increased serum triglycerides, fatty acid and insulin levels. However, they only start to develop hyperglycaemia at 6 weeks of age and do not stabilise until the age of 10-12 weeks[152, 153], which means it could be costly in terms of both time and money. There are the Otsuka Long-Evans Tokushima Fatty rats which have a late onset of hyperglycaemia of 18 weeks[154], which is not ideal from a time and cost perspective. The Zucker diabetic fatty rats, on the other hand, develop cardiac hypertrophy[155], have dramatically altered substrate metabolism

and impaired cardiac contractility[156-158], similarly displaying an extreme end-stage phenotype of type 2 diabetes, rather than that present in most patients with type 2 diabetes, Given the disadvantages of the existing diabetic rat models, a novel model, which is inexpensive, takes a short time to generate and closely resembles the human type 2 diabetic pathophysiology is needed. The comparison between models made was based on severity and development time of the pathology of the models instead of cardiac metabolic characterisations as not all of these models have their cardiac metabolism characterised. For example, The ZDF rats have been shown to have decreased cardiac PDH activity[159] but metabolic studies on GK rats has been primarily on its pancreatic islet metabolism[160, 161]. Also, while the mouse models were better characterised metabolically, as mentioned before, the heart tissue they provide is insufficient for mitochondrial experiments. Therefore the aim of this chapter was to develop a diabetic rat model and then characterise its cardiac metabolism to further reaffirm our choice of model. Table 3.1 highlights the disadvantages of existing diabetic rodent models to date.

Table 3.1: Existing diabetic rodent models and their disadvantages for this study.

Diabetes model	Disadvantages
Zucker Fatty rats	Model for obesity but not hyperglycaemic and develop cardiac hypertrophy with unaltered cardiac efficiency and CA^{2+} handling
Zucker Diabetic Fatty rats	Type 1 model inbred from Zucker Fatty rats, becomes too severe too soon
Otsuka Long-Evans Tokushima Fatty rats	Type 2 model with late onset of hyperglycemia (18 weeks)
Goto-kakizaki (GK) rats	Type 2 non-obese model, hence not representing human pathophysiology
Db/db mouse	Type 2 model with cardiac hypertrophy and reduced cardiac output, LV-develop pressure and cardiac power which may affect perfusions. Heart tissue insufficient for mitochondrial experiments
Ob/ob mouse	Type 2 model with obesity but develops cardiac hypertrophy which may affect cardiac function. Heart tissue insufficient for mitochondrial experiments
Akita diabetic mouse	Type 1 model with onset 5 to 6 weeks (human 15-25 years). Heart tissue insufficient for mitochondrial experiments
OVE26 mouse	Type 1 model with diabetes development in first week postpartum. Heart tissue insufficient for mitochondrial experiments

A model using the combination of high fat diet and streptozotocin (STZ) has been recently used by Srinivasan *et al.*[136]. The high fat diet has been shown to cause insulin resistance and obesity, while STZ is a drug that impairs insulin secretion via its ability to induce selective necrosis in pancreatic β-cells[162]. STZ is selectively accumulated in pancreatic β-cells via GLUT2, methylating and fragmenting DNA molecules, leading to the depletion of cellular energy stores in the attempt to repair the DNA damage, which ultimately leads to β-cell necrosis[163-166]. This appears to be a good alternative rat model

to the commonly used genetic models. However, there is very little knowledge on cardiac metabolism in this rat model to date. It was decided that the model based on Srinivasan *et al.*is more suitable for this study based on the pathology of the model but Wistar rats were used instead of Sprague-Dawley rats as most of our research group's previous studies on cardiac metabolism used Wistar rats and therefore would allow a more direct comparison. The possibility of substituting high fat diet with western diet was also discussed but it was decided that since this model is based on a publication, changing more variables on top of the strain of rat used may not produce comparable results. In addition, another limitation of this study is that glucose tolerance test was not performed on the diabetic rats as the protocol was not establish in the group's project license. It would have been reaffirming that the model was indeed diabetic should the test be carried out and this could possibly be a future experiment to further validate the model.

It was also made aware that the STZ model also has its limitations including potential for extrapancreatic genotoxic effects, which may downregulate genes in other tissues such as the liver which are irrelevant to the development of type 2 diabetes and cause other detrimental effects[167]. In the heart, STZ may directly impair cardiac contractile function through a p38 MAP kinase-dependent oxidative stress mechanism[168]. In addition, STZ may produce varying severity of the disease depending on the consistency of administration procedure and mitochondrial dysfunction may only develop in rats with ketosis despite the equivalent degrees of hyperglycaemia[169], although this is more relevant to type 1 diabetic STZ models.

A model that not only resembles human type 2 diabetes systemically, but also displays the cardiac phenotype of human type 2 diabetes is required. In the diabetic heart, there is an increase in fatty acid metabolism and decrease in glucose metabolism[84, 170].

Peroxisome proliferator-activated receptor α (PPARα) is a nuclear receptor which increases transcription of fatty acid metabolism enzymes and is activated in the diabetic mouse hearts, shown by increases in the expression of PPARα gene and its downstream target genes[88]. One of the downstream products of PPARα is the mitochondrial uncoupling protein 3 (UCP3), which is increased in diabetic rat hearts[90] and in high fat fed mouse hearts[61]. In addition, medium-chain acyl-CoA dehydrogenase (MCAD), another PPARα target involved in the mitochondrial fatty acid oxidation pathway, was elevated in high fat fed rat hearts[61].

Glucose enters the cells via glucose transporter (GLUT) and studies on animal models have shown that diabetic hearts have decreased GLUT4 concentrations[83, 84]. An important enzyme in glucose metabolism is pyruvate dehydrogenase (PDH), which catalyses the decarboxylation of pyruvate to produce acetyl-CoA for the TCA cycle. Type 2 diabetic rat hearts have a significant decrease in PDH flux, reaffirming that glucose metabolism is downregulated in diabetes[86].

Therefore, the aim of this chapter is to develop a new rat model resembling human type 2 diabetes which displays insulin resistance, obesity, hyperglycaemia, and dyslipidaemia. We will use the combination of STZ and high fat diet. The optimal dose of STZ to be administered needed to be determined so as not to completely inhibit insulin production, producing a type 1 diabetic model[171]. Secondly, cardiac metabolism will be characterised to determine if this model displays the increase in fatty acid metabolism and decrease in glucose metabolism seen in type 2 diabetic patients[33, 39]. This model will be used in later chapters to investigate the metabolic and contractile response to chronic and acute hypoxia in diabetic rat hearts and the signalling pathways involved.

3.3 METHODS

3.3.1 Animal handling and generation of type 2 diabetic model

All animal studies conducted conformed to the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act of 1986 and to University of Oxford institutional guidelines as described in Chapter 2. Diabetes was induced by feeding Wistar rats high fat diet for two weeks followed by an overnight fast and then a single intraperitoneal injection of STZ at between 09:00 and 10:00. The rats were maintained for another week on high fat diet, while control rats were fed chow for the three week protocol. Different doses of STZ were used to determine the optimal dose to develop type 2 diabetes: ranging from 0, 15, 20, 25 and 30 mg/kg, where 0 mg/kg represents the group of rats maintained on high fat diet only without STZ.

3.3.2 Problem solving - insulin stimulated glycolytic rates in isolated perfused hearts

Detailed description of *ex vivo* Langendorff heart perfusion is given in Chapter 2. We aimed to optimise the protocol for measuring insulin-stimulated glycolytic rates in control rat hearts. Hearts were perfused with Krebs-Henseleit (KH) buffer containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.97 mM $CaCl_2$, 0.5 mM EDTA, 25 mM $NaHCO_3$, 11 mM glucose and 1.2 mM K_2HPO_4. Firstly hearts were perfused with KH buffer (without palmitate or bovine serum albumin (BSA)) and 3 U/l insulin was added, as described in previous perfusion studies[146, 172]. In a second group, hearts were perfused with KH with 1.5% BSA alone and 3 U/l insulin. A third group of hearts were perfused with KH buffer containing 1.5% BSA bound to 0.4 mM palmitate, and 3 U/l insulin. A lower concentration of insulin (0.3 U/l) was also used, to determine its

effect on glycolytic rates. In addition, hearts from fasted or fed rats were perfused to determine the effect on rates of glycolysis.

3.3.3 Statistical analysis

Results are expressed as means ± standard error of the mean (SEM). Data were analysed using one way analysis of variance (ANOVA) and $p < 0.05$ was taken as the level of statistical significance.

3.4 RESULTS

3.4.1 Physical parameters

Control rats gained 69 ± 3g of body weight during the 3 week protocol (Table 3.2). The lower doses of STZ (0, 15, 20, and 25 mg/kg) did not cause any significant change in total body weight gain, whereas a significant body weight loss was observed in the final week following the injection of 30 mg/kg STZ compared to the control group and other doses ($p < 0.05$). There was no change in body weight gain between groups during the first 2 weeks of diet modification alone but after the STZ injection at the end of the second week, the group with highest dose of STZ lost weight compared to the other groups. Epididymal fat pad weight is an indicator of total body adiposity, and there was a significant increase in epididymal fat pad weight and fat pad to body weight ratio in the higher doses of STZ compared with controls ($p < 0.05$). There was no significant change in the heart weight or heart to body weight ratio between groups, indicating there was no significant cardiac hypertrophy.

Table 3.2: Physical parameters of control and diabetic rats, induced using high fat diet and low dose STZ.

	STZ doses					
	Controls	**0 mg/kg**	**15 mg/kg**	**20 mg/kg**	**25 mg/kg**	**30 mg/kg**
Total body weight gain (g)	69 ± 3	82 ± 7	88 ± 15	93 ± 5	77 ± 9	50 ± 9 $^{\#}$
Body weight change during 2 weeks of diet modification (g)	46 ± 1	49 ± 7	56 ± 12	60 ± 2	55 ± 8	55 ± 7
Body weight change in final week following STZ or control (g)	23 ± 4	33 ± 5	32 ± 2	33± 3	22 ± 4	-5 ± 4 *†
Epididymal fat pad weight (g)	4.8 ± 0.4	7.0 ± 0.7	5.7 ± 0.4	7.6 ± 0.7	9.8 ± 0.6 $^{*\#}$	8.3 ± 0.7 *
Epididymal fat pad to body weight ratio (%)	1.5 ± 0.1	2.0 ± 0.2	1.8 ± 0.1	2.4 ± 0.2 *	2.5 ± 0.1 *	2.3 ± 0.2 *
Heart weight (g)	1.19 ± 0.08	1.20 ± 0.03	1.08 ± 0.06	1.10 ± 0.08	1.04 ± 0.06	1.13 ± 0.11
Heart to body weight ratio (x 10^3)	36 ± 0.2	3.5 ± 0.1	3.4 ± 0.2	3.4 ± 0.2	3.2 ± 0.2	3.1 ± 0.1

*p < 0.05 vs. controls, †p < 0.05 vs. all other doses, #p < 0.05 vs. 15 mg/kg STZ. *n* = 11 for control group, n = 4 for diabetic groups. STZ; streptozotocin

3.4.2 Plasma metabolites in the fed state

Blood glucose concentrations were not significantly different between the control group, high fat diet alone (0 mg/kg) and 15 mg/kg STZ, but there was a significant increase with higher doses of STZ compared with controls ($p < 0.05$), with the 30 mg/kg STZ group having a significantly higher blood glucose concentration compared

to all other groups (Table 3.3). There was a significance decrease in plasma insulin concentration observed in the 30 mg/kg STZ group, with no difference in the other groups compared with controls. Non-esterified fatty acids (NEFA) concentrations were increased in 30 mg/kg STZ compared with controls, 15 and 25 mg/kg STZ groups ($p < 0.05$). Triglycerides (TAG) and β-hydroxybutyrate (β-OHB) concentrations were also increased significantly with 30 mg/kg STZ compared to all other groups. Cholesterol concentrations were elevated following 25 mg/kg STZ compared to controls and lower doses of STZ. Regression analysis showed positive relationships between STZ dose and plasma glucose ($r^2 = 0.37$), TAG ($r^2 = 0.30$), β-OHB ($r^2 = 0.20$) and significant negative relationship between STZ dose and plasma insulin ($r^2 = -0.25$) ($p < 0.05$ for all).

Table 3.3: Plasma metabolite concentrations of control and diabetic rats in the fed state, induced using high fat diet and low dose STZ.

	STZ doses					
Metabolites	**Controls**	**0 mg/kg**	**15 mg/kg**	**20 mg/kg**	**25 mg/kg**	**30 mg/kg**
Glucose (mM)	12.7 ± 0.3	14.6 ± 1.8	14.7 ± 0.6	17.2 ± 1.2 *	16.9 ± 1.5 *	25.1 ± 1.1 *†
Insulin (µg/L)	3.03 ± 0.51	2.68 ± 0.57	2.00 ± 0.31	1.59 ± 0.36	1.93 ± 0.26	1.04 ± 0.22*
NEFA (mM)	0.28 ± 0.06	0.29 ± 0.05	0.29 ± 0.02	0.29 ± 0.01	0.14 ± 0.04	0.59 ± 0.07 *€
Triglycerides (mM)	1.79 ± 0.20	1.13 ± 0.22	0.87 ± 0.06	1.41 ± 0.15	1.79 ± 0.35	7.25 ± 1.33 *†
β-OHB (mM)	0.26 ± 0.04	0.33 ± 0.04	0.45 ± 0.07	0.77 ± 0.06	0.98 ± 0.21	5.39 ± 0.59 *†
Cholesterol (mM)	2.46 ± 0.13	2.07 ± 0.21	1.68 ± 0.15	2.11 ± 0.27	3.87 ± 0.58*$	3.02 ± 0.44 #

*$p < 0.05$ vs. controls, †$p < 0.05$ vs. all other doses, #$p < 0.05$ vs. 15 mg/kg STZ, \$$p < 0.05$ vs. 0, 15 and 25 mg/kg, €$p < 0.05$ vs. 15 and 25 mg/kg. *n* = 11 for control group, n = 4 for diabetic groups. STZ; streptozotocin

3.4.3 Hepatic intracellular substrate stores

Hepatic triglyceride and glycogen concentrations were measured to investigate the changes in liver metabolism following high fat diet in combination with low dose of STZ (Figure 3.1). High fat diet alone did not change hepatic glycogen or triglyceride concentrations. High fat diet with STZ at doses of 15, 20 and 30 mg/kg decreased hepatic glycogen concentrations compared with controls. High fat diet in combination with all doses of STZ increased hepatic triglycerides concentrations, compared with controls and high fat diet alone. Hepatic triglyceride correlated positively, while glycogen correlated negatively, with STZ dose (glycogen r^2 = -0.23, triglycerides r^2 = 0.39, $p < 0.05$).

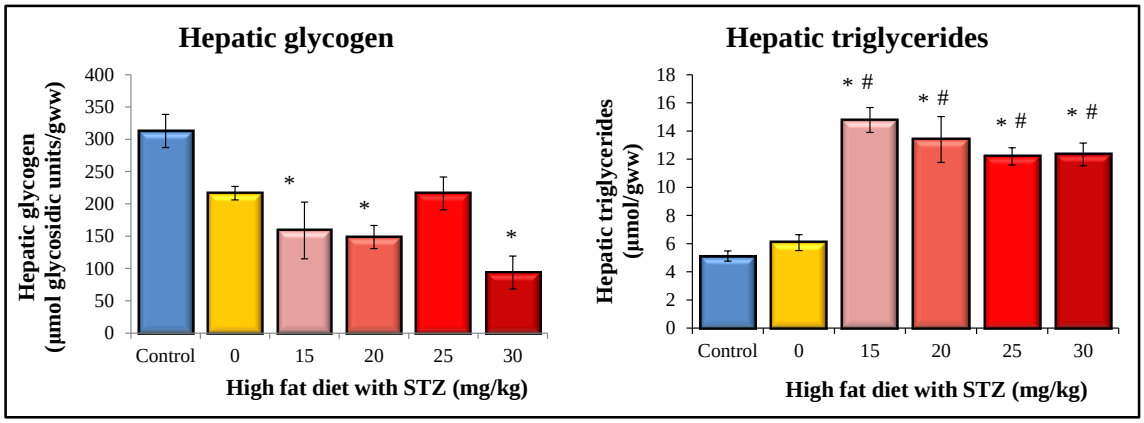

Figure 3.1: Hepatic glycogen and triglyceride concentrations in control and diabetic rats following high fat feeding in combination with increasing doses of STZ. *$p < 0.05$ vs. control, #$p < 0.05$ vs. high fat only, n = 10 for control group, n = 4 for diabetic groups.

3.4.4 Cardiac intracellular substrate stores

The aim of this study was to develop a type 2 diabetic rat model, which exhibited not only the systemic phenotype of type 2 diabetes, but also the cardiac metabolic alterations observed in humans and other animal models[38, 173, 174]. Cardiac glycogen concentrations were decreased by high fat diet alone and high fat diet in combination

with 15 and 20 mg/kg STZ, but higher doses of STZ (25 and 30 mg/kg) had no significant effect compared with controls (Figure 3.2). The highest dose of STZ (30 mg/kg) increased cardiac glycogen significantly compared to high fat diet alone and lower doses of STZ (15 and 20 mg/kg) ($p < 0.05$). Similar to hepatic triglyceride concentrations, high fat diet alone did not affect cardiac triglyceride concentrations, but high fat diet in combination with all doses of STZ increased cardiac triglycerides significantly compared to controls. Both glycogen and triglycerides positively correlated with STZ dose (glycogen $r^2 = -0.33$, triglycerides $r^2 = 0.25$, $p < 0.05$).

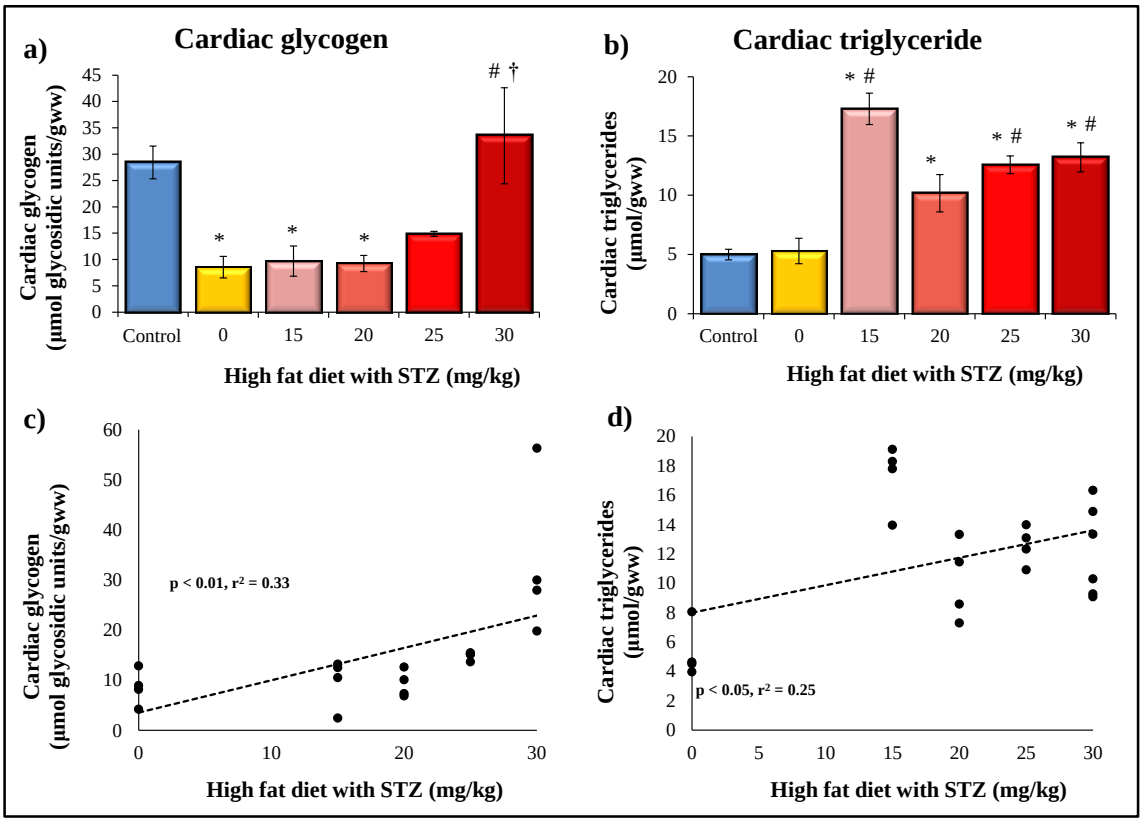

Figure 3.2: Cardiac glycogen (a) and triglyceride (b) concentrations in control and diabetic rats, and correlation between STZ doses and cardiac glycogen (c) and triglycerides (d). * $p < 0.05$ vs. control, # $p < 0.05$ vs. high-fat only, † $p < 0.05$ vs. 15 and 20 mg/kg STZ, n = 9 for control group, n = 4 for diabetic groups.

3.4.5 Cardiac enzyme activities

Pyruvate dehydrogenase (PDH) is a key enzyme in glucose metabolism, which catalyses the irreversible oxidative decarboxylation of pyruvate to acetyl-CoA to enter the TCA cycle. Cardiac PDH activity was decreased by 68% in diabetic rats with the highest dose of STZ (30 mg/kg) compared with controls (Figure 3.3) ($p < 0.05$). While there were no significant decreases in PDH activity with lower doses of STZ, a significant negative correlation between PDH activity and STZ dose was demonstrated. There was no change in mitochondrial enzyme, citrate synthase (CS) activity, suggesting no change in mitochondrial content. MCAD, an enzyme involved in mitochondrial fatty acid oxidation, was increased by 43% in diabetic rats with 30 mg/kg STZ compared with controls ($p < 0.05$).

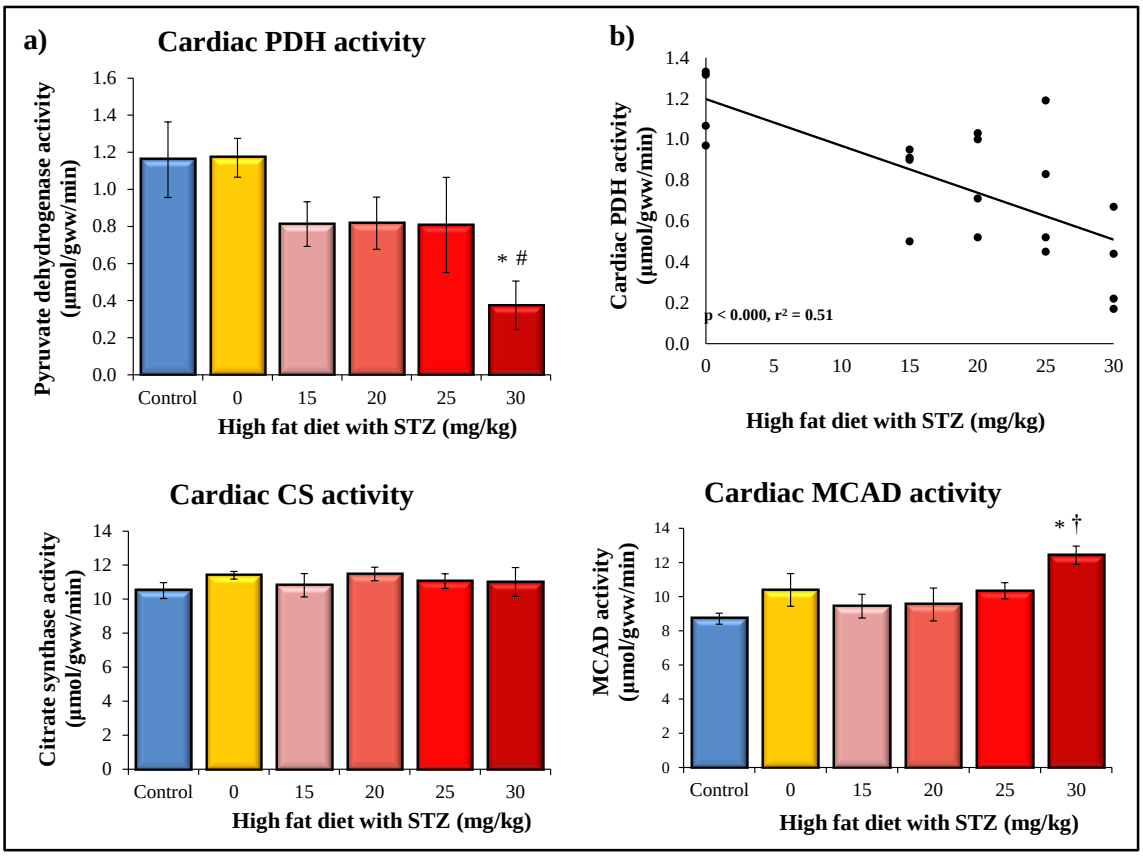

Figure 3.3: Cardiac pyruvate dehydrogenase (PDH) (a) and its correlation with STZ doses (b), citrate synthase (CS) and medium chain acyl-dehydrogenase (MCAD) activities in control and diabetic rats, induced using high fat diet in combination with low dose of STZ. *$p < 0.05$ vs. control, #$p < 0.05$ vs. high fat only, †$p < 0.05$ vs. 15 and 20 mg/kg STZ, n = 4 – 5 per group.

3.4.6 Cardiac metabolic proteins

The changes in cardiac enzyme activities suggested that there may be a STZ dose-dependent metabolic shift, characterized by an increased reliance on fatty acids for energy production and decreased contribution from glucose. In order to further reaffirm and characterise these changes in the type 2 diabetic heart, cardiac metabolic protein levels were measured. All doses of STZ decreased GLUT4 protein levels compared with controls (Figure 3.4) ($p < 0.05$). GLUT4 is the predominant cardiac isoform of the glucose transporter and is responsible for insulin-stimulated glucose uptake, therefore,

the decrease in GLUT4 concentrations implies a decrease in glucose uptake in the type 2 diabetic heart. There was no change in GLUT1 protein levels between groups. Pyruvate dehydrogenase kinase 4 (PDK4), which inhibits PDH activity and glucose oxidation, was increased in diabetic hearts compared with controls, with the highest dose (30 mg/kg) being significantly increased compared to all other doses.

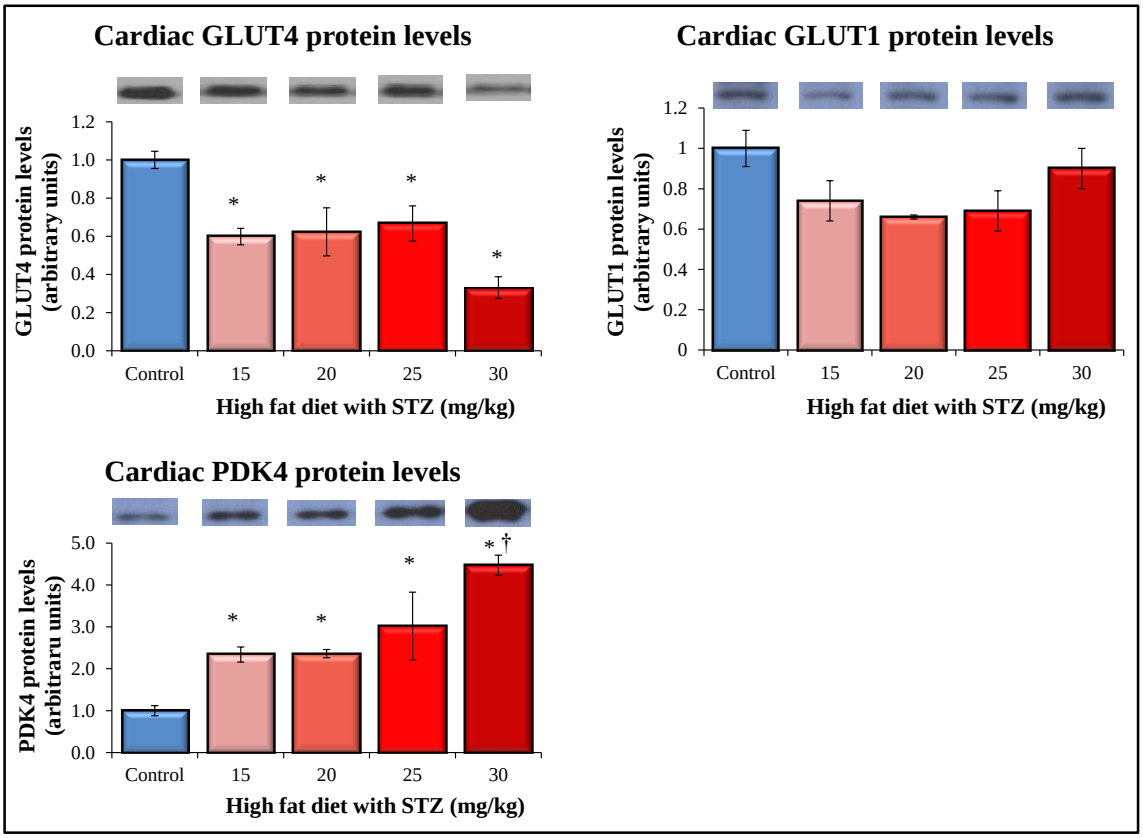

Figure 3.4: Cardiac glucose transporters (GLUT) 4 and 1, and pyruvate dehydrogenase kinase 4 (PDK4) protein levels in control and diabetic rats, induced using high fat diet in combination with low dose of STZ. *$p < 0.05$ vs. control, †$p < 0.05$ vs. all other doses of STZ, n = 6 for control group, n = 4 for diabetic groups.

Metabolic proteins associated with fatty acid metabolism were also measured to determine if the decrease in glucose metabolism-related proteins was coupled with an upregulation of fatty acid metabolism. Uncoupling protein 3 (UCP3) is a protein regulated by fatty acids[61] and its levels were increased by all doses of STZ in a dose-dependent manner compared with controls ($r^2 = 0.34$, $p < 0.05$) (Figure 3.5). There was

no significant difference in monocarboxylate transporter 1 (MCT1) and fatty acid translocase (FAT/CD36) protein levels between groups, but there was a general trend for lower MCT1 levels and higher FAT/CD36 levels in diabetic hearts.

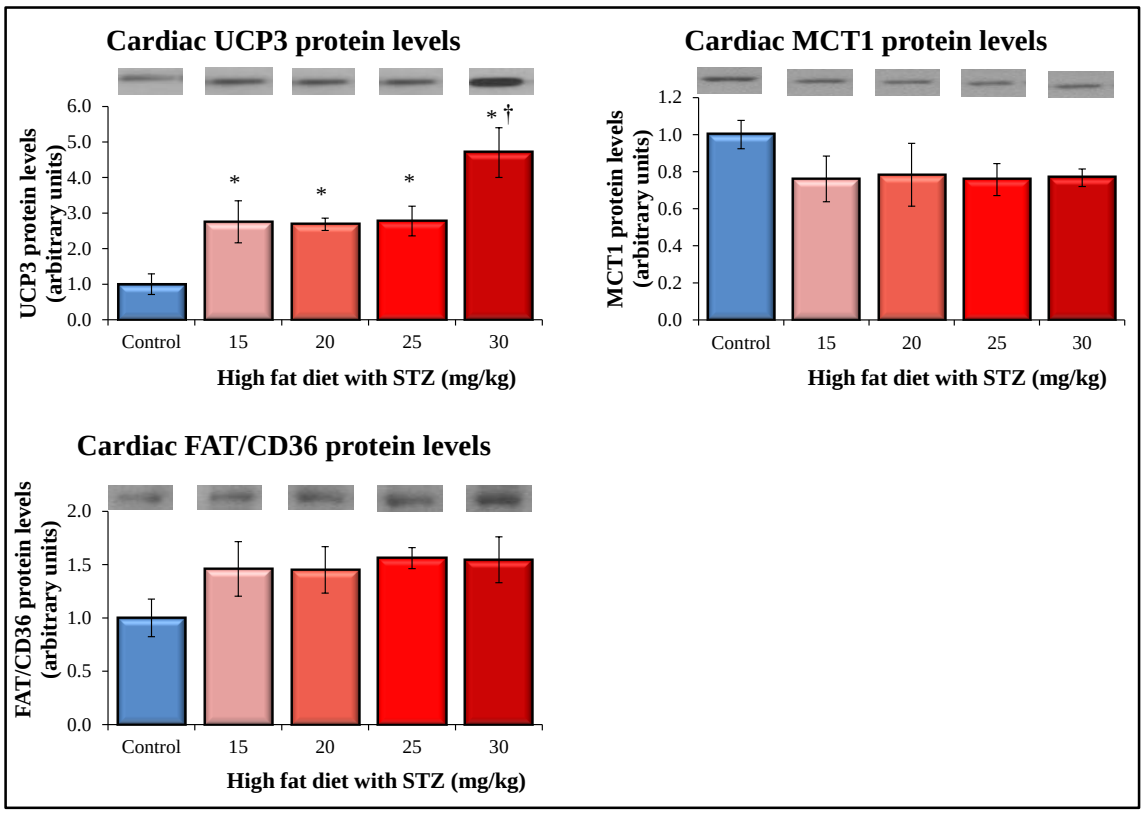

Figure 3.5: Cardiac uncoupling protein 3 (UCP3), monocarboxylate transporter 1 (MCT1) and fatty acid translocase (FAT/CD36) protein levels in control and diabetic rats, induced using high fat diet in combination with low dose of STZ. *$p < 0.05$ vs. control, †$p < 0.05$ vs. all other doses of STZ, n = 6 for control group, n = 4 for diabetic groups.

3.4.7 Cardiac glycolytic rates from 15 mg/kg STZ high fat fed diabetic rats

In the diabetic heart, there is an increase in fatty acid metabolism and decrease in glucose metabolism[84, 170]. The data obtained so far suggested that 15 mg/kg may be the lowest dose sufficient to produce a type 2 diabetic model which develops the pathophysiology of the disease without making the condition too severe. In order to investigate substrate metabolism in the diabetic heart further, hearts from high fat diet

and 15 mg/kg STZ diabetic rats were perfused with 3 U/l insulin and 11 mM glucose for the measurement of insulin-stimulated glycolytic rates. There were 2 groups of diabetic rats, consisting of a group of rats perfused at three weeks and another group perfused at six weeks. Isolated heart perfusion data showed that there was no significant difference in glycolytic rates between groups (Figure 3.6).

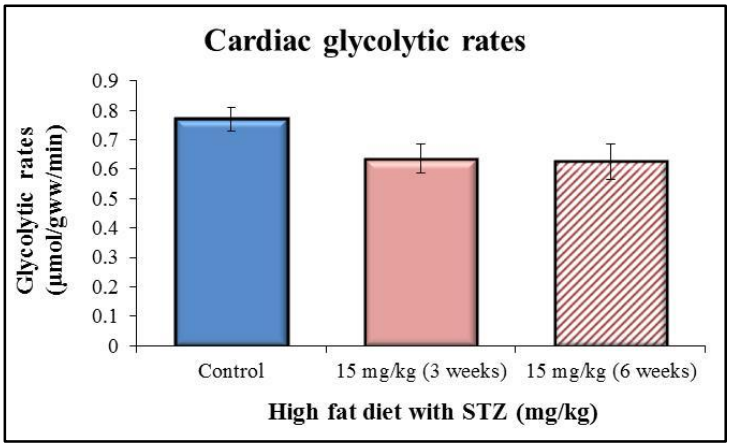

Figure 3.6: Cardiac glycolytic rates of control and diabetic rats sacrificed after 3 and 6 weeks following high fat diet in combination with 15 mg/kg STZ.

3.4.8 Problem solving – insulin-stimulated glycolytic rates in perfused hearts

The perfusions to compare control hearts and diabetic hearts (15 mg/kg) in the previous section were conducted using KH buffer containing glucose but without palmitate. It would be preferable to use KH-glucose buffer containing palmitate bound to albumin be used, as it would represent the physiological state more closely. However, we found that perfusing with palmitate and BSA present was preventing the insulin-stimulated increase in glycolytic rates that would be predicted to occur. Therefore, perfusions were conducted with the aim of identifying and solving the problem by process of elimination (Table 3.4). The response to insulin in these perfusions was defined as an increase in glycolytic rates after insulin was added into the recirculating buffer, compared with the glycolytic rates during the first 20 minutes of perfusion in the

absence of insulin. There was an increase in glycolytic rates with insulin when hearts from fed rats were perfused with KH-glucose only and KH-glucose containing BSA (Sigma, UK). However, when palmitate was introduced into KH-glucose and BSA (Sigma, UK), there was no increase in glycolytic rates with insulin stimulation. This was rectified when hearts from fasted rats were perfused using KH-glucose containing BSA (Sigma, UK) and palmitate, but an experimental design using fed animals was needed for future studies due to experimental limitations. Therefore BSA from different manufacturers (Apollo Scientific, UK and Lampire Biological Laboratories, USA) and concentrations of insulin were used to identify the underlying problem. Perfusions using BSA from Apollo did not have an insulin response at either insulin concentrations. In contrast, hearts perfused using BSA from Lampire and the lower concentration of insulin (0.3 U/l) produced an effect of insulin on the glycolytic rates. All subsequent studies involving this technique were conducted using this finalised protocol.

Table 3.4: Fed or fasted isolated heart perfusions using different buffer compositions (with 0.4 mM palmitate and 1.5% BSA where applicable). Different insulin concentrations were administered and their effects on glycolytic rates were recorded.

Buffer composition	Fed or fasted state	Insulin concentration (U/l)	Is there an increase in glycolytic rates with insulin?
KH-glucose only	Fed	3	Yes
KH-glucose + albumin	Fed	3	Yes
KH-glucose + albumin (Sigma) + palmitate	Fed	3	No
KH-glucose + albumin (Sigma) + palmitate	Fasted	3	Yes
KH-glucose + albumin (Apollo) + palmitate	Fed	3	No
KH-glucose + albumin (Apollo) + palmitate	Fed	0.3	No
KH-glucose + albumin (Lampire) + palmitate	Fed	3	No
KH-glucose + albumin (Lampire) + palmitate	Fed	0.3	Yes

3.5 DISCUSSION

The pathology of type 2 diabetes is multifactorial and complex, with multiple stages involving insulin resistance of different organs and the changing ability of pancreatic β-cells to produce insulin to sufficiently meet the body's needs. Therefore animal models resembling the pathology of human type 2 diabetes are of great value. As described before, the existing models were either too severe, mimicking type 1 diabetes, too lean, expensive or took too long to develop the disease. The aim of this chapter was to develop a novel rat model which mimics the pathology of type 2 diabetes, which was both time and cost effective. In this chapter, findings demonstrated that high fat diet in combination with low dose STZ induced adiposity, hypercholesterolaemia and mild hyperglycaemia without compromising insulin secretion, and exhibited cardiac metabolic changes that mirrored the well characterised shift from glucose to fatty acid metabolism in type 2 diabetes. These characteristics were mostly STZ dose dependent and hence this model is easily modifiable to achieve the severity as required in different studies.

3.5.1 Plasma metabolites

The metabolic profiling in this study showed most of the parameters measured were dose-dependent. The rats with the highest dose, 30 mg/kg STZ, were found to have plasma metabolite profiles that included hyperglycaemia, hyperlipidaemia, hyperketonaemia and hypoinsulinaemia. This resembles the characteristics of type 1 diabetes, as shown in studies which used STZ in the generation of type 1 diabetic models[90, 171, 175, 176]. In contrast, lower doses of STZ displayed no difference in insulin, triglycerides, NEFA and β-OHB. Therefore, care must be taken on the dose used to avoid producing a model that is too severe. It is also important to consider the effect of this method on different strains of rats as it might have a different response to high fat

diet, as has been shown previously in mice[177]. We used Wistars in contrast to Srinivasan *et al*. who used Sprague-Dawleys and the most obvious difference is that we require a lower dose of STZ (15 – 25 mg/kg), compared to their 35 mg/kg, to generate the type 2 diabetic model.

3.5.2 Effect of STZ on plasma insulin

In other models such as the Zucker diabetic fatty rats, an increase in plasma insulin concentrations compared with controls was observed[158] but in our model, there was no difference in insulin concentrations between the lower doses of STZ (15-25 mg/kg) compared with controls. However, there was a decrease of insulin concentrations with the highest dose of STZ (30 mg/kg) compared with controls. Srinivasan *et al.* suggested that high fat diet alone causes insulin resistance and subsequently a rise in plasma insulin concentrations as a coping mechanism, but the insulin concentrations were normalised to control levels with the injection of STZ[136]. This shows that STZ has a specific mechanism of action which targets and damages the β-cells, decreasing insulin secretion. In addition, when combined with high fat diet, it produces a type 2 diabetic model which not only has a systemic impact on plasma insulin concentrations but also induces insulin resistance which affect peripheral organs. The highest dose of STZ used in this study (30 mg/kg) may represent a later stage of type 2 diabetes when β-cell function starts to become compromised and no longer matches the increased demand for insulin.

3.5.3 Increased lipid metabolism in the type 2 diabetic heart

There was an increase in triglyceride concentrations in diabetic hearts and livers, which is consistent with other studies[178-182], but this only occurred due to the combination of high fat diet and STZ, as we did not see an increase in rats on high fat diet alone. There was an increase in MCAD activity, suggesting that these diabetic

hearts not only store more lipids but also oxidise more fatty acids in their mitochondria. The increase in fatty acid metabolism is associated with PPARα activation as we and others have shown increased protein concentrations of UCP3 and PDK4, downstream targets of PPARα[61].

3.5.4 Decreased glucose metabolism in the type 2 diabetic heart

According to the Randle cycle, the increase in fatty acid metabolism in diabetes would inhibit glucose metabolism[63]. Our results showed decreased cardiac PDH activity in diabetic rats with the highest dose of STZ, which could be explained by the significant increase in protein levels of PDK4, which inhibits PDH. This agrees with Le page *et al.* who showed a decrease in PDH flux in type 2 diabetic rat hearts[86]. In addition, GLUT4 protein levels were also decreased in the diabetic heart, suggesting a decreased capacity of insulin-stimulated glucose uptake. This is consistent with results from previous studies in both humans[183] and rats[84]. There was a dose-dependent relationship between STZ dose, and cardiac and hepatic glycogen concentrations, albeit in opposite directions between the two organs, where there was an increase in the heart with a decrease in the liver. The increase in cardiac glycogen concentrations is supported by previously documented findings of Nakao *et al*. who observed a marked increase in cardiac glycogen in similar type 2 diabetic model but with a higher dose of STZ (50 mg/kg)[184]. The data on glycogen concentrations suggests that the regulatory mechanisms involved in the heart may be different to that involved in the liver. Taken together, the hearts from our type 2 diabetic rats developed using high fat feeding and low dose STZ displayed a metabolic shift from glucose towards lipid metabolism, replicating that seen in type 2 diabetic patients[38].

3.5.5 Mild type 2 diabetes pathology in 15 mg/kg STZ high fat fed diabetic rats

Based on the cardiac metabolic protein levels, one would predict a decrease in glucose utilisation pathways, such as glycolysis. At 3 weeks, 15 mg/kg STZ diabetic hearts showed significant decrease in glycolytic rates compared with control hearts using t-test analysis but when data from 6-week old hearts were included in the analysis using one-way ANOVA, the significant difference was lost. This suggests that the severity of diabetes induced by the lower dose may be insufficient to alter substrate flux in the pathway, indicating that the lower dose may be not meet the requirements of our future studies to investigate the abnormal substrate metabolism in the diabetic heart. In addition, the increased adiposity and elevated cholesterol following the 25 mg/kg STZ are characteristics shown of other type 2 diabetic models[149, 185, 186], suggesting 25 mg/kg may be the optimal dose to induce both systemic and cardiac-specific features of type 2 diabetes, ideal for future work. Therefore, we will use 25 mg/kg STZ in combination with high fat diet for future studies to induce hypercholesterolaemia, hyperglycaemia and adiposity without compromising insulin secretion, while exhibiting the substrate metabolic shift from glucose metabolism to fatty acid metabolism, which is a characteristic of type 2 diabetes. In addition, this model is inexpensive, easy to induce and can be modified to achieve a range of severities of diabetes as required by the study.

CHAPTER 4

EFFECT OF CHRONIC HYPOXIA ON CARDIAC METABOLISM AND FUNCTION IN THE TYPE 2 DIABETIC HEART

4.1 ABSTRACT

Metabolic changes and abnormal hypoxic signalling pathways in the diabetic heart were hypothesised to decrease cardiac recovery following a myocardial infarction. Therefore, the aim of this study was to investigate the cardiac metabolic adaptation to physiological hypoxia in type 2 diabetic rats. Diabetic rats were generated using high fat diet and low dose streptozotocin (25 mg/kg), and both control and diabetic rats were housed in hypoxia (11% oxygen) or normoxia for three weeks. Following hypoxia, control hearts upregulated anaerobic glycolysis by 25%, increased glycogen content by 4.6 µmol/gww and suppressed fatty acid oxidation by 15% compared to normoxic control hearts. However this effect of hypoxia was not seen in diabetic hearts as glycolytic rates were decreased by 28% and fatty acid oxidation rates remained 36% higher in hypoxic diabetic hearts compared with hypoxic control hearts. Peroxisome proliferator-activated receptor α (PPARα) is a transcription factor that positively regulates fatty acid metabolism, and PPARα-regulated targets were downregulated following hypoxia in the control hearts. PPARα target proteins were upregulated in normoxic diabetic hearts and remained elevated in diabetic rats following hypoxia. Cardiac mRNA expression of the hypoxia-inducible factor (HIF) target genes, prolyl hydroxylase 3, heme oxygenase 1 and VEGF, were regulated in control and diabetic hearts to the same extent by chronic hypoxia, suggesting that there were no defects in hypoxia signalling or HIF in diabetic hearts. In conclusion, the abnormal metabolic adaptation to hypoxia in diabetes is associated with an inability of hypoxia to suppress PPARα targets, and occurs independently of changes in HIF signalling pathways. This maintained fat metabolism to the detriment of glucose metabolism in hypoxic diabetic hearts.

4.2 INTRODUCTION

The healthy heart predominantly uses oxygen-dependent mitochondrial oxidative phosphorylation to generate ATP for contraction, and is thus sensitive to changes in oxygen concentrations. During a myocardial infarction, the heart undergoes an ischaemic assault, causing a shortage of substrates and oxygen needed for cellular metabolism, with the accumulation of waste products, such as lactate, in the cardiomyocytes. Hypoxia is a component of ischaemia where the heart is deprived of adequate oxygen supply. It activates the evolutionarily-conserved transcription factor, hypoxia-inducible factor (HIF), which is a central transcription factor that regulates a large number of genes responsible for oxygen delivery, angiogenesis, cell proliferation, cell differentiation, and metabolism[96, 97]. HIF is a heterodimer composed of an alpha subunit, such as HIF-1α, and a β subunit that is constitutively expressed. HIF-1α is regulated by prolyl hydroxylase (PHD) and factor inhibiting HIF (FIH) enzymes, which use oxygen as their substrate for the hydroxylation of HIF-1α, leading to its ubiquitination and proteosomal degradation. In hypoxia, HIF-1α escapes degradation as there is insufficient oxygen available for the PHD and FIH enzymes. Therefore HIF-1α can translocate to the nucleus to dimerise with HIF-1β, and initiate transcription via hypoxia response elements (HREs) within the target genes[104, 105]. This allows the cell to adapt to the decreased oxygen availability and promotes cell survival. From metabolism perspective, HIF activates proteins associated with increased glycolysis, decreased fatty acid oxidation and mitochondrial respiration.

Some studies demonstrated that the HIF signalling pathway is impaired in the type 1 diabetic heart[120, 121, 135]. Marfella *et al*. showed that there was an increase in infarct size in type 1 diabetic rat hearts due to hyperglycaemia, associated with decreased HIF-1α expression[120] and then later demonstrated that human type 2 diabetes is associated with

reduced expression of HIF-1α and VEGF following ischaemia[121]. In addition, Chou *et al.* showed decreased cardiac expression of vascular endothelial growth factor (VEGF) in both diabetic patients and rats[135]. This suggests that the diabetic heart may be more susceptible to ischaemia-reperfusion injury due to defects in the HIF signalling pathway. Therefore, the aim was to investigate if the type 2 diabetic model we developed had a defect in its ability to metabolically adapt to hypoxia and its hypoxic signalling pathway, by exposing the rats to chronic hypoxia.

However, the defects in the HIF signalling pathway in the diabetic heart may not be the only possible explanation for the increased risk of ischaemia-reperfusion injury in the diabetic population. As shown in the previous chapter, the diabetic model that we developed had increased cardiac fat metabolism and decreased glucose metabolism, as supported by other studies in diabetic mice[170, 173]. The term 'metabolic inflexibility' was associated with the diabetic heart and, defined as the impaired capacity to switch between fat and glucose as the primary fuel in response to different physiological stressors[94]. This flexibility is especially crucial during chronic hypoxia, a major physiological stressor of myocardial substrate selection, as a healthy heart should upregulate anaerobic ATP generation via glycolysis and downregulate fatty acid oxidation, which is less oxygen efficient[35, 36, 187]. Failure to adapt metabolically to hypoxia could result in anaerobic ATP depletion, inefficient use of limited oxygen and oxidative damage.

Using a physiological hypoxia environment, metabolic adaptation to hypoxia and the HIF signalling pathway in the diabetic heart were investigated. Physiological hypoxia is a stressor that requires high metabolic flexibility as an adaptation mechanism. This study investigated whether the type 2 diabetic heart is less flexible metabolically following chronic hypoxia compared with control hearts.

4.3 METHODS

4.3.1 Chronic hypoxic housing of type 2 diabetic rats

The type 2 diabetic rat model was generated using a combination of high fat diet and 25 mg/kg streptozotocin (STZ) as described in detail in Chapter 2. On day 20 of modified feeding, control and diabetic rats had fasting blood collected from the saphenous vein for plasma metabolite analysis, to confirm diabetic status prior to hypoxia (Figure 4.1). On day 21, which is a week after STZ injection, half the control and diabetic rats were transferred to a normobaric hypoxic chamber (Biospherix)[119]. The chamber was connected to gas cylinders containing 95% N_2 and 5% CO_2 (Figure 4.2) that was used to displace the oxygen in the chamber to produce a hypoxic environment. During the first 7 days, the oxygen concentration was decreased gradually from 21% to 11% as an adaptation period, then the rats were maintained under 11% oxygen thereafter for a further 2 weeks before the being sacrificed. The chamber was also monitored twice daily to ensure that the oxygen concentration was stable, carbon dioxide concentration was not excessive and both temperature and humidity were optimal. The rats were also checked at the same time to ensure no drastic change of behaviour or weight loss occurred while in the chamber.

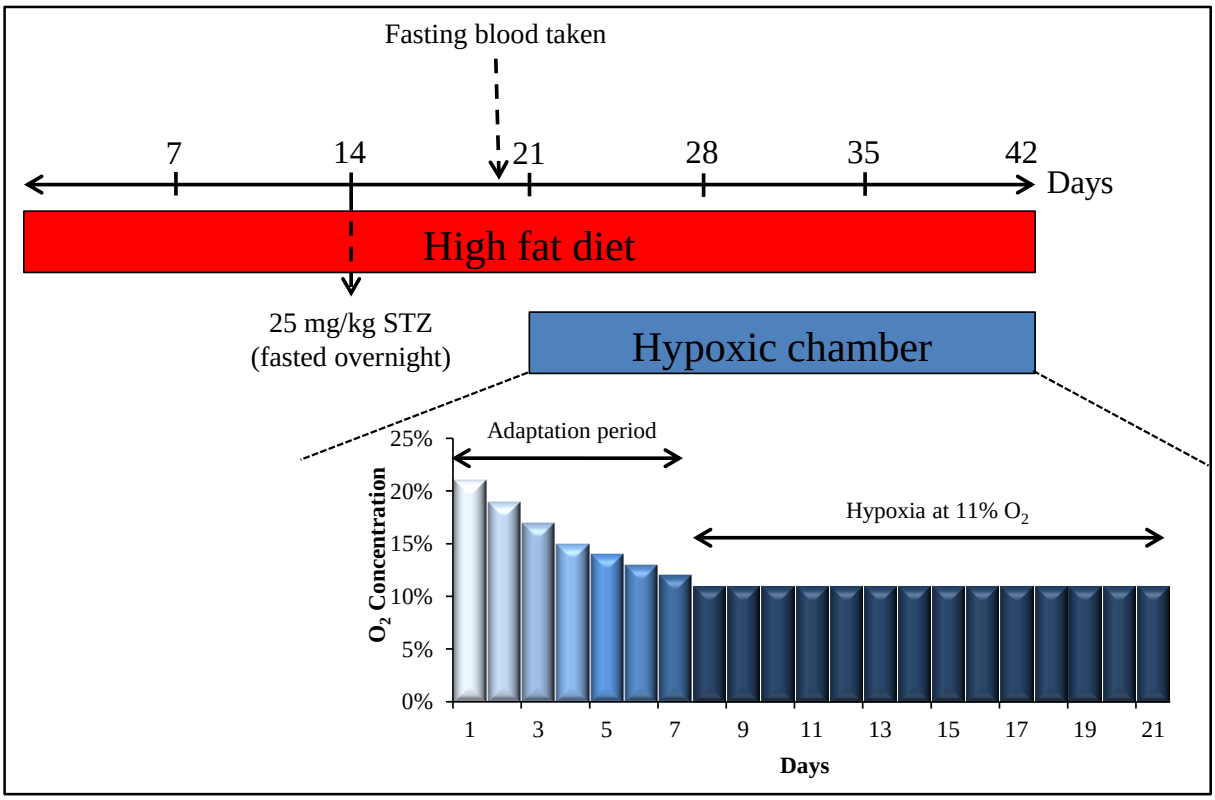

Figure 4.1: Timeline of chronic hypoxic housing of the type 2 diabetic rat model.

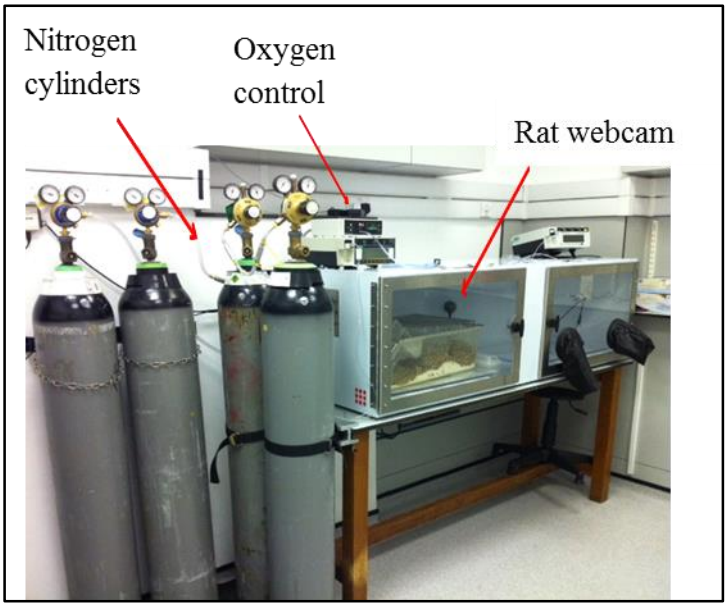

Figure 4.2: Set up of the normobaric hypoxic chamber.

4.3.2 Mitochondrial isolation and respiration

Rat hearts were excised, heart weight measured and mitochondria were isolated based on the original protocol of Palmer *et al*.[188]. All isolated mitochondria experiments were performed by Dr. Lisa Heather with my assistance. The interfibrillar and subsarcolemmal mitochondria were isolated using a modified Chappell–Perry buffer (100 mM KCl, 50 mM MOPS, 5 mM $MgSO_4.7H_2O$, 1 mM EDTA, 1 mM ATP, and 0.2% BSA, pH 7.4)[189]. The interfibrillar mitochondria were isolated using 5 mg/gww trypsin digestion followed by further homogenisation, while the subsarcolemmal mitochondria were isolated by Polytron and Potter–Elvejhem homogenization. Mitochondria were resuspended in KME medium (100 mM KCl, 50 mM MOPS, and 0.5 mM EGTA, pH 7.4) and then incubated in respiratory media (100 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM KH_2PO_4, and 1 mg/mL BSA, pH 7.4) before respiration was measured with a Clark-type oxygen electrode using palmitoyl CoA (40 μM) with carnitine (5 mM) and malate (5 mM), pyruvate (10 mM) with malate, glutamate (20 mM), and succinate (20 mM) with rotenone (3.7 μM) (30). State 3 (100 nmol ADP-stimulated) respiration, state 4 (ADP-limited) respiration, maximal ADP-stimulated (1000 nmol) respiration (max ADP), and respiratory control ratios (RCR, state 3/state 4 respiration rates) were also measured.

4.3.3 Aconitase activity assay

The spectrophotometer was set at 240 nm wavelength and zeroed with 1 ml 50mM Tris against 1 ml H_2O. A volume of 1 ml of assay buffer (50 mM Tris-HCl (pH 7.5), 20 mM cis-aconitate) was added to a quartz cuvette and its absorbance was determined for 1 minute to ensure stability. The aconitase assay reaction started when 75 μg mitochondrial protein was added to the cuvette. Immediately, the absorbance was measured at 240 nm at 37 ºC for two minutes. The reaction rates were calculated from

the initial change in absorbance using the extinction coefficient for cis-aconitate (3600 $\mu M^{-1}cm^{-1}$).

4.3.4 Real-time quantitative PCR

This experiment was carried out by Prof. Ellen Aasum's group from the University of Tromsø, Norway. Left ventricular tissue was immersed in RNAlater (Qiagen, Germany), and total RNA was extracted according to the RNeasy Fibrous Tissue kit protocol (Qiagen, Norway). Real-time PCR (qPCR) was performed in an ABI PRISM 7900 HT fast real-time thermal cycler as previously described[190]. In order to address the correction for the multiple housekeeping genes, we analysed 5 housekeeping genes; GAPDH, RPL13A, HMBS, Cyclo, HPRT and found that GAPDH, RPL13A and HMBS was the most stable genes and calculated the geomean Ct value of these using methods involving GeNorm described by Vandesompele *et al.*[191]. In addition, mRNA expression of the genes of interest was adjusted to the geomean of the three most stable housekeeping genes. This value was used for adjusting the gene expression of the genes of interest using the ΔΔCt method. Primer sequences used are given in Table 4.1.

Table 4.1: Real-time quantitative PCR primers.

Real-time quantitative PCR primers		
Target	NM	Primer sequence
Heme oxygenase 1	NM_012580.2	Fp: GTC-AGG-TGT-CCA-GGG-AAG-G Rp: CTC-TTC-CAG-GGC-CGT-ATA-GA
VEGF	NM_031836.2	Fp: CAA-GCC-AAG-GCG-GTG-AGC-CA Rp: TCT-GCC-GGA-GTC-TCG-CCC-TC
PHD3	NM_019371.1	Fp: CTA-TGT-CAA-GGA-GCG-GTC-CAA Rp: GTC-CAC-ATG-GCG-AAC-ATA-ACC
HIF-1α	NM_024359.1	Fp: TGC-TTG-GTG-CTG-ATT-TGT-GA Pp: GGT-CAG-ATG-ATC-AGA-GTC-CA
GAPDH	NM_017008	FP: TGG-GAA-GCT-GGT-CAT-CAA-C Rp: GCA-TCA-CCC-CAT-TTG-ATG-TT
Cyclophilin A	NM_017101.1	Fp: CTG-ATG-GCG-AGC-CCT-TG Rp: TCT-GCT-GTC-TTT-GGA-ACT-TTG-TC
Ribosomal protein L13a	NM_173340.2	Fp: CCC-TCC-ACC-CTA-TGA-CAA Rp: GGT-ACT-TCC-ACC-CGA-CCT-C
Hydroxymethylbilane synthase	NM_013168.2	Fp: TCC-CTG-AAG-GAT-GTG-CCT-AC Rp: ACA-AGG-GTT-TTC-CCG-TTT-G
Hypoxanthine-guanine phosphoribosyltransferase 1	NM_012583.2	Fp: GAC-CGG-TTC-TGT-CAT-GTC-G Rp: ACC-TGG-TTC-ATC-ATC-ACT-AAT-CAC

VEGF; Vascular endothelial growth factor, PHD3; Prolyl hydroxylase 3, HIF-1α; Hypoxia-inducible factor 1α, GAPDH; Glyceraldehyde 3-phosphate dehydrogenase.

4.3.5 Statistical analysis

Results are expressed as means ± standard error of the mean (SEM). Data were analysed using two way analysis of variance (ANOVA) with diabetes and hypoxia being the 2 factors and should there be any effect, post-hoc t-testing was conducted. $p < 0.05$ was taken as the level of statistical significance.

4.4 RESULTS

4.4.1 Physical parameters

There were no significance differences in terminal body weights between groups (Table 4.2). There was an increase of heart weight, and heart weight to body weight ratio, in the normoxic diabetic hearts compared with normoxic control hearts ($p < 0.05$), but these parameters were not affected by hypoxia. Both epididymal fat pad weight and fat pad to body weight ratio, indicators of adiposity, were increased in diabetic rats both in normoxia and hypoxia compared with their respective control groups ($p < 0.05$), but there was no effect of hypoxia on adiposity.

Table 4.2: Physical characteristics from control and diabetic rats housed in normoxia or hypoxia.

	Normoxic Control	**Normoxic Diabetic**	**Hypoxic Control**	**Hypoxic Diabetic**
Starting body weight (g)	270 ± 13	270 ± 8	270 ± 6	265 ± 7
Terminal body weight (g)	380 ± 13	395 ± 8	366 ± 5	376 ± 10
Heart weight (g)	1.21± 0.09	1.47 ± 0.06 *	1.34 ± 0.09	1.52 ± 0.05
Heart weight to body weight ratio ($x10^3$)	3.14 ± 0.14	3.66 ± 0.18 *	3.56 ± 0.19	4.01 ± 0.10
Epididymal fat pad weight (g)	5.9 ± 0.6	10.3 ± 0.3 *	5.7 ± 0.8	8.4 ± 0.4 *
Fat pad to body weight ratio (x 10^2)	1.46 ± 0.13	2.59 ± 0.09 *	1.60 ± 0.19	2.30 ± 0.12 *

* $p < 0.05$ vs. control under the same oxygen concentration. Body weights n = 12 – 16, heart weights n = 8 – 10, fat pads n = 4 – 8.

4.4.2 Plasma metabolites

Prior to entering the hypoxic chamber at day 21, fasting plasma from the diabetic rats showed an increase in both blood glucose and plasma insulin concentrations compared with controls ($p < 0.05$) (Table 4.3). Terminal blood plasma from the fed state illustrated that normoxic diabetic rats had increased blood glucose concentrations, but hypoxia decreased blood glucose in both controls and diabetics compared with their normoxic counterparts ($p < 0.05$). A decrease in plasma insulin concentrations was observed in the hypoxic diabetics compared with the hypoxic controls. Non-esterified fatty acids (NEFA) concentrations were elevated in the normoxic diabetics compared with normoxic controls, and chronic hypoxia was seen to further increase NEFA in both controls and diabetics ($p < 0.05$). While diabetes increased β-hydroxybutyrate (β-OHB) concentrations compared with controls, hypoxia had an additive effect, resulting in even higher concentration of β-OHB in hypoxic diabetic plasma compared with both hypoxic controls and normoxic diabetes ($p < 0.05$). There was no effect of diabetes on plasma triglyceride concentrations but hypoxia increased triglyceride concentrations in both controls and diabetics compared with their normoxic counterparts.

Table 4.3: Plasma metabolites from control and diabetic rats housed in normoxia or hypoxia.

	Normoxic Control	Normoxic Diabetic	Hypoxic Control	Hypoxic Diabetic
Fasting plasma metabolites				
Glucose (mmol/l)	6.18 ± 0.43	8.08 ± 0.61 *		
Insulin (ug/l)	0.31 ± 0.03	0.44 ± 0.04 *		
Plasma metabolites in the fed state				
Glucose (mmol/l)	12.7 ± 0.3	14.1 ± 0.3 *	10.1 ± 0.5 #	11.5 ± 0.7 #
Insulin (ug/l)	1.90 ± 0.26	1.88 ± 0.22	1.56 ± 0.21	1.11 ± 0.16 *
NEFA (mmol/l)	0.05 ± 0.01	0.09 ± 0.01 *	0.11 ± 0.02 #	0.15 ± 0.02 #
β-OHB (mmol/l)	0.32 ± 0.02	0.57 ± 0.03 *	0.39 ± 0.01 #	0.78 ± 0.04 *#
TAG (mmol/l)	1.44 ± 0.18	1.18 ± 0.11	2.15 ± 0.27 #	1.81 ± 0.19 #

* $p < 0.05$ vs. control under the same oxygen concentration, # $p < 0.05$ vs. normoxic group with the same disease status. Fasting metabolites obtained prior to entry into the hypoxia chamber after 3 weeks (n = 5 – 9), fed metabolites after 6 weeks (n = 12 – 17). NEFA, non-esterified fatty acids; β-OHB, β-hydroxybutyrate; TAG, triglycerides.

4.4.3 Cardiac function

There was no significance difference in coronary flow rates between groups (Table 4.4). There was no significance difference in rate pressure product, the multiple of heart rate and developed pressure, between groups, demonstrating that overall systolic cardiac function was not affected by diabetes or chronic exposure to hypoxia.

Table 4.4: Cardiac function from isolated control and diabetic hearts of rats housed in normoxia or hypoxia.

	Normoxic Control	**Normoxic Diabetic**	**Hypoxic Control**	**Hypoxic Diabetic**
Coronary flow rates (ml/min)	14 ± 1	12 ± 1	12 ± 1	13 ± 1
Developed pressure (mmHg)	134 ± 2	136 ± 6	149 ± 6 #	149 ± 7
Heart rate (beats/min)	280 ± 8	276 ± 10	258 ± 8	277 ± 11
Rate pressure product (mmHg/min x 10^3)	38 ± 2	38 ± 3	38 ± 1	41 ± 1

# $p < 0.05$ vs. normoxic group with the same disease status, n = 7 – 10.

4.4.4 Cardiac glucose metabolism

In normoxia, diabetes decreased cardiac glycolytic rates by 30% compared with control hearts (Figure 4.3). Following chronic hypoxia, the glycolytic rates of both control and diabetic hearts were increased by 25% compared to their normoxic counterparts ($p < 0.05$). Even though chronic hypoxia increased the glycolytic rates in both groups, the glycolytic rates remained 28% lower in the hypoxic diabetic hearts compared to that of hypoxic control hearts. The changes in cardiac glycolytic rates were mirrored by changes in net lactate efflux. The diabetic hearts had decreased lactate efflux by 40% compared with control hearts ($p < 0.05$). Following hypoxia, control hearts had an increase of 8.6 x 10^{-3} μmol/min/gww in lactate efflux while diabetic hearts increased their lactate efflux by 7.6 x 10^{-3} μmol/min/gww. In hypoxia the lactate efflux of diabetic hearts remained 38% lower compared to the control hearts ($p < 0.05$). There was a significant correlation between glycolytic rates and lactate efflux rates.

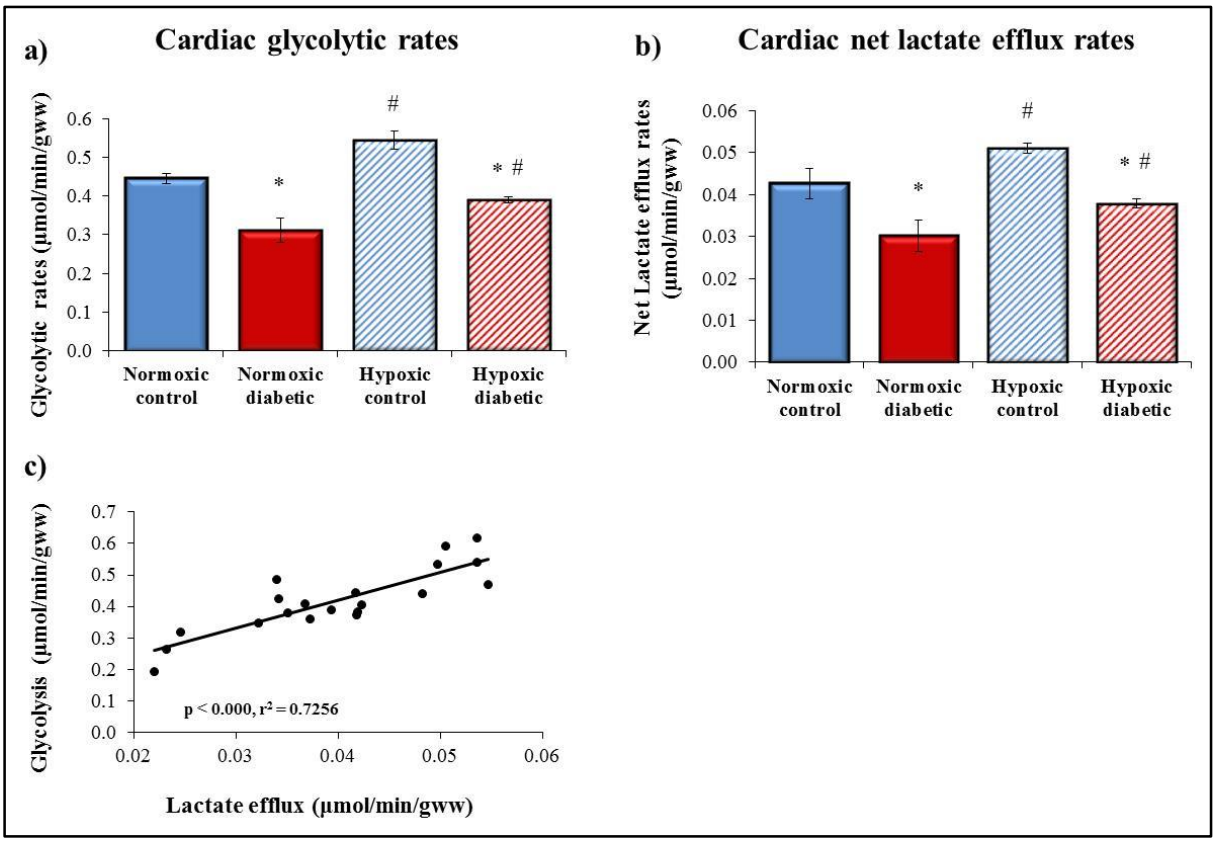

Figure 4.3: Glycolytic rates (a), lactate efflux rates (b), and correlation between glycolysis and lactate efflux (c) in control and diabetic hearts following adaptation to chronic hypoxia or normoxia. * $p < 0.05$ vs. control group under the same oxygen concentrations, # $p < 0.05$ vs. normoxic group with the same disease status, $n = 5 – 9$ per group.

Hypoxia increased myocardial glycogen concentrations in both controls and diabetics, but the effect was blunted in diabetic hearts (Figure 4.4). Control rats increased cardiac glycogen content by 4.6 µmol/gww following chronic hypoxia but hypoxic diabetic rats only increased their cardiac glycogen content by 3.2 µmol/gww, 30% less than control hearts ($p < 0.05$). There was a decrease in GLUT4 protein levels in normoxic diabetic hearts compared with control hearts ($p < 0.05$), but there was no effect of hypoxia observed in either control or diabetic hearts.

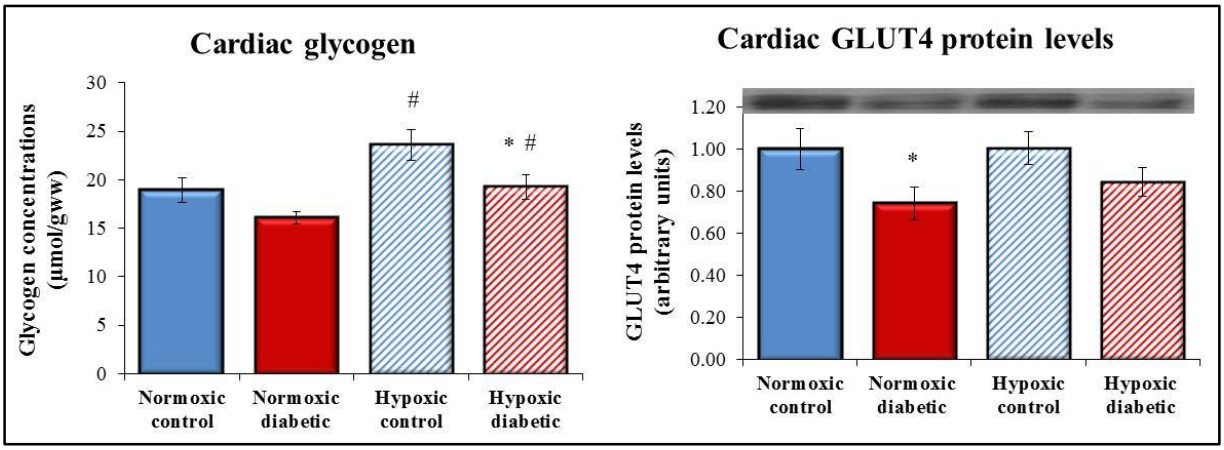

Figure 4.4: Cardiac glycogen and GLUT4 protein levels in control and diabetic hearts following adaptation to chronic hypoxia or normoxia. * $p < 0.05$ vs. control group under the same oxygen concentrations, # $p < 0.05$ vs. normoxic group with the same disease status, $n = 5 – 9$ per group.

There was decreased PDH activity in the diabetic hearts compared to control hearts under normoxic conditions (Figure 4.5). In hypoxia, the PDH activity in control hearts decreased significantly compared with normoxic control hearts, to a point lower than that of hypoxic diabetic hearts ($p < 0.05$). Hypoxia had no additional effect on PDH activity in the diabetic hearts. The CS activity in control hearts during hypoxia also decreased significantly compared to the control group in normoxia ($p < 0.05$). In contrast the CS activity in diabetic hearts was not affected by hypoxia.

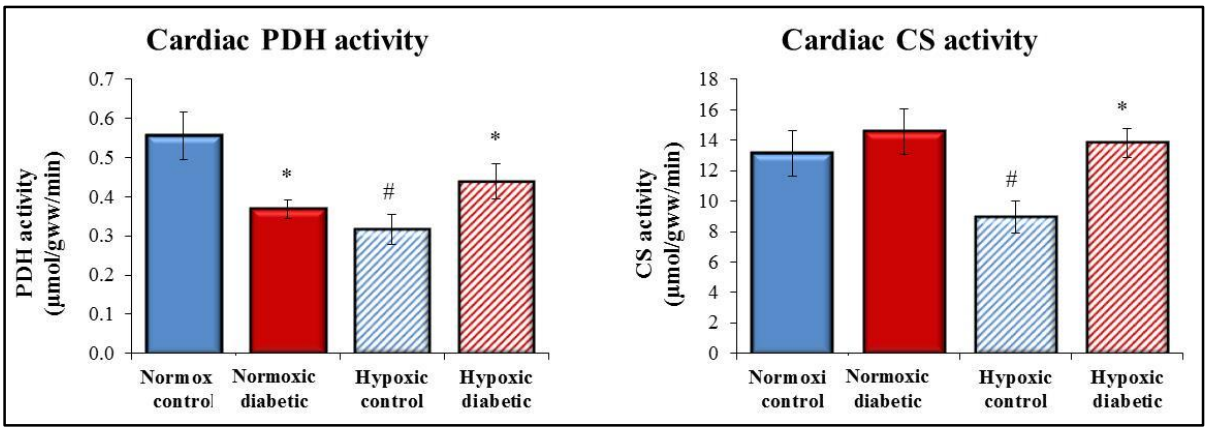

Figure 4.5: Cardiac PDH and CS activity in control and diabetic hearts following adaptation to chronic hypoxia or normoxia. * $p < 0.05$ vs. control group under the same oxygen concentrations, # $p < 0.05$ vs. normoxic group with the same disease status, n = 5 – 6 per group.

4.4.5 Cardiac lipid metabolism

In normoxia, diabetes increased fatty acid oxidation rates by 31% compared with controls ($p < 0.05$) (Figure 4.6). Upon exposure to hypoxia, fatty acid oxidation in control hearts decreased by 15%, but remained elevated in diabetic hearts, resulting in 36% higher oxidation rates in the hypoxic diabetic hearts compared to hypoxic control hearts ($p < 0.05$). Cardiac FAT/CD36 protein levels mirror the fatty acid oxidation rates where normoxic diabetic hearts have significantly higher FAT/CD36 protein levels compared to normoxic controls and the levels remained elevated in hypoxia. There was no significant difference in cardiac triglyceride concentrations and plasma membrane fatty acid-binding protein (FABPpm) protein levels between groups.

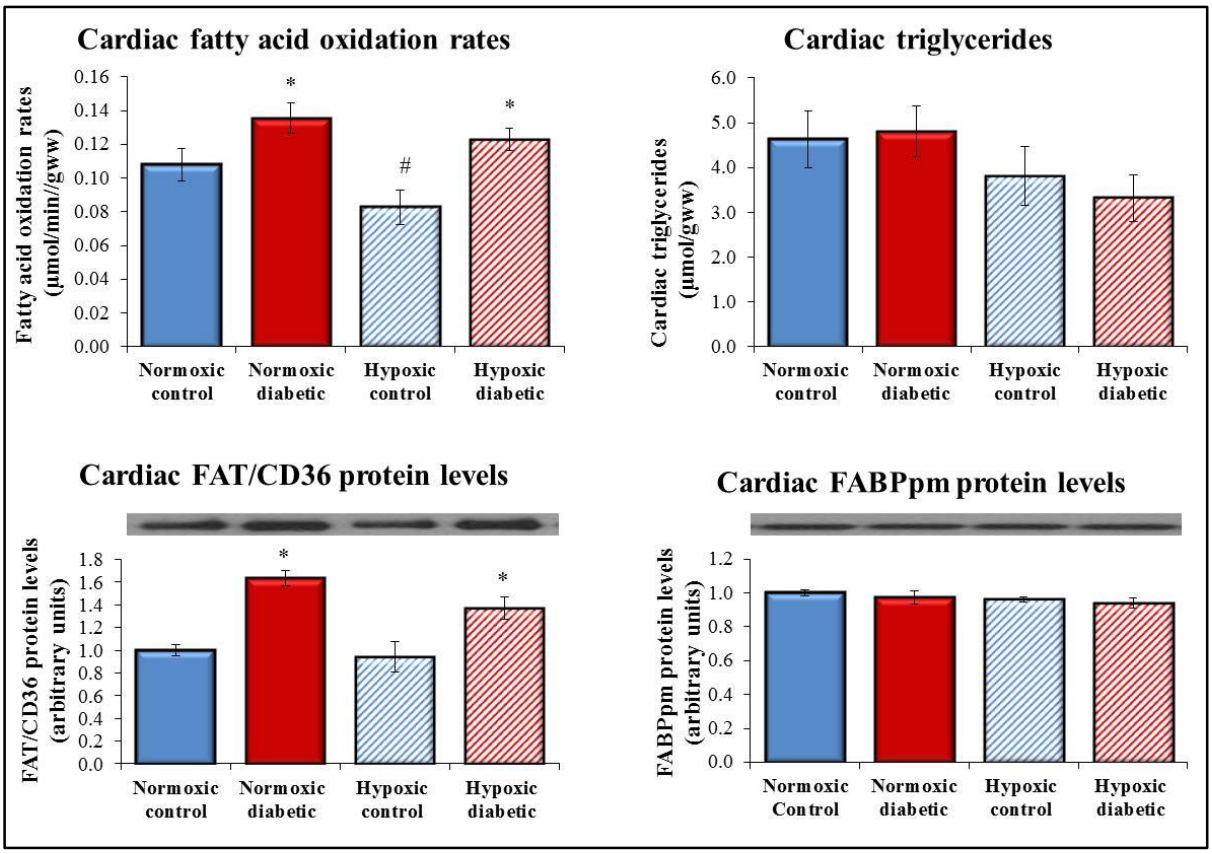

Figure 4.6: Fatty acid oxidation rates, myocardial triglycerides, fatty acid transporters FAT/CD36 and FABPpm protein levels in control and diabetic hearts following adaptation to chronic hypoxia or normoxia. *$p < 0.05$ vs. control group under the same oxygen concentrations, # $p < 0.05$ vs. normoxic group with the same disease status, n = 5 – 9 per group.

4.4.6 Cardiac mitochondrial respiration

The elevated fatty acid oxidation in diabetic hearts in hypoxia suggests that oxygen consumption was maintained in the diabetic hearts, even in unfavourable hypoxic conditions. Therefore, respiration was measured in isolated interfibrillar (IFM) and subsarcolemmal (SSM) mitochondria from control and diabetic rat hearts following normoxia or hypoxia. Following hypoxia, state 3 respiration rates in control hearts with palmitoyl-CoA, a fatty acid substrate, were decreased by 28% and 26% in IFM and SSM, respectively ($p < 0.05$) (Figure 4.7). However, the IFM and SSM from diabetic hearts maintained state 3 respiration rates on palmitoyl-CoA following hypoxia. There

was also no change in state 4 respiration rates, a measure of ATP production-independent oxygen consumption rates, using palmitoyl-CoA as a substrate in any groups.

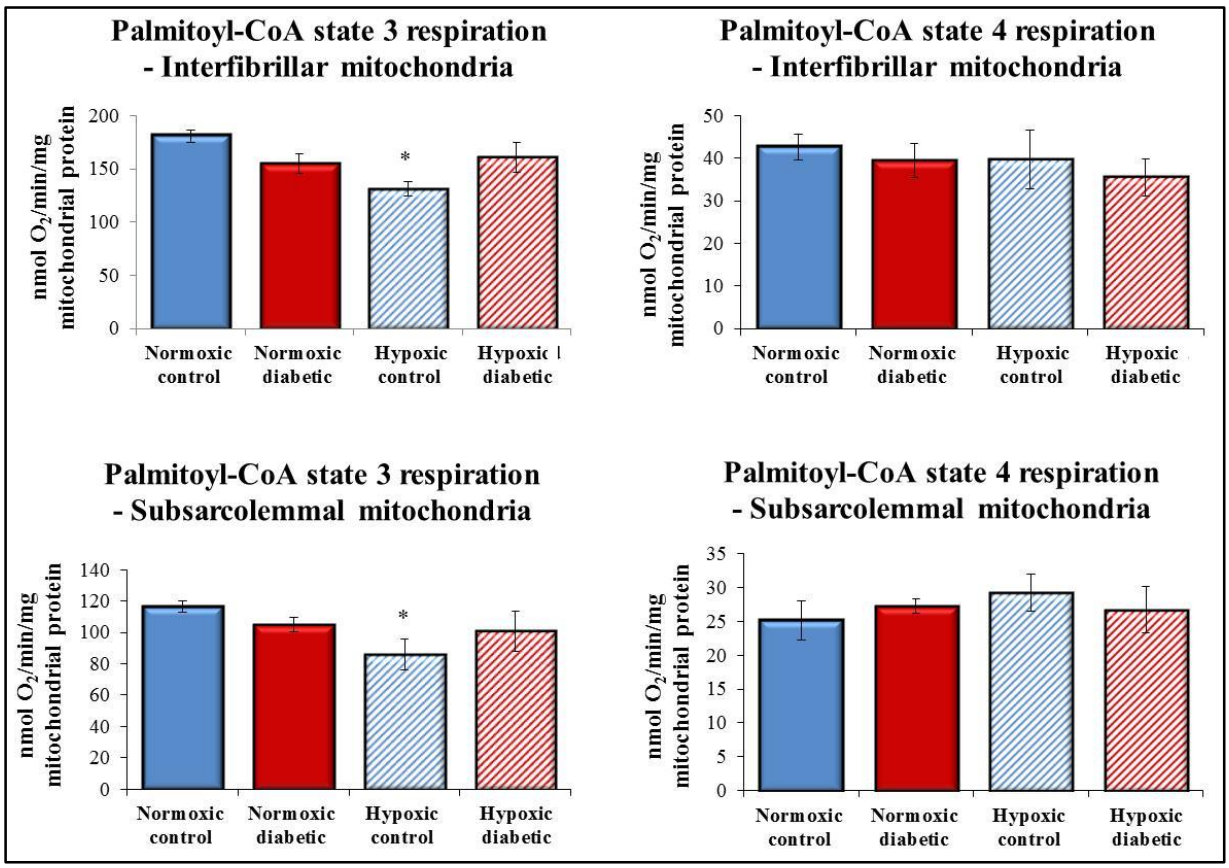

Figure 4.7: Mitochondrial respiration rates using palmitoyl-CoA as substrate, under ADP-stimulated state 3 and ADP-depleted state 4 conditions, in interfibrillar and subsarcolemmal mitochondria from control and diabetic hearts following adaptation to chronic hypoxia or normoxia. * $p < 0.05$ vs. control group under the same oxygen concentrations, $n = 4 – 8$ per group.

There was no effect of diabetes or hypoxia observed on state 3 respiration rates using succinate, a TCA cycle intermediate, as substrate for IFM and SSM (Figure 4.8). In contrast, in diabetic IFM, ATP-independent state 4 respiration rates were increased compared with controls, and the rates were further increased following hypoxia. This hypoxic response was not seen in the control hearts. In general, hypoxia decreased mitochondrial palmitoyl-CoA state 3 respiration in control mitochondria and had no

effect on state 4 ATP-independent respiration. In contrast, diabetic rats did not decrease their rates of fatty acid respiration following hypoxia while increasing oxygen consumption independent of ATP production.

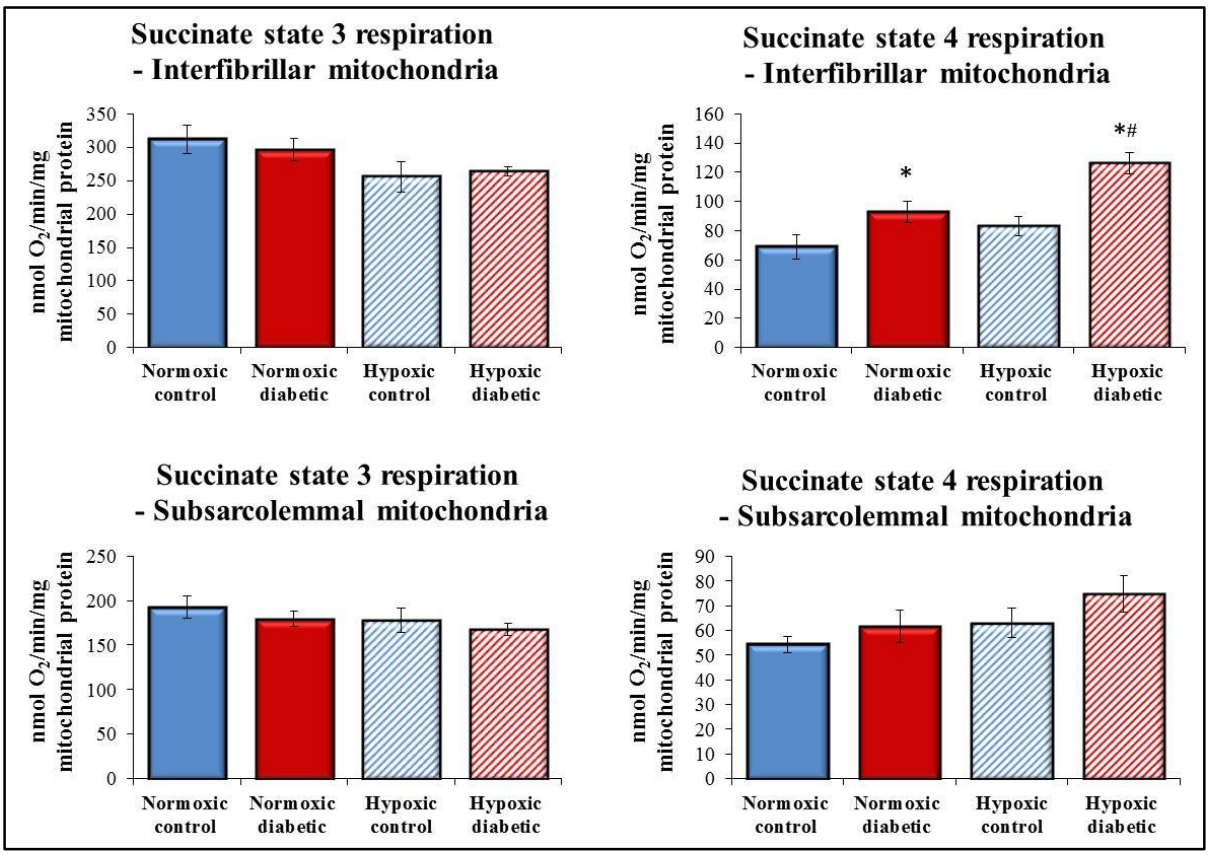

Figure 4.8: Mitochondrial respiration rates using succinate as substrate, under ADP-stimulated state 3 and ADP-depleted state 4 conditions, in interfibrillar and subsarcolemmal mitochondria from control and diabetic hearts following adaptation to chronic hypoxia or normoxia. * $p < 0.05$ vs. control group under the same oxygen concentrations, # $p < 0.05$ vs. normoxic group with the same disease status, $n = 4 – 8$ per group.

4.4.7 Cardiac HIF signalling pathway

The abnormal response to hypoxia in diabetes may be due to a defect in the HIF signalling pathway, and hence mRNA expression and protein levels of components involved in this pathway were investigated. The increase in haematocrits in both control and diabetic rats housed in hypoxia showed that there is no systemic defect in hypoxic

sensing or signalling in diabetes (Figure 4.9). The decrease in cardiac aconitase activity, a HIF metabolic target[192], following hypoxia in both control and diabetic hearts showed that there was no defect in downstream hypoxic signalling in the heart. The mRNA expressions of other downstream targets of HIF signalling, prolyl hydroxylase 3 and heme oxygenase 1, were both increased in hypoxia to the same extent in control and diabetic rat hearts, while cardiac VEGF mRNA showed no difference between control and diabetic rats (Table 4.5). There were no significant differences in HIF1α mRNA expressions between groups, but there was a trend towards an increase following hypoxia for both control and diabetic hearts. These data suggests that there was no prior abnormality in the HIF signalling pathway due to type 2 diabetes, and no abnormalities in the HIF-induced changes following hypoxia in type 2 diabetes.

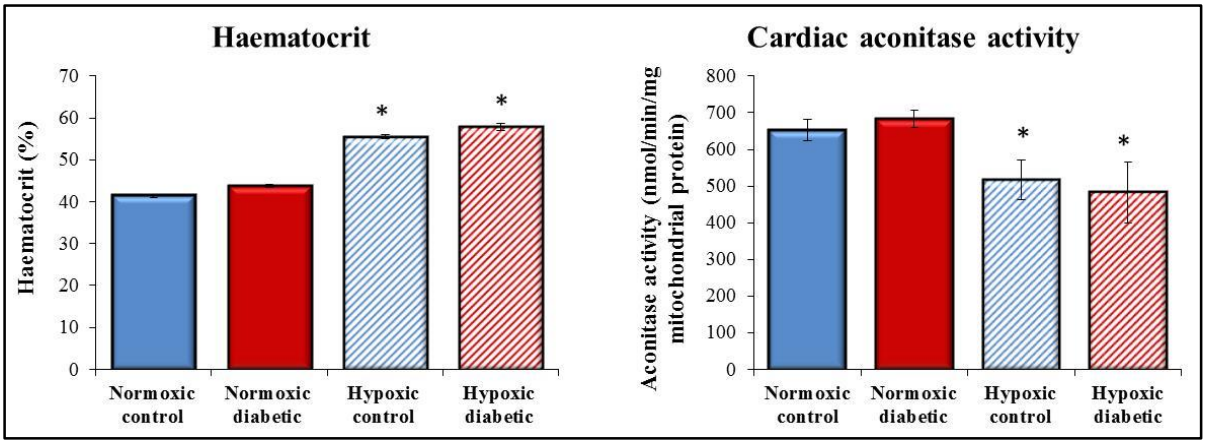

Figure 4.9: Haematocrit and HIF-target enzymes, aconitase activity from control and diabetic hearts following adaptation to chronic hypoxia or normoxia. * $p < 0.05$ vs. normoxic group with the same disease status, $n = 5 – 12$ per group.

Table 4.5: Cardiac mRNA expressions of HIF1α, HIF-target genes and enzymes from control and diabetic hearts following adaptation to chronic hypoxia or normoxia.

Cardiac mRNA expression	Normoxic Control	Normoxic Diabetic	Hypoxic Control	Hypoxic Diabetic
Prolyl hydroxylase 3	1.00 ± 0.09	0.96 ± 0.10	1.58 ± 0.10 #	1.37 ± 0.10 #
Heme oxygenase 1	1.00 ± 0.14	0.81 ± 0.12	1.52 ± 0.14 #	1.21 ± 0.11 #
VEGF	1.00 ± 0.09	1.09 ± 0.14	1.19 ± 0.15	1.02 ± 0.19
HIF1α	1.00 ± 0.09	1.11 ± 0.16	1.25 ± 0.14	1.24 ± 0.12

Arbitrary units. # $p < 0.05$ vs. normoxic group with the same disease status, $n = 5 – 12$ per group.

4.4.8 Cardiac PPARα signalling pathway

PPARα is a transcription factor associated with lipid metabolism and an increase in its expression activity was shown to be associated with increased fatty acid metabolism[193]. PPARα and HIF have a reciprocal relationship, as PPARα expression was shown to be suppressed by HIF[117, 194] and HIF signalling was demonstrated to be suppressed by PPARα in cancer cells[195]. Following the increased lipid metabolism observed in the diabetic heart, we hypothesised that diabetic hearts are not able to downregulate PPARα in chronic hypoxia, which may explain the abnormal hypoxic response. A few PPARα-regulated targets such as medium chain acyl-coenzyme A dehydrogenase (MCAD), pyruvate dehydrogenase kinase 4 (PDK4), uncoupling protein 3 (UCP3) and fatty acid transport protein 1 (FATP1) were measured to test the hypothesis. MCAD is an enzyme involved in β-oxidation and there was an increase in MCAD activity in normoxic diabetic hearts compared with normoxic control hearts (Figure 4.10). Following hypoxia, MCAD activity was decreased in both control and diabetic hearts, but remained 24% higher in hypoxic diabetic hearts compared with hypoxic control hearts. In addition, mitochondrial PPARα targets, PDK4 and UCP3 protein levels were

increased in normoxic diabetes compared with normoxic controls, but decreased following hypoxia in both control and diabetic hearts. However, the protein levels of PDK4 and UCP3 remained significantly elevated in the hypoxic diabetic hearts, compared with hypoxic control hearts. Similarly, FATP1, a sarcolemmal PPARα target, decreased in controls in response to hypoxia, but not in diabetics, demonstrating maintained signalling through this pathway. The maintained PPARα activation in hypoxia would promote fatty acid utilisation and maintain mitochondrial respiration and, via the Randle effect[63], inhibit the glycolytic pathway, as shown in our type 2 diabetic hearts following hypoxia.

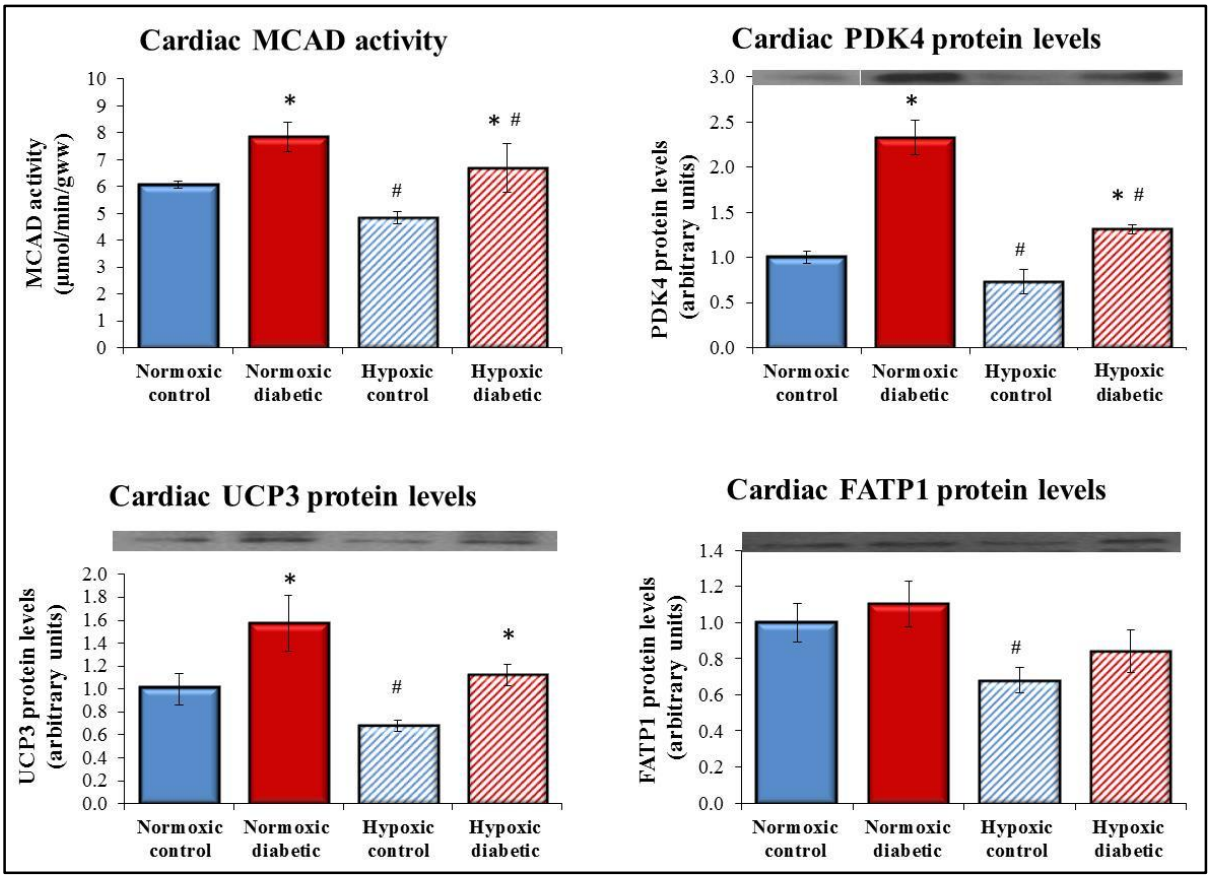

Figure 4.10: PPARα targets; MCAD activity, PDK4, UCP3 and FATP1 protein levels from control and diabetic hearts following adaptation to chronic hypoxia or normoxia. * $p < 0.05$ vs. control group under the same oxygen concentrations, # $p < 0.05$ vs. normoxic group with the same disease status, n = 5 – 8 per group.

4.5 DISCUSSION

The findings of this study showed that the diabetic heart has impaired metabolic response to chronic hypoxia. Control hearts upregulated anaerobic glycolytic rates and increased glycogen stores, and at the same time decreased fatty acid oxidation and respiration rates following chronic hypoxia. In contrast, diabetic hearts had blunted glycolytic rates and maintained fatty acid oxidation, with mitochondria displaying inefficient oxygen consumption following exposure to chronic hypoxia. This abnormal response to hypoxia observed in diabetic hearts was not associated with any defects in the HIF signalling pathway. The increased lipid metabolism in diabetic hearts was accompanied by an overexpression of PPARα target proteins in hypoxia.

4.5.1 Plasma metabolites

Upon exposure to chronic hypoxia, the control rats had decreased blood glucose concentration and elevated lipid metabolites such as NEFA, β-OHB and triglycerides. This reflects the systemic response to hypoxia, involving the upregulation of a more oxygen efficient mechanism to generate ATP via glucose metabolism and downregulate lipid metabolism, which has been demonstrated in mice[196, 197] and humans[198]. Chronic hypoxia normalised the blood glucose concentration in the diabetic rats, implying it might have ameliorated the disease state, but the increased lipid metabolites, ketone bodies and decreased insulin concentrations suggested that chronic hypoxia exacerbates the disease state instead.

4.5.2 Alterations of glucose metabolism in hypoxia

In hypoxia, there was an increase in glycolytic rates in healthy hearts and this has also been well documented in cancer cells[199, 200]. Mazer *et al.* saw similar increases in glycolysis and lactate production in dog hearts in hypoxia and in addition, detected an

increase in lactate oxidation[201]. Papandreou *et al.* demonstrated that in hypoxic cells, HIF actively decreases mitochondrial oxygen consumption via activation of PDK1, which in turn decreases the influx of pyruvate into the tricarboxylic acid (TCA) cycle[202]. This diverts the pyruvate produced from increased glycolytic rates into lactate production instead of going into the TCA cycle and subsequent oxidation. Therefore HIF upregulates glycolysis in hypoxia, via inhibition of PDH activity, by activating PDK1 and increased activation of HIF-responsive glycolytic enzymes.

In normoxia, diabetic hearts had decreased glycolytic rates, PDH activity and GLUT4 protein concentrations compared to control hearts, which suggests that glucose metabolism was suppressed in the diabetic heart. In ob/ob mouse hearts, glycolytic rates were suppressed and did not increase with insulin stimulation[150]. The PDH activity has been shown to be decreased both *in vitro*[143] and *in vivo*[203] in the diabetic heart. The decreased PDH activity may contribute to the accumulation of TCA cycle intermediates, which increases the export of citrate to the cytosol, which in turn inhibits phosphofructokinase, reducing flux through glycolysis and the generation of pyruvate[65]. In addition, decreased insulin-stimulated glucose uptake also plays a role in the decrease in glucose metabolism in the diabetic heart. Menard *et al*. showed decreased GLUT4 mRNA expression in their type 2 diabetic rats, which supports our findings of decreased GLUT4 protein concentrations in diabetic hearts[204].

In hypoxia, even though there was an increase in glycolytic rates and lactate efflux in diabetic hearts compared to normoxic diabetic hearts, the rates were still lower compared to hypoxic control hearts. This suggests that even though there was a response to hypoxia, it was not sufficient to upregulate of glycolysis in diabetic hearts to the same extent as control hearts. Furthermore, PDH activity remained the same in the hypoxic diabetic hearts compared to the normoxic diabetic hearts, suggesting that

either diabetes prevents the full response of the HIF signalling pathway to hypoxia or that PDH is already maximally suppressed in diabetes under normoxic condition.

4.5.3 Increased lipid metabolism in the type 2 diabetic heart in normoxia and hypoxia

In general, control hearts decrease lipid metabolism in hypoxia to decrease oxygen consumption whereas diabetic hearts maintain an elevated fat metabolism even when oxygen is scarce. Control hearts had decreased fatty acid oxidation accompanied by decreased state 3 respiration using palmitoyl-CoA in both subfractions of mitochondria, IFM and SSM, following chronic hypoxia. Heather *et al.* investigated metabolic changes in cardiac mitochondria following chronic hypoxia and the results agreed with our study as the authors observed 17 - 18% decreased state 3 respiration rates with fatty acid[119].

In normoxia, the increased fatty acid oxidation rates in the diabetic hearts were mirrored by increases in FAT/CD36 with no changes in FABPpm protein levels between groups. In agreement, Bonen *et al.* demonstrated an increase in sarcolemmal palmitate transport rates and sarcolemmal FAT/CD36 but no change in FABPpm expression in type 2 diabetic humans[205]. Similarly, Luiken *et al.* used a STZ-induced type 1 diabetic model to show an increase in cardiac FAT/CD36 protein levels with no changes in cardiac FABPpm levels[206].

In hypoxia, the diabetic hearts maintained elevated fatty acid oxidation rates and state 3 respiration rates with fatty acid, suggesting that diabetic hearts have a different response to hypoxia compared to control hearts. High fat diet-induced cardiac dysfunction in mice has been tied to the increase of prolyl hydroxylase-2, which contributes to the degradation of HIF-1α, suppressing the response to hypoxia[207]. Therefore the abnormal

response to hypoxia with elevated oxygen consumption despite the lack of oxygen seen in the diabetic hearts may be detrimental to these hearts.

The inability of diabetic hearts to upregulate glucose metabolism (specifically the glycolytic pathway in hypoxia) may suggest that the diabetic heart either produces less ATP, which could be detrimental in that condition, or produces ATP via other means. The diabetic hearts maintained their PDH and CS activities, state 3 respiration rates, but increased the ATP-independent oxygen consumption via state 4 respiration in hypoxia, which suggests that the diabetic hearts were unable to switch on the hypoxic response seen in control hearts to optimise oxygen consumption for the highest possible yield of ATP. This is in agreement with How *et al.,* who demonstrated in type 1 diabetic hearts, there was decreased cardiac efficiency caused by oxygen waste in normoxia for non-contractile purposes[208]. In another study, Buchanan *et al.* showed that ob/ob and db/db mice had increased oxygen consumption coupled with decreased cardiac efficiency in normoxia[185].

4.5.4 Normal HIF-1α pathways in type 2 diabetic hearts in response to hypoxia

There are a number of publications claiming that the HIF-1α signalling pathway is impaired in the diabetic heart. Katavetin *et al.* showed that hyperglycaemia blunts the VEGF response to hypoxia in cultured cells[209], but the cells were cultured in 25 mM glucose, a much higher concentration than blood glucose in our diabetic rats and in most type 2 diabetic patients. Studies have demonstrated an increase in infarct size due to low levels of heme oxygenase-1[210], a HIF-targeted enzyme, and decreased HIF-1α expression in type 1 diabetic rat hearts[120]. In addition, Marfella *et al.* demonstrated impaired HIF-1α and VEGF expressions in diabetic patients. Sasso *et al.* discovered contrasting findings as they measured an increase in VEGF expression but a decrease in

VEGF signal transduction in their type 2 diabetic patient populations with coronary heart disease[211].

4.5.5 Overactivation of PPARα signalling in type 2 diabetes underpins the abnormal metabolic response

Based on our results, the type 2 diabetic heart does not have a defect in the HIF signalling pathway, which is known to be responsible to activate a cascade of regulatory mechanisms to maintain ATP production under limited oxygen availability, while decreasing oxygen consumption. However, the metabolic response of the diabetic heart to hypoxia is certainly abnormal compared with control hearts. PPARα is associated with increased lipid metabolism as it upregulates many enzymes of fatty acid metabolism, including long and medium chain acyl co-enzyme A dehydrogenases, thereby upregulating the capacity for fatty acid utilisation. PPARα has also been shown to be activated in diabetes following the increase in lipids in blood plasma and fatty acid utilisation in the heart[212]. Since PPARα was shown to be suppressed by HIF activation[117, 194], it plays a key role in answering our question on the abnormal response of diabetic hearts in hypoxia. Retinoid X receptor α (RXRα) is an obligate partner of PPARα which dimerises with the latter and the dimer complex binds with the PPAR responsive element on DNA, followed by the upregulation of enzymes involved in lipid metabolism[194]. Huss *et al.* proposed that fatty acid metabolism may be supressed in hypoxia via the decrease in RXRα level as they found that RXRα levels were decreased in both nuclear and whole cell fractions with no significant changes in nuclear PPARα protein concentrations[117]. The results of the PPARα target protein expressions demonstrated the effect of both hypoxia and diabetes at work. The data suggests that HIF is unable to suppress the overactivation of PPARα in the diabetic hearts, which is shown by the elevated fatty acid oxidation, UCP3 and PDK4 protein levels, and MCAD

activity even in hypoxic conditions. Even though PPARα targets were shown to be elevated in diabetic hearts, PPARα protein levels could not be measured with our lab equipment. Multiple attempts have been conducted to measure PPARα protein levels but to no avail, we have also discussed this issue with experts and there are opinions that PPARα proteins could not be measured with the current technology. However, PPARα mRNA was measured and there was no difference between groups (Table 4.6).

Table 4.6: Cardiac mRNA expressions of PPARα from control and diabetic hearts following adaptation to chronic hypoxia or normoxia.

Cardiac mRNA expression	**Normoxic Control**	**Normoxic Diabetic**	**Hypoxic Control**	**Hypoxic Diabetic**
PPARα	1.00 ± 0.09	0.93 ± 0.08	0.93 ± 0.08	0.85 ± 0.06

Despite the limitations on measuring PPARα protein levels in this study, it is still evident that the downstream targets of PPARα were elevated in diabetic hearts and that hypoxia did not manage to suppress its overexpression. This data supports the current use of PPARα agonist to treat the abnormal substrate metabolism in type 2 diabetes. Fibrates, such as bezafibrate or gemfibrozil, are known PPARα agonists used to treat dyslipidaemia, hence reducing risk of cardiovascular disease. The VA-HIT (Veterans Affairs –HDL-C Intervention Trial) suggested that patients with diabetes derive greater benefits from fibrate therapy than nondiabetic subjects by examining a clinical population that included 25% patients with diabetes[213, 214]. St. Mary's, Ealing, Northwick Park Diabetes Cardiovascular Disease Prevention (SENDCAP) showed that patients treated with bezafibrate significantly reduced the combined incidence of probable ischemic change on resting electrocardiogram and documented myocardial infacrtion[215]. In addition the Fenofibrate Intervention and Event Lowering in Diabetes

(FIELD) study showed the optimal benefit of fenofibrate observed in patients with marked dyslipidaemia; relative 27% risk reduction in cardiovascular disease[216].

4.5.6 Role of AMPK in hypoxia and potential target for type 2 diabetes treatment

AMP-activated protein kinase (AMPK) is a nutrient sensor that plays a vital role in regulating the systemic and cellular energy balance. AMPK is activated by an increase in AMP:ATP ratio during stress such as starvation, exercise, ischaemia and hypoxia[217]. AMPK was shown to increase survivability by restoring energy balance in hypoxic fibroblast cells[218] and induce cardioprotection in cardiomyocytes during hypoxia via attenuation of endoplasmic reticulum stress[219]. In addition, AMPK was also shown to be critical for HIF-1 transcriptional activity as the inhibition of AMPK attenuated target gene expressions, VEGF secretion and glucose uptake[220]. This suggests that activation of AMPK could also be a potential route to treat the abnormal metabolic response of diabetic hearts to chronic hypoxia. This is especially relevant as it has been suggested that type 2 diabetic patients had diminished AMPK activity[221] and this could be detrimental under physiological stress such as hypoxia. The compound 5-aminoimidazole-4-carboxamide riboside (AICAR) was the first compound identified to activate AMPK although it is unlikely to be used as treatment in humans due to poor bioavailability and short half-life. However, administration of AICAR was shown to reverse some aspect of metabolic syndrome in *ob/ob* mice and high fat fed rats[222, 223]. Short term activation of AMPK in liver led to normalised blood glucose levels and decreased adipose tissue mass in diabetic mice[224]. Shaw *et al.* showed that AMPK is also the therapeutic target of metformin[127], an oral medication widely used for glycaemic control in type 2 diabetic patients.

In conclusion, cardiac adaptation to chronic hypoxia in type 2 diabetes is blunted, with diabetic hearts unable to upregulate glycolysis and glycogen content, and unable to

suppress fatty acid oxidation and mitochondrial oxygen consumption to the same extent as control hypoxic rats. This abnormal metabolic adaptation to hypoxia in diabetes occurs independently of changes in HIF signalling pathways, and is associated with an inability of hypoxia to suppress PPARα target proteins. Coupled with existing clinical data using PPARα agonist, PPARα may be promising potential therapeutic target to treat the abnormal cardiac substrate metabolism and subsequently decreasing the risk for cardiovascular disease within the type 2 diabetic populations.

CHAPTER 5

EFFECT OF ACUTE HYPOXIA ON CARDIAC METABOLISM AND FUNCTION IN THE TYPE 2 DIABETIC HEART

5.1 ABSTRACT

Acute hypoxia is a key component of a myocardial ischaemia, to which the heart must rapidly respond to ensure cardiomyocyte survival. Therefore this study was aimed to investigate the metabolic adaptation of diabetic hearts in acute hypoxia and its effect on cardiac function. Control and diabetic rat hearts were perfused for a total of 72 minutes comprising 16 minutes of normoxia, 36 minutes of hypoxia and 20 minutes of reoxygenation and freeze-clamped at the end of the experiment for tissue analyses. Glycolytic rates were increased by 0.78 µmol/gww/min in control hearts and 0.49 mmol/gww/min in diabetic hearts during acute hypoxia compared with their respective normoxic rates. Fatty acid oxidation rates in diabetic hearts were 37% greater than in control hearts in normoxia and there was no difference between groups during hypoxia. However, upon reoxygenation, fatty acid oxidation rates were increased in diabetic hearts compared to control hearts. Triglyceride concentrations of diabetic hearts increased by 55% compared with control hearts following reoxygenation. Cardiac function, as defined by rate pressure product (RPP), decreased by 84% in diabetic hearts compared to a decrease of 68% in control hearts during hypoxia. Upon reoxygenation, control hearts recovered RPP to 75% of normoxic rates, whereas diabetic hearts only recovered to 63%. There was a significant increase in left ventricular end-diastolic pressure coupled with ventricular ectopic beats, a form of arrhythmia, in diabetic hearts compared with control hearts during hypoxia. The inability of diabetic hearts to upregulate glycolysis during acute hypoxia and deposition of triglycerides upon reoxygenation supports the notion of metabolic inflexibility in diabetic hearts with high reliance on lipid metabolism. The detrimental effects of metabolic inflexibility in diabetic hearts were reflected by decreased cardiac function during acute hypoxia and reoxygenation.

5.2 INTRODUCTION

Acute hypoxia is a component of myocardial ischaemia, and the ability to sense and instantly respond to the changes in oxygen is essential for the heart to survive a myocardial infarction[225]. Type 1 diabetic rats were shown to have an increased infarct size compared to controls[120, 210], suggesting that the diabetic heart may have defects in adapting and responding to ischaemia and hypoxia.

In hypoxia, cells upregulate glycolysis and decrease fatty acid oxidation to conserve oxygen and maintain ATP production anaerobically[199, 201, 202]. Glycolytic ATP is considered to be especially important for the maintenance of cellular ion homeostasis and diastolic relaxation[226], and complete oxidation of carbohydrates provides 8–11% more ATP per mole of oxygen consumed than fatty acid oxidation, making carbohydrate metabolism more oxygen efficient under oxygen-scarce conditions[227]. In Chapter 4, it was demonstrated that chronic hypoxia failed to suppress PPARα overactivation in diabetic hearts, resulting in increased fatty acid oxidation and decreased glycolytic rates in hypoxic diabetic hearts compared with control hearts. This metabolic dysfunction in the diabetic heart when a physiological stressor such as hypoxia was present suggests that the diabetic heart was not metabolically flexible and unable to switch substrates even if it would be more favourable in the given conditions. This chapter will investigate if this cardiac metabolic inflexibility of the diabetic heart is also present in response to acute hypoxia and if it has detrimental effects on cardiac function during hypoxia and upon reoxygenation.

In addition to metabolic changes, acute hypoxia and reoxygenation may reveal any contractile dysfunction in the diabetic heart, as type 1 diabetic patients exhibit impaired recovery of contractile function following myocardial infarction[25]. It has also been

demonstrated in STZ type 1 diabetic rats that there was a marked impairment of systolic and diastolic functions in diabetic hearts compared with control hearts[228, 229]. Similar contractile dysfunction was observed in Goto-Kakizaki type 2 diabetic rats model[151]. However, the Goto-Kakizaki is a lean type 2 diabetic model which is different from our STZ and high fat diet model, which was aimed to closely resemble the human diabetic pathophysiology. In addition, as El Omar *et al.* suggested but did not investigate, the contractile dysfunction in the Goto-Kakizaki rats had a primarily metabolic basis[151] and this chapter will be investigating the metabolic changes of the diabetic hearts during hypoxia and upon reoxygenation. This contractile dysfunction was also reinforced in other studies using diabetic cardiomyocytes, demonstrating contractile abnormalities in these cells[230-233].

Therefore, the aims of this chapter were firstly to investigate whether there is an altered metabolic response to acute hypoxia and reoxygenation in type 2 diabetic hearts compared to control hearts. Secondly, it is essential to determine if the hearts of our type 2 diabetic model have decreased cardiac function in acute hypoxia and recovery upon rexoygenation as there are currently no studies on the effect of acute hypoxia on low dose STZ type 2 diabetic rat models available. Finally, to establish a link between the metabolic response and any contractile function changes in diabetic hearts during hypoxia and reoxygenation.

5.3 METHODS

5.3.1 Acute hypoxic isolated heart perfusion

Rat hearts were perfused in Langendorff mode as described in Chapter 2. However, in this study, insulin was not added to the buffer and the perfusion lasted 72 minutes instead of the standard 52 minutes in previous experiments. Hearts were perfused under normoxic conditions (95% O_2, 5% CO_2) for 16 minutes before acute hypoxia was induced by replacing the gas supply with 95% N_2, 5% CO_2, decreasing the oxygen partial pressure (pO_2) from 413 ± 15 mmHg to 90 ± 10 mmHg. Following 36 minutes of acute hypoxia, hearts were reoxygenated for 20 minutes by returning the O_2 gas supply to the recirculating buffer to pre-hypoxic levels (95% O_2, 5% CO_2) as shown in Figure 5.1. Throughout the perfusion, the buffer composition was not altered and only the degree of oxygenation was changed to induce acute hypoxia. Metabolic rates, cardiac function and coronary flow rates were measured during normoxia, hypoxia and reoxygenation. Hearts were freeze-clamped after reoxygenation. In a separate batch of animals, hearts were freeze-clamped at the end of normoxia or at the end of hypoxia, to allow tissue analysis at the end of the three different conditions. Some of the fatty acid extractions and data analyses were done with the assistance Miss Georgina Yea during her undergraduate project.

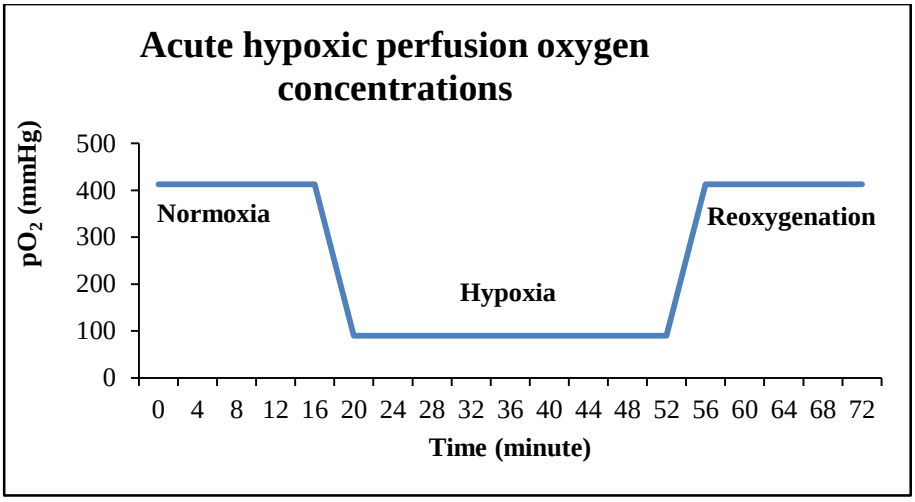

Figure 5.1: Oxygen concentration changes in KH buffer measured during the acute hypoxic perfusion protocol to induce normoxic, hypoxic and reoxygenation conditions.

5.3.2 Statistical analysis

Results are expressed as means ± standard error of the mean (SEM). Glycolytic rates, fatty acid oxidation rates and cardiac function were measured in the same heart in normoxia, hypoxia and reoxygenation in both control and diabetic groups. Therefore they were analysed using two way repeated measured ANOVA with a post hoc Holm-Sidak test. All other data were from separate hearts collected at the end of normoxia, end of hypoxia or end of reoxygenation, and were analysed using two way ANOVA with post hoc t-tests. Independent t-tests were carried out on physical characteristic and plasma metabolite data. $p < 0.05$ was taken as the level of significance.

5.4 RESULTS

5.4.1 Physical parameters

There were no significant differences in terminal body weights, heart weights and heart weight to body weight ratios between control and diabetic rats (Table 5.1). Both epididymal fat pad weight and fat pad to body weight ratio, indicators of adiposity, were increased in diabetic rats compared with control rats. Both glucose and insulin concentrations in the fasted state were increased significantly in the diabetic rats compared to the control rats, replicating the hyperglycaemia and hyperinsulinaemia seen in type 2 diabetic patients[234, 235]. There were significant increases in fed plasma glucose, NEFA and β-OHB concentrations in diabetic rats compared to controls, whereas insulin, triglyceride and cholesterol concentrations were not different between the two groups.

Table 5.1: Physical characteristics, plasma metabolites in fed and fasted states from control and diabetic rats.

Physical characteristics	**Control**	**Diabetic**
Terminal body weight (g)	377 ± 8	390 ± 10
Heart weight (g)	1.40 ± 0.03	1.46 ± 0.04
Heart weight to body weight ratio ($x10^3$)	3.74 ± 0.09	3.73 ± 0.07
Epididymal fat pad weight (g)	5.6 ± 0.3	8.5 ± 0.7 *
Fat pad to body weight ratio ($x10^2$)	1.49 ± 0.1	2.22 ± 0.2 *
Plasma metabolites in the fasted state		
Glucose (mmol/l)	5.0 ± 0.3	6.4 ± 0.5 *
Insulin (ug/l)	0.34 ± 0.03	0.94 ± 0.17 *
Plasma metabolites in the fed state		
Glucose (mmol/l)	13.1 ± 0.3	16.0 ± 0.6 *
Insulin (ug/l)	2.19 ± 0.29	2.09 ± 0.35
NEFA (mmol/l)	0.06 ± 0.01	0.11 ± 0.01 *
β-OHB (mmol/l)	0.32 ± 0.02	0.57 ± 0.03 *
TAG (mmol/l)	1.43 ± 0.19	1.25 ± 0.16
Cholesterol (mmol/l)	1.33 ± 0.05	1.25 ± 0.07

* $p < 0.05$ vs. control group, Physical characteristics n = 16 – 28 per group, Plasma metabolites in the fed state n = 16 per group, plasma metabolites in the fasted state n = 6 per group. NEFA, non-esterified fatty acids; β-OHB, β-hydroxybutyrate; TAG, triglycerides.

5.4.2 Cardiac glucose metabolism

In normoxia, diabetic hearts have significantly lower glycolytic rates compared with control hearts (Figure 5.2). Acute hypoxia significantly increased glycolytic rates by 0.78 µmol/gww/min in control hearts and 0.49 mmol/gww/min in diabetic hearts, with diabetic hearts retaining significantly lower glycolytic rates compared with controls ($p < 0.05$). Despite this, the percentage increase in glycolytic rates in diabetic hearts was greater compared with control hearts in hypoxia, suggesting that there may be a maximum threshold that the control hearts have reached and further *in vivo* data will be needed to further elucidate this data. Upon reoxygenation, glycolytic rates in both control and diabetic hearts returned to rates similar to normoxic levels, with the diabetic hearts having lower glycolytic rates compared to control hearts.

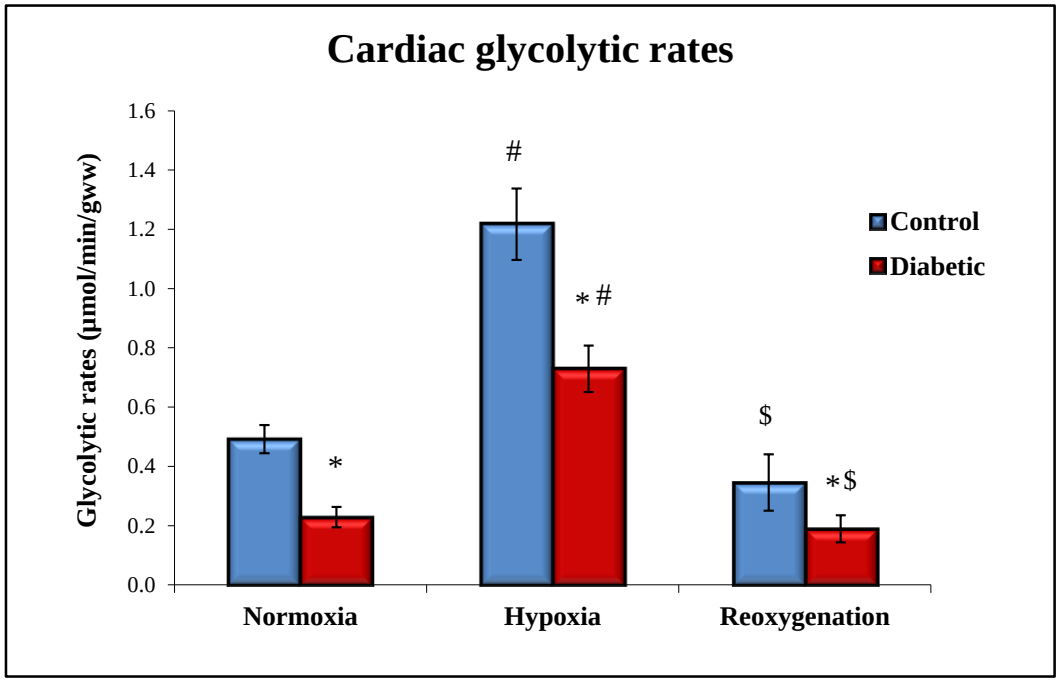

Figure 5.2: Cardiac glycolytic rates of isolated control and diabetic hearts perfused in normoxia, hypoxia and reoxygenation. * $p < 0.05$ vs. control group under the same oxygen concentrations, # $p < 0.05$ vs. same disease state at normoxia, $ $p < 0.05$ vs. same disease state at hypoxia, $n = 5 – 7$ per group.

Lactate efflux during acute hypoxia was increased significantly in both control (291%) and diabetic hearts (234%), mirroring changes in glycolytic rates in acute hypoxia (Figure 5.3). However, upon reoxygenation, lactate efflux in both control and diabetic hearts decreased significantly compared to rates in acute hypoxia. The 2 way ANOVA repeated measures analysis indicated that there was an effect of diabetes and hypoxia but there was no interaction between the variables, so no post hoc t-test was performed between control and diabetic hearts at specific oxygen concentrations. Instead, the symbol '‡' was given to indicate that as a grouped data, diabetic hearts were significantly different compared to control hearts.

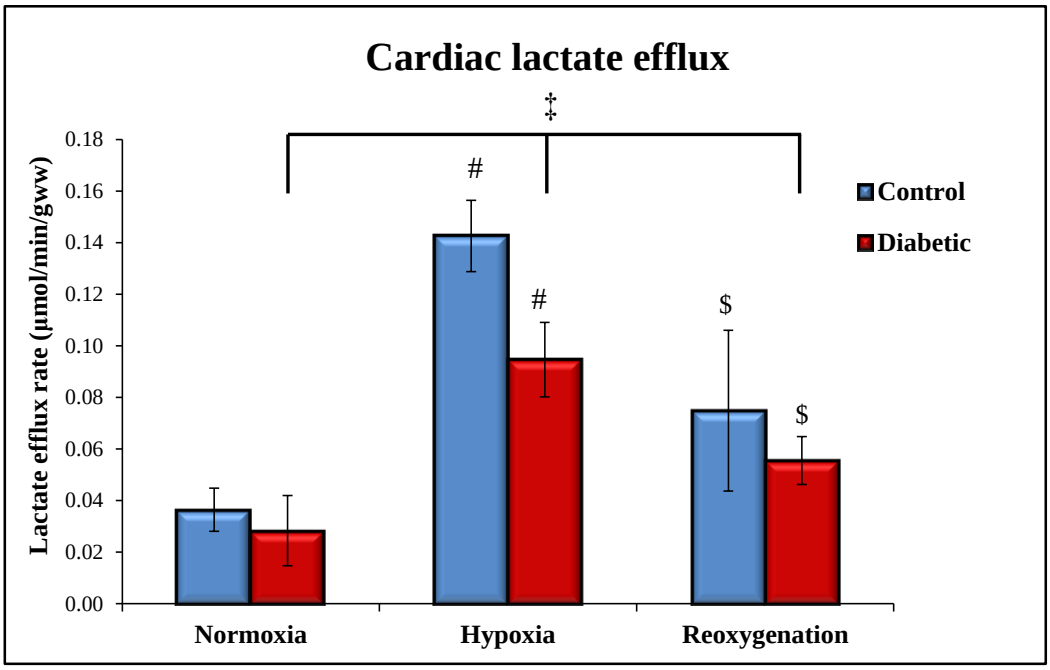

Figure 5.3: Cardiac lactate efflux rates of isolated control and diabetic hearts perfused in normoxia, hypoxia and reoxygenation. ‡ $p < 0.05$ vs. control group, # $p < 0.05$ vs. same disease state at normoxia, $ $p < 0.05$ vs. same disease state at hypoxia, $n = 4 – 6$ per group.

In normoxia, cardiac glycogen concentrations in diabetic hearts were significantly decreased compared with control hearts (Figure 5.4). Acute hypoxia decreased glycogen concentrations of both control and diabetic hearts, and the difference between

the control and diabetic hearts was lost. Upon reoxygenation, the glycogen content of both control and diabetic hearts increased significantly compared to their respective values in hypoxia but they remained lower compared to their normoxic values. The difference in glycogen concentrations between control and diabetic hearts was also reinstated upon reoxygenation, suggesting that diabetic hearts may handle its carbohydrate storage differently compared to control hearts.

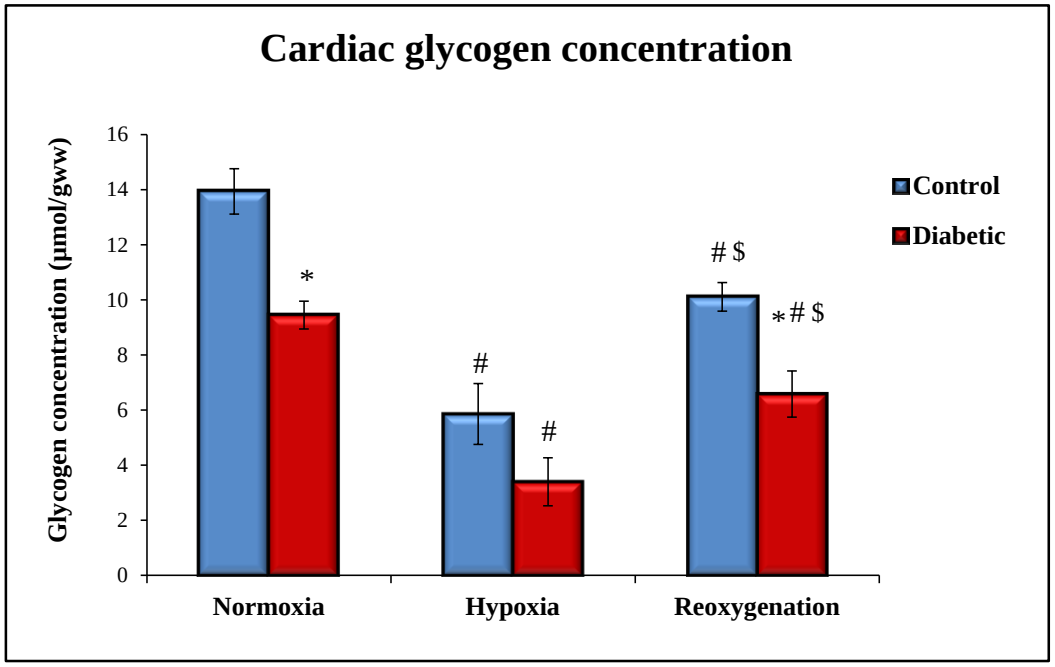

Figure 5.4: Cardiac glycogen concentrations of isolated control and diabetic hearts perfused in normoxia, hypoxia and reoxygenation. *p < 0.05 vs. control group under the same oxygen concentrations, # p < 0.05 vs. same disease state at normoxia, $ p < 0.05 vs. same disease state at hypoxia, n = 5 – 6 per group.

There was no significant difference in cardiac PDH activity between control and diabetic hearts in normoxia (Figure 5.5). In hypoxia, PDH activity was significantly higher in the control hearts compared with diabetic hearts, but there was no difference in both groups compared to their respective normoxic levels. There was an increase in PDH activity in control hearts upon reoxygenation compared to the activity in normoxia, whereas PDH activity in diabetic hearts remained unchanged throughout the

3 different conditions. There was also a significant difference in PDH activity between control and diabetic hearts upon reoxygenation.

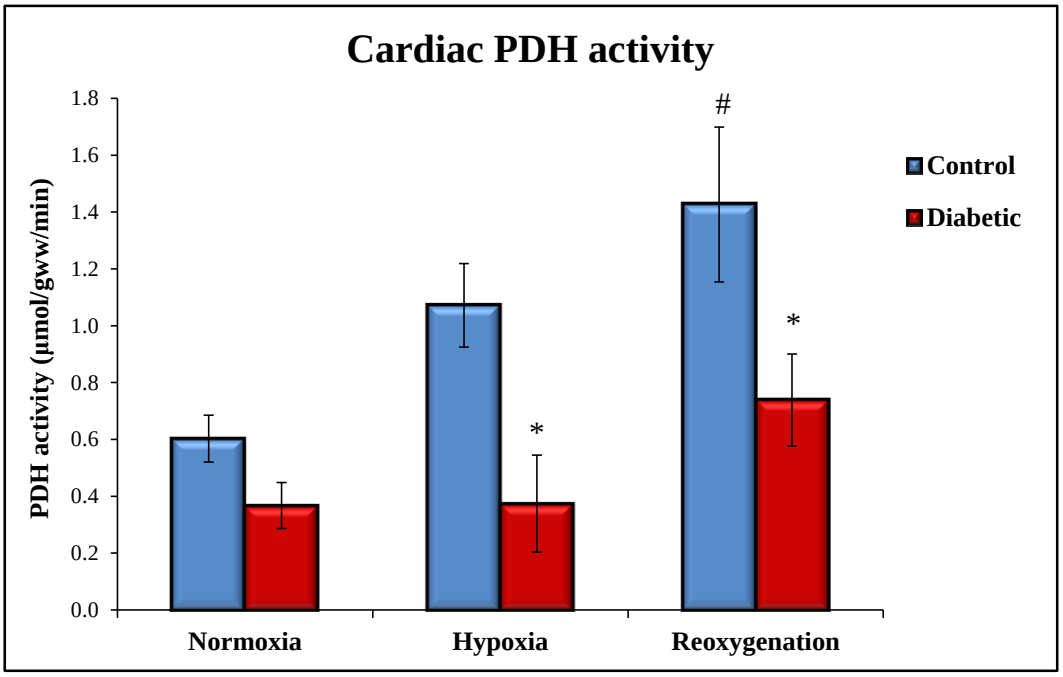

Figure 5.5: Cardiac PDH activity of isolated control and diabetic hearts perfused in normoxia, hypoxia and reoxygenation. *p < 0.05 vs. control group under the same oxygen concentrations, # p < 0.05 vs. same disease state at normoxia, n = 5 – 6 per group.

5.4.3 Cardiac lipid metabolism

In normoxia, diabetic hearts had 37% greater rates of palmitate oxidation compared with controls (p < 0.05) (Figure 5.6). Acute hypoxia decreased fatty acid oxidation in both control and diabetic hearts to similar levels. Upon reoxygenation, fatty acid oxidation rates returned to rates similar to their normoxic levels, with diabetic hearts showing significantly increased rates compared to controls.

There was no difference in cardiac triglyceride conentrations between control and diabetic hearts in both normoxia and hypoxia (Figure 5.7). Upon reoxygenation, the triglyceride concentrations of diabetic hearts increased by 55% compared with control

hearts ($p < 0.05$), which was significantly greater than the cardiac triglyceride concentration in diabetic hearts in normoxia.

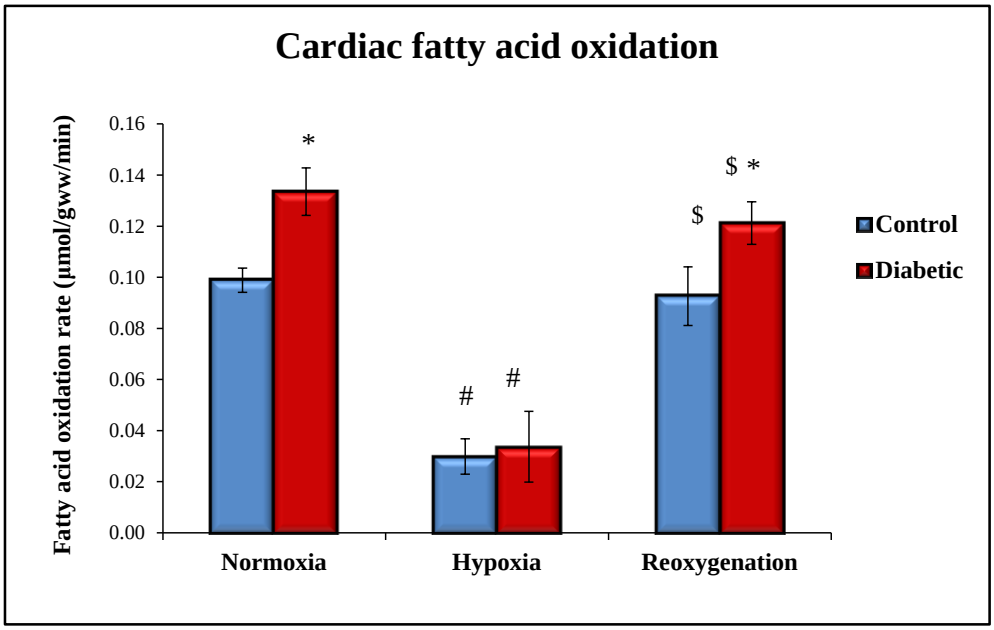

Figure 5.6: Cardiac fatty acid oxidation rates of isolated control and diabetic hearts perfused in normoxia, hypoxia and reoxygenation. *$p < 0.05$ vs. control group under the same oxygen concentrations, # $p < 0.05$ vs. same disease state at normoxia, $ $p < 0.05$ vs. same disease state at hypoxia, $n = 6 – 8$ per group.

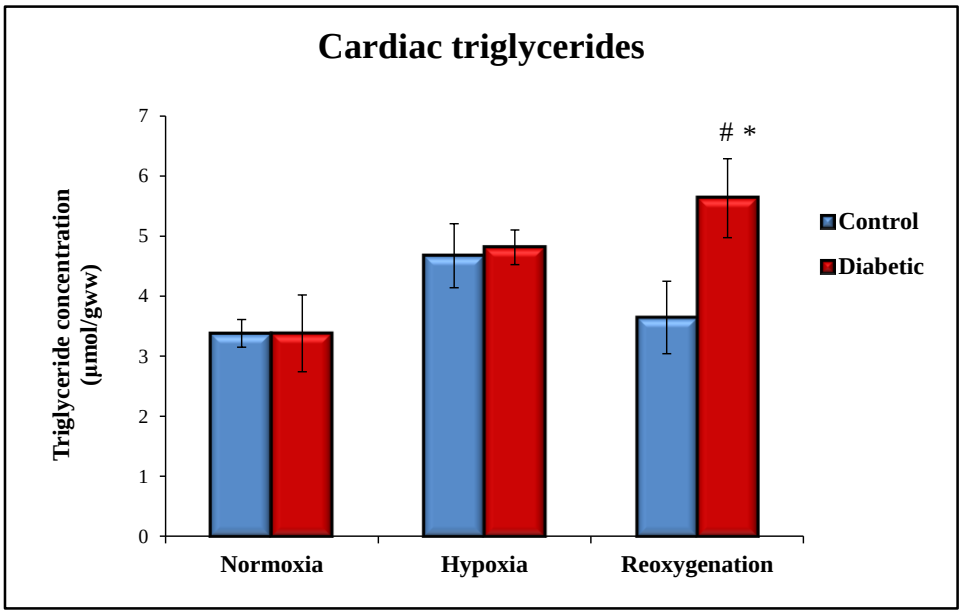

Figure 5.7: Cardiac triglyceride concentrations of isolated control and diabetic hearts perfused in normoxia, hypoxia and reoxygenation. *$p < 0.05$ vs. control group under the same oxygen concentrations, # $p < 0.05$ vs. same disease state at normoxia, $n = 5 – 6$ per group.

5.4.4 Cardiac function

In control hearts, coronary flow rates were increased significantly during hypoxia and continue to increase upon reoxygenation (Table 5.2). In the diabetic hearts, coronary flow rates were increased only upon reoxygenation. There was no difference between control and diabetic coronary flow rates.

Table 5.2: Coronary flow rates of control and diabetic hearts in normoxia, hypoxia and reoxygenation conditions during perfusion.

Coronary flow rates (ml/min)	**Control**	**Diabetic**
Normoxia	20 ± 1	22 ± 1
Hypoxia	23 ± 1 #	22 ± 2
Reoxygenation	25 ± 1 #$	24 ± 2 $

# $p < 0.05$ vs. same disease state at normoxia, $ $p < 0.05$ vs. same disease state at hypoxia, n = 7 – 8 per group.

Acute hypoxia decreased heart rates of control hearts by 23% and diabetic hearts by 75% ($p < 0.05$) (Figure 5.8). Upon reoxygenation, there was full recovery of heart rates to normoxic levels in control hearts, whereas diabetic hearts showed only an 83% recovery ($p < 0.05$).

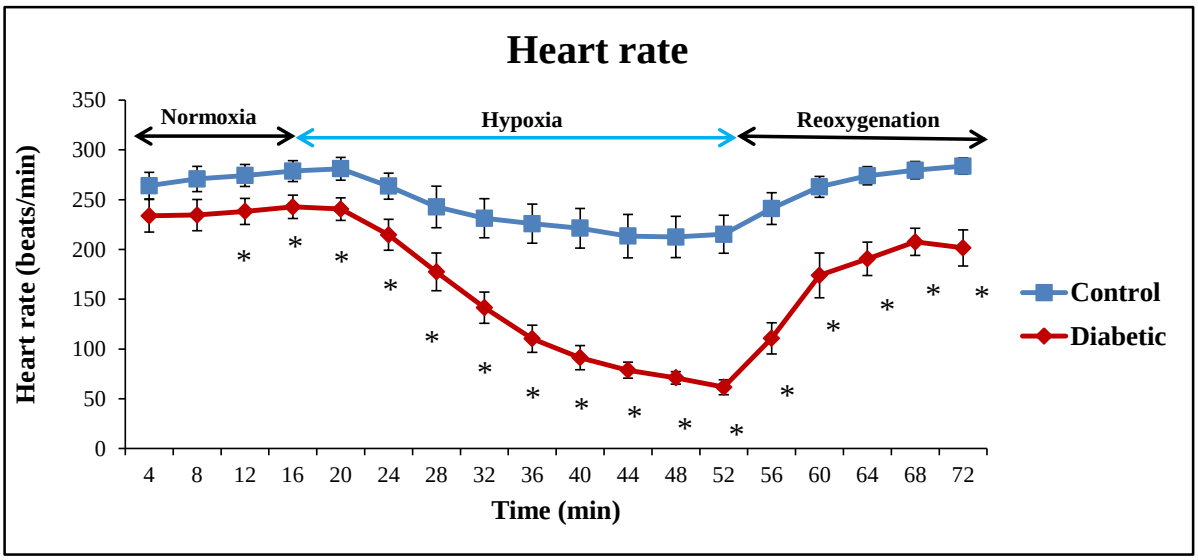

Figure 5.8: Graph showing heart rates of isolated control and diabetic hearts perfused in normoxia, hypoxia and reoxygenation. * $p < 0.05$ vs. control group at same time point. n = 7 – 10 per group.

End-diastolic pressure is the pressure in the ventricle following filling at the end of diastole (i.e. at the start of systole). Thus the relationship between the end-diastolic volume and the resulting end-diastolic pressure is determined by the compliance of the ventricular wall. Acute hypoxia increased the end-diastolic pressure of both control and diabetic hearts, with a more pronounced effect on the diabetic hearts as end-diastolic pressure was significantly higher in diabetic hearts compared with control hearts by the end of hypoxia (Figure 5.9). Given that the end-diastolic volume was fixed, represented as the volume of the intraventricular balloon, the increase in end-diastolic pressure at the end of hypoxia suggests that diabetic hearts were stiffer (lower compliance) and not able to fully relax compared with control hearts during acute hypoxia.

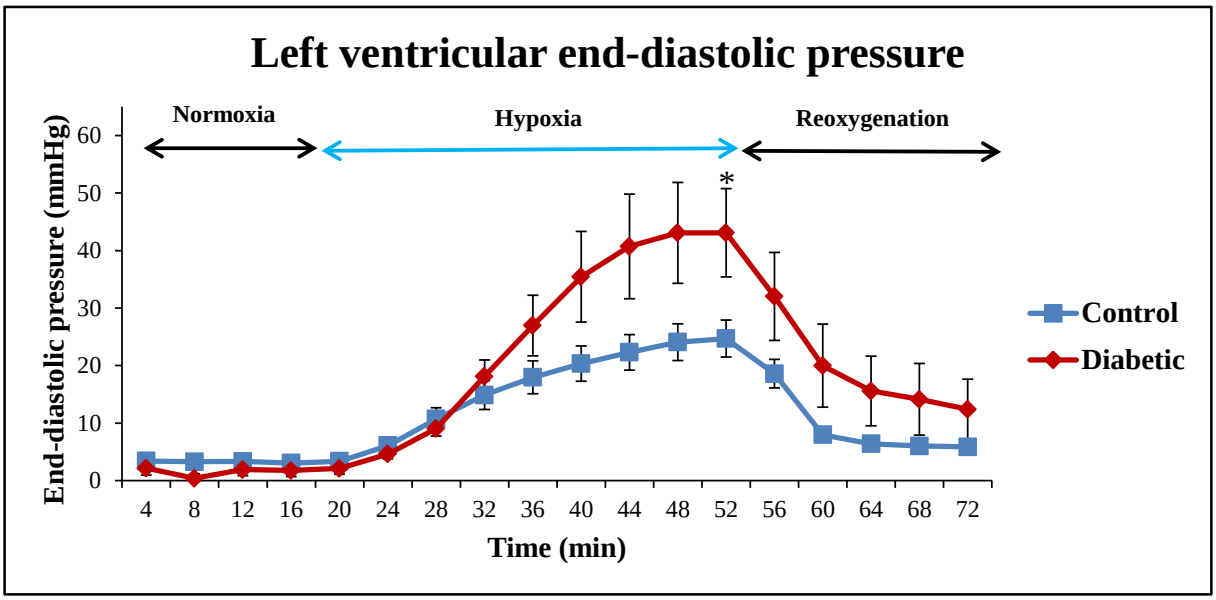

Figure 5.9: Graph showing left ventricular end-diastolic pressure at fixed end-diastolic volume of isolated control and diabetic hearts perfused in normoxia, hypoxia and reoxygenation. * $p < 0.05$ vs. control group at same time point. n = 7 – 10 per group.

Unexpectedly, diabetic hearts generated significantly greater left ventricular developed pressure than control hearts during acute hypoxia, but there was no significant difference between groups in normoxia and upon reoxygenation (Figure 5.10).

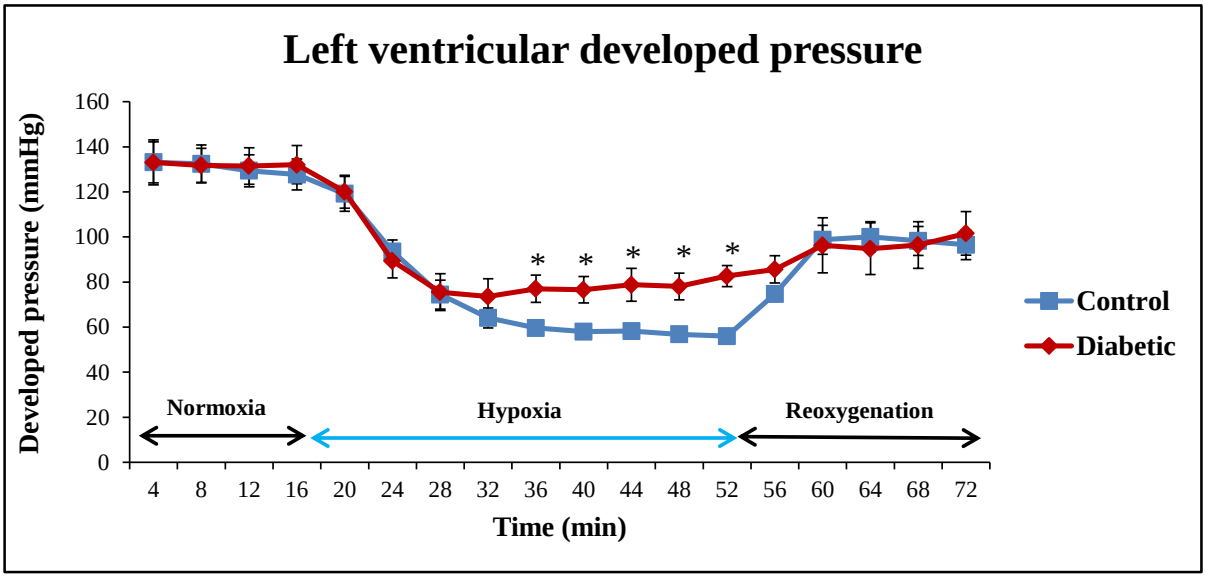

Figure 5.10: Graph showing left ventricular developed pressure of isolated control and diabetic hearts perfused in normoxia, hypoxia and reoxygenation. * $p < 0.05$ vs. control group at same time point. n = 7 – 10 per group.

A representative example of a cardiac function trace shows that during hypoxia, the control hearts maintained higher heart rate (normal intrinsic rhythm) with physiological left ventricular developed pressure (Figure 5.11). Figure 5.11a is an example of the cardiac pressure trace from a control heart obtained throughout the experiment and Figure 5.11b highlights the trace towards the end of hypoxia. Diabetic hearts however, had a higher left ventricular developed pressure with decreased heart rates (Figure 5.12), indicating that during hypoxia, each contraction was more powerful but much less frequent. Asterisk (*) was marked in both Figure 5.11 and 5.12 to identify the significant decrease in heart rates between control and diabetic hearts during hypoxia.

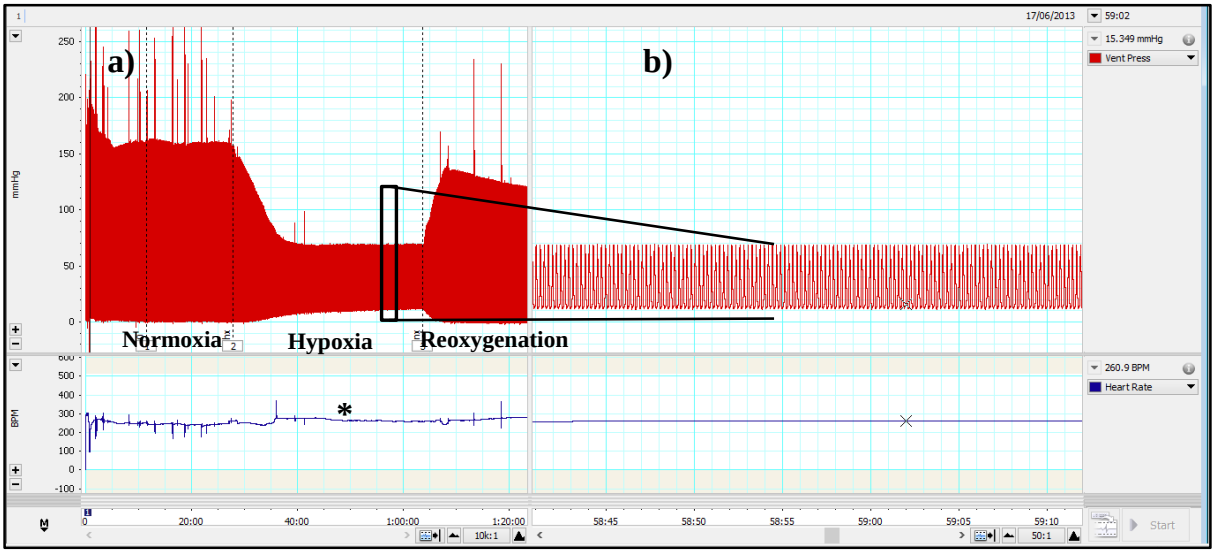

Figure 5.11: Example of cardiac function trace, showing pressure and heart rate of an isolated control heart perfused in normoxia, hypoxia and reoxygenation. **a)** Trace throughout the 72-minute experiment. **b)** Highlighted section of the trace towards the end of hypoxia. * Heart rates during hypoxia.

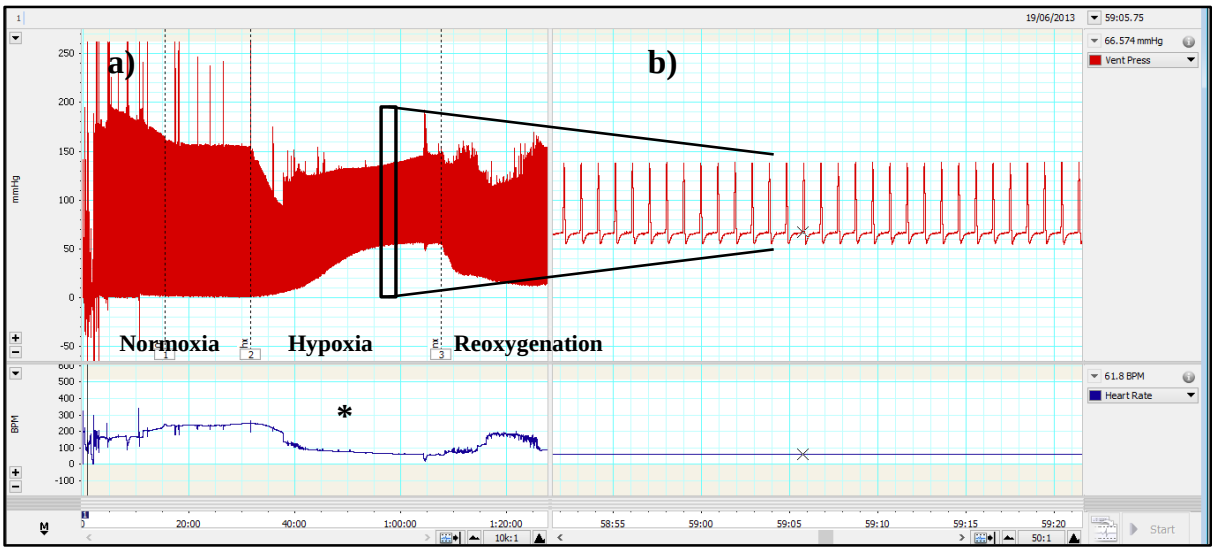

Figure 5.12: Example of cardiac function trace, showing pressure and heart rate of an isolated diabetic heart perfused in normoxia, hypoxia and reoxygenation. **a)** Trace throughout the 72-minute experiment. **b)** Highlighted section of the trace towards the end of hypoxia. * Heart rates during hypoxia.

The rate pressure product, calculated as the product of left ventricular developed pressure and heart rate, was decreased in both control and diabetic hearts during hypoxia by 68% and 84%, respectively (Figure 5.11). Upon reoxygenation, the rate pressure product recovered by 75% in control hearts while diabetic hearts only managed to recover by 63%. The rate pressure product at the end of reoxygenation in the diabetic hearts remained 26% lower than that of control hearts at the same time point.

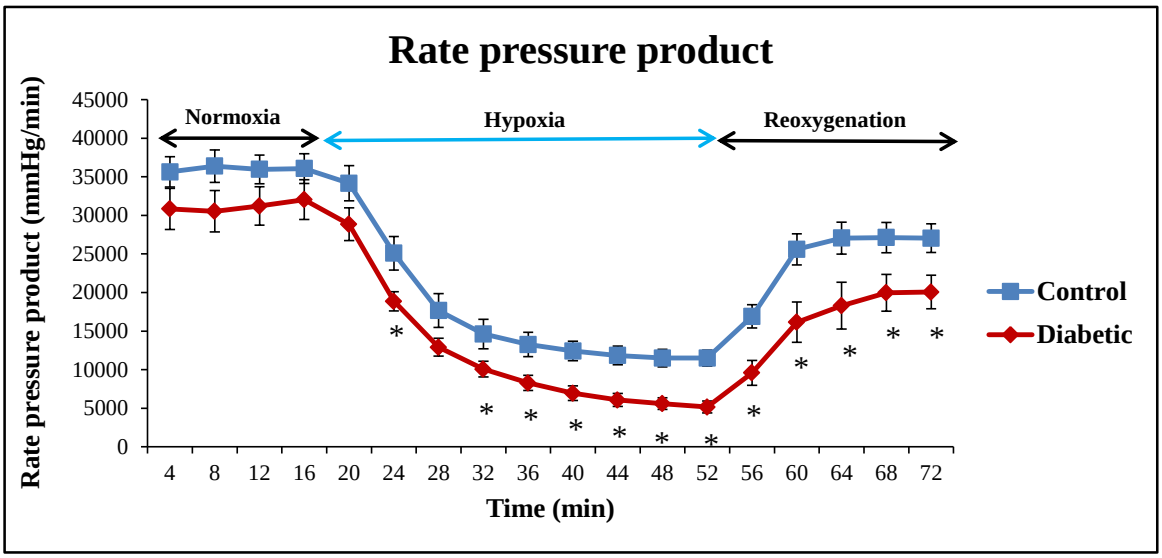

Figure 5.13: Graph showing rate pressure product of isolated control and diabetic hearts perfused in normoxia, hypoxia and reoxygenation. * $p < 0.05$ vs. control group at same time point. n = 7 – 10 per group.

5.5 DISCUSSION

This chapter demonstrated that the diabetic heart had impaired metabolic adaptation to acute hypoxia, after investigating the changes in substrate metabolism and cardiac function during acute hypoxia and upon reoxygenation. In acute hypoxia, the control hearts upregulated glycolysis and decreased fatty acid oxidation, in contrast to the diabetic hearts which were unable to upregulate glycolysis to the same extent during hypoxia, and increased triglyceride deposition at reoxygenation. This difference in metabolic adaptation to acute hypoxia and reoxygenation may have contributed to the severe bradycardia and increased end-diastolic pressure seen in diabetic hearts in hypoxia, and limited recovery of cardiac function upon reoxygenation.

5.5.1 Metabolic inflexibility of the diabetic heart in hypoxia and reoxygenation

In the healthy heart, acute hypoxia increased glycolysis and decreased fatty acid oxidation, to decrease its oxidative capacity and promote ATP generation anaerobically. As acute hypoxia is a component of ischaemia, our findings are in line with data in healthy Wistar rats during ischaemia[139], which showed increased glycolysis and decreased fatty oxidation rates. Thus, it is possible that hypoxia is the driving force behind the metabolic changes in ischaemia. The lack of oxygen may drive the production of AMP as ATP generation via oxidative phosphorylation was limited, which would consequently activate AMP-activated protein kinase (AMPK). AMPK has been proposed to increase glycolysis during hypoxia and ischaemia via increased GLUT4 translocation[236], and phosphorylation and activation of PFK-2[237].

The diabetic heart was unable to upregulate glycolysis to the same extent as control heart, despite the need for anaerobic ATP generation in hypoxia. This metabolic inflexibility is in agreement with altered cardiac metabolism in *db/db* mice[170, 173, 174].

These mice hearts were also shown to develop ventricular dysfunction with age, and since cardiac metabolic alterations in type 2 diabetes preceded the development of cardiac dysfunction, Aasum *et al.* proposed that the cardiac metabolic alteration may contribute to cardiac dysfunction, including increased susceptibility to ischaemia-reperfusion injury[149].

In control hearts, glycogen concentrations were decreased during hypoxia and replenished upon reoxygenation. This has also been shown in healthy rat hearts in ischaemia, where the isolated rat heart decreased its glycogen concentration during ischaemia and increased its glycogen concentrations to a level similar to its pre-ischaemic concentrations[139]. Diabetic hearts did not increase their glycogen concentrations to the same extent as control hearts upon reoxygenation, supporting the concept of cardiac metabolic inflexibility in type 2 diabetes.

There was no change in cardiac triglycerides in acute hypoxia and reoxygenation in control hearts which is consistent with findings of Saddik and Lopaschuk, as they showed no difference in triglyceride concentrations between pre-ischaemia, ischaemia and reperfusion in healthy rat hearts[238]. In diabetic hearts, there was no change in normoxia or during acute hypoxia, but upon reoxygenation there was a significant deposition of cardiac triglycerides. This increase in triglyceride deposition may be detrimental to the heart as it may increase the content of toxic lipid intermediates such as diacylglycerols and ceramides[239, 240]. In addition, high lipid concentrations in the heart have been suggested to increase the incidence of arrhythmias, which could be fatal, especially after myocardial infarction[55, 241]. Saddik and Lopaschuk showed that in diabetic rat hearts myocardial tricylglyceride lipolysis is significantly increased compared with controls, and triglycerides can readily be used as a source of fatty acids for mitochondrial β-oxidation[242]. Therefore, the diabetic hearts in our study may be

driven by hypoxia-reoxygenation to increase deposition of triglyceride stores, given the preference of the diabetic heart to oxidise fatty acid instead of glucose for ATP generation. Heather *et al.* showed that the sarcolemmal fatty acid transporter, FAT/CD36 in control hearts translocates away from the sarcolemma during ischaemia and reperfusion, and we hypothesise that this process may be defective in diabetic hearts in response to reoxygenation[139]. This hypothesis is strongly supported by other studies in normoxia, which showed an increase in both sarcolemmal FAT/CD36 content as well as its mediated uptake of long chain fatty acids in cardiac myocytes from obese Zucker rats[152, 243]. In addition, in type 2 diabetic humans, triglyceride accumulation was also shown to be associated with the increase in sarcolemmal FAT/CD36[205].

5.5.2 Decreased cardiac function of the diabetic heart during hypoxia and reoxygenation

Cardiac function analyses were conducted to investigate whether the abnormalities in cardiac metabolism in diabetic hearts were associated with changes in cardiac function during hypoxia and upon reoxygenation. In diabetic hearts, there was decreased heart rate, increased end-diastolic pressure, and decreased rate pressure product in hypoxia and reoxygenation compared with control hearts. El Omar *et al.* showed in another diabetic rat model, the Goto-Kakizaki lean diabetic rat model, that their diabetic rats had increased end-diastolic pressure and decreased cardiac function in acute hypoxia[151]. Therefore this study was designed to investigate the effect of acute hypoxia under more physiological conditions on our STZ and high fat diet model which has a closer resemblance to human type 2 diabetes pathophysiology.

Decreased heart rate and rate pressure product are indicative of reduced contractile systolic function, suggesting diabetic hearts cannot functionally adapt to acute hypoxia as well as control hearts. The increased end-diastolic pressure in diabetic hearts

indicates the inability of the diabetic heart to fully relax, which is an ATP-dependent process, suggesting this abnormal response to acute hypoxia is likely due to decreased ATP production during hypoxia. This decreased ATP production may be a result of the impaired ability of diabetic hearts to increase glycolytic rates in acute hypoxia, causing ATP depletion and consequently decreasing cardiac function and recovery upon reoxygenation. In order to confirm if the diabetic hearts produce less ATP during hypoxia, another perfusion study using the NMR technique was conducted (Appendix 4). This experiment was set up to replicate the acute hypoxic perfusions but with the added challenge of perfusing the heart in the bore of an 11.7T magnet to allow measurement of ^{31}P energetics. Unfortunately, the experiment did not succeed due to technical difficulties. The presence of albumin in the adapted magnet perfusion rig created micro air bubbles, which eventually accumulated and formed an air block along the magnet umbilical, which decreased the flow of buffer into the heart. As a result of this, cardiac functions decreased with time in perfused hearts under normoxic conditions. Most importantly, due to the air blockage during the 36 minutes of hypoxia, most of the hearts did not recover upon reoxygenation (Appendix 4a). This was confirmed by examining the ^{31}P ATP concentrations, which decreased during hypoxia and did not recover upon reoxygenation (Appendix 4b).

The diabetic hearts had an increased developed pressure compared to control hearts in hypoxia, suggesting they may have had better systolic function. Upon closer inspection however, the higher developed pressure in diabetic hearts was very likely caused by ventricular ectopic beats with premature ventricular contraction, characterised by an early beat, followed by a compensatory pause, with the following beat is considerably greater in force then the preceding ectopic beat. This suggests that the ventricular pressure was artefactually increased by the dysrhythmic bradycardia. During hypoxia,

the diabetic heart had much higher end-diastolic pressure, increased developed pressure and much lower heart rate compared to the control heart (Figure 5.9a). Figure 5.9b indicates that the pause between each beat was longer and the low heart rate with high pressure is a hallmark of ectopic activity[34]. In addition, the cardiac trace of the diabetic heart upon reoxygenation showed arrhythmia (Figure 5.12) compared with the more stable function in control hearts (Figure 5.11). It has been long established that high levels of plasma free fatty acids are a strong predictor of the vulnerability of patients with myocardial infarction to arrhythmias[55, 244]. In perfused rat hearts, high plasma free fatty acid:albumin molar ratio can produce arrhythmia even in non-ischaemic conditions[241]. Our type 2 diabetic rats had higher plasma free fatty acids and cardiac fatty acid oxidation rates. Since the KH buffer used for isolated heart perfusions had a fixed fatty acid concentration of 0.4 mM, these arrhythmias in diabetic hearts were likely to be associated with chronic exposure to elevated lipids and not any acute changes in lipid availability. Anzawa *et al*. demonstrated that the type 2 diabetic Otsuka Long-Evans Tokushima fatty rat hearts had 100% incidence of reperfusion-induced arrhythmia with significantly longer duration compared with controls, even when perfused without fatty acids in the buffer[245]. This suggests that in our type 2 diabetic rats, which had high plasma free fatty acids and increased cardiac lipid metabolism, it is very likely that the diabetic hearts will have arrhythmia upon reoxygenation associated with chronic exposure to elevated lipids.

In conclusion, the diabetic hearts had an abnormal response to acute hypoxia, defined by the inability to upregulate glycolysis in hypoxia and the accumulation of triglycerides upon reoxygenation. This metabolic inflexibility may be the cause of ATP starvation and lipotoxicity, which eventually led to the decreased cardiac function in hypoxia and blunted recovery upon reoxygenation. There was also evidence of

arrhythmia in the form of ventricular ectopic beats in the diabetic hearts during hypoxia, but further studies using electrocardiography (ECG) must be conducted to confirm this.

CHAPTER 6

EFFECT OF SULFO-N-SUCCINIMIDYL OLEATE ON CARDIAC METABOLISM AND FUNCTION IN THE TYPE 2 DIABETIC HEART

6.1 ABSTRACT

In Chapter 5, the altered metabolism in diabetic hearts was associated with decreased cardiac function in response to acute hypoxia. Therefore the aim of this study was to use sulfo-N-succinimidyl oleate (SSO), a FAT/CD36 inhibitor, to inhibit fatty acid uptake rebalance substrate metabolism in diabetic hearts, and it was hypothesised that this metabolic modulation may improve cardiac function of diabetic hearts during acute hypoxia and reoxygenation. Isolated hearts were perfused for a total of 72 minutes (16 minutes of normoxia, 36 minutes of hypoxia and 20 minutes of reoxygenation) and freeze-clamped at the end of the experiment for tissue analyses. SSO was added at 12 minutes, 4 minutes prior to acute hypoxia. SSO normalised fatty acid oxidation rates and total myocardial triglyceride concentrations in diabetic hearts to control heart levels upon reoxygenation. SSO also significantly increased glycolytic rates in diabetic hearts compared to untreated diabetic hearts during hypoxia. Glycogen content was unchanged with SSO treatment. The rate pressure product (RPP) in SSO-treated diabetic hearts was improved and there was no longer a difference between SSO-treated control and diabetic hearts. In addition, RPP of SSO-treated diabetic hearts were significantly higher than that of untreated diabetic hearts. In conclusion, SSO is a fast-acting and specific metabolic modulator, targeting sarcolemmal FAT/CD36, the primary protein in cardiac lipid metabolism, to inhibit fatty acid oxidation. SSO upregulated glycolysis in hypoxia, and prevented triglyceride deposition upon reoxygenation in diabetic hearts, thus, resulting in an improvement in cardiac function in diabetic hearts following acute hypoxia and reoxygenation.

6.2 INTRODUCTION

In cardiomyocytes, FAT/CD36 is responsible for 50 – 60% fatty acid uptake via transport of fatty acids across the sarcolemma[38]. Bonen *et al*. demonstrated that acute regulation of fatty acid uptake involves the translocation of FAT/CD36 from intracellular stores to the sarcolemma[246], which means FAT/CD36 plays a vital role in response to acute physiological stressors such as ischaemia[139]. In ischaemia, FAT/CD36 in control hearts was shown to translocate away from the sarcolemma, as described by Heather *et al.*[139], to decrease fatty acid uptake and metabolism while increasing glycolytic rates to ensure survivability. We demonstrated in Chapter 4 that total protein levels of FAT/CD36 were increased in our type 2 diabetic hearts compared to control hearts, and this is in agreement with the elevated FAT/CD36 mRNA and protein levels in cardiomyocytes from STZ-induced type 1 diabetic rats and high fat fed rat hearts[247, 248]. In addition, in type 2 diabetic humans, myocardial triglyceride accumulation was also shown to be associated with the increase in sarcolemmal FAT/CD36[205]. Therefore FAT/CD36 could be a potential target of metabolic modulation, to increase metabolic flexibility in diabetic hearts and subsequently improve cardiac function during hypoxia and upon reoxygenation.

Sulfo-N-succinimidyl oleate (SSO) (Figure 6.1) is a FAT/CD36 inhibitor that has been widely used in studies in isolated cells to inhibit FAT/CD36-mediated fatty acid uptake and oxidation. Recently, it has been confirmed that SSO binds irreversibly to Lysine 164 in the fatty acid binding site of FAT/CD36[249]. SSO has been shown to decrease fatty acid uptake in rat adipocytes[250] as well as cardiomyocytes[60]. These data demonstrate that SSO acts at the level of sarcolemmal membrane and FAT/CD36 is a major fatty acid uptake protein and upstream target; its inhibition will lead to a decrease in fatty acid and triglyceride concentrations as well.

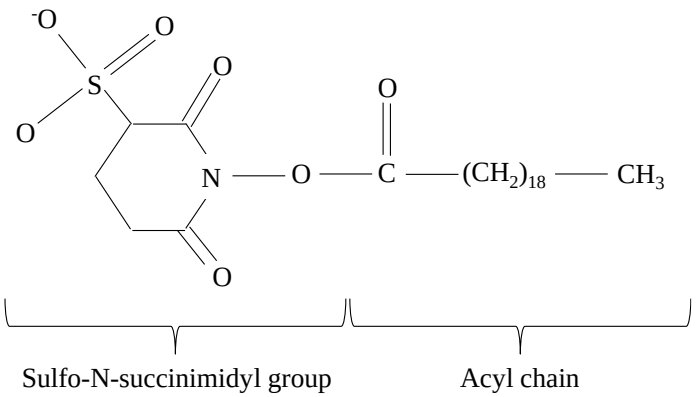

Figure 6.1: The structure of sulfo-N-succinimidyl oleate. The molecule consists of a reactive sulfo-N-succinimidyl head group and an acyl chain tail. Adapted from Coort *et al.*[251].

Although the vital role of FAT/CD36 in acute regulation of fatty acid uptake and the specificity of SSO in binding FAT/CD36 have been well documented, it is still uncertain if SSO will have a direct effect on cardiac metabolism and function in isolated perfused hearts, as past studies have mainly investigated the effect of SSO in cells. Therefore in this chapter, SSO was chosen as a metabolic modulator to inhibit fatty acid uptake, forcing the diabetic heart to downregulate fatty acid oxidation while upregulating glycolysis during hypoxia and upon reoxygenation. Subsequently, this may increase the cardiac metabolic flexibility in the diabetic heart, resulting in better adaptation to acute hypoxia and improved cardiac function upon reoxygenation.

6.3 METHODS

6.3.1 Acute hypoxic heart perfusion with SSO

The SSO used in this chapter was kindly synthesised and provided by Will Coumans from the groups of Prof. Jan Glatz and Dr. Joost Luiken from Maastricht University, the Netherlands. In all experiments, the powdered form of SSO had to be dissolved in 200 µl dimethyl sulphoxide (DMSO) immediately before being used in the experiments, as it is relatively unstable in solution.

A pilot study was conducted to investigate whether SSO had an effect on substrate metabolism in the intact perfused hearts, and the dose at which it had any effect. Hearts from two batches of control male Wistar rats were perfused to measure fatty acid oxidation and glycolytic rates, respectively (radioactive perfusions for metabolic rates measurements as described in Chapter 2). In the fatty acid oxidation rate perfusions, hearts were perfused with ^{3}H-palmitate under basal conditions for 20 minutes, followed by introduction of 200 µl DMSO containing 0.5 mM SSO or 1 mM SSO, and then hearts were perfused for another 32 minutes. Fatty acid oxidation rates were compared between groups to determine if SSO dissolved in DMSO, or DMSO alone had effects on metabolism and cardiac function, and if increasing the concentration of SSO decreased fatty acid oxidation rates further. In the glycolytic rate perfusions, hearts were perfused with ^{3}H-glucose under basal conditions for 20 minutes, then 200 µl DMSO or 0.5 mM SSO (dissolved in 200 µl DMSO) was added into the perfusate and the hearts were perfused for another 32 minutes. The cardiac glycolytic rates before and after the introduction of SSO were compared.

In the main study of this chapter, rat hearts were perfused in Langendorff mode identical to the protocol used in Chapter 5 except 0.5 mM SSO was added into the 200

ml recirculating buffer at 12 minutes, and 4 minutes later the oxygen supply was replaced with nitrogen to produce a hypoxic environment for the perfused heart (Figure 6.2). At 52 minutes, oxygen supply was restored to reoxygenate the perfused heart. Perfusate samples were taken every 4 minutes for substrate metabolic rate measurements and hearts were freeze-clamped at the end of perfusions for further tissue analyses.

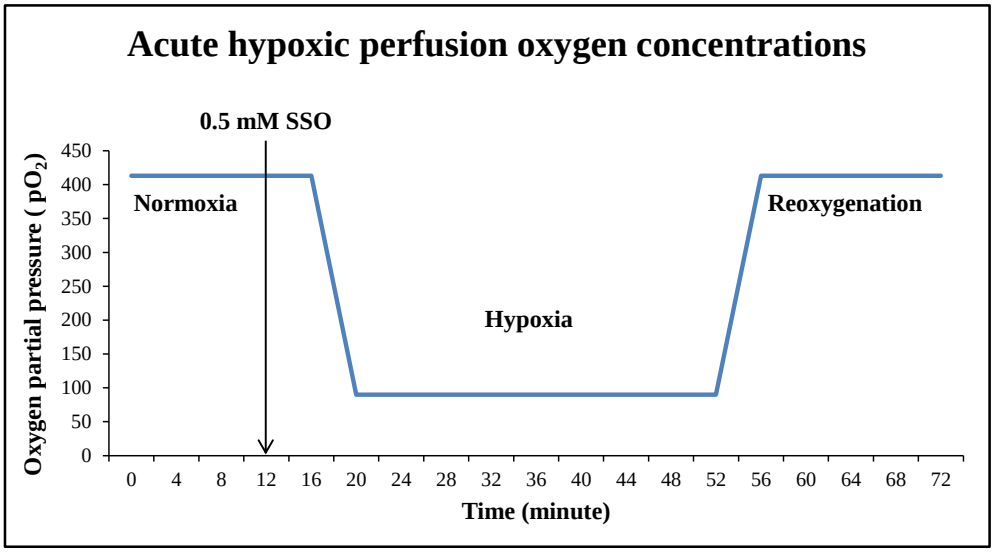

Figure 6.2: Oxygen concentration changes in KH buffer measured during the acute hypoxic perfusion protocol to induce normoxic, hypoxic and reoxygenation conditions 0.5 mM (final concentration) SSO dissolved in 200 µl DMSO was added at 12 minutes.

In order to maximise cost effectiveness, SSO was only included in experiments that last for the entire acute hypoxic perfusion protocol, so all the freeze-clamped heart tissues with SSO experienced the full duration of normoxia, hypoxia and reoxygenation. Therefore, analyses on cardiac glycogen, triglyceride and lipid incorporation only compared groups after reoxygenation.

6.3.2 Thin layer chromatography (TLC)

Firstly, Folch extraction was carried out to extract total lipids from freeze-clamped perfused hearts. Radioactive (^{3}H-palmitate) frozen heart tissues (40-50 mg) were

weighed out and added to 10 ml glass test tubes containing 5 ml Folch solution (2:1 chloroform:methanol v/v). An additional 3ml Folch solution was added and the mixture was mixed on a lab Stuart rotator SB3 at 40 rpm for 1 hour. Then 2 ml water was added and mixed for another hour. Samples were left overnight to separate. The upper phase was removed and discarded leaving the lower inorganic phase, which was dried under air. Next, 1 ml ethanol was added and mixed thoroughly, and a duplicate of 100 µl sample was taken and left overnight to evaporate. The evaporated sample was resuspended with 0.2 ml of ice-chilled chloroform.

A thin layer chromatography plate (silica gel G) was prepared and marked with 6 lanes with a razor blade (Figure 6.3). Small aliquots (20 µl) of the resuspended sample in chloroform were sequentially spotted on the plate, and the process was repeated until all of the 0.2 ml of solvent was spotted onto the labelled lane. The plate was then placed in a TLC tank containing a final volume of 106 ml of solvent, made up of 70 ml hexane, 30 ml diethyl ether and 1.6 ml glacial acetic acid (prepared and left to equilibrate 30 minutes prior). As the solvent slowly travels up the plate, the different lipids travel at different rates and the lipid mixture separated into different bands. The plate was removed from the tank when the solvent front is approximately 1 cm from the top of the plate. Rhodamine 6G in acetone vehicle was sprayed evenly onto the plate in a fumehood as a visualisation agent. The bands on the plate were carefully marked under UV light and then the band along with the adsorbent were scraped from the glass plate into a scintillation vial and read using the scintillation counter. Lipid concentrations were calculated using cpm values from the scintillation counter and the specific activity of the perfusate.

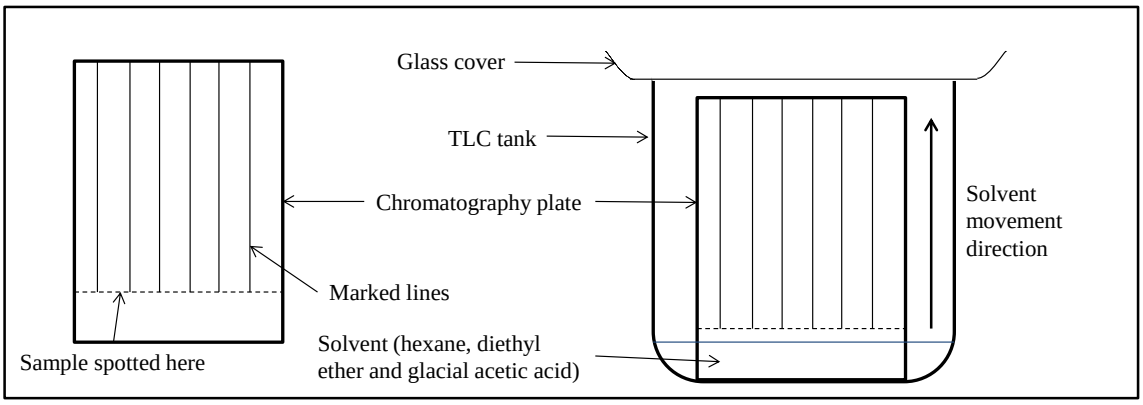

Figure 6.3: Schematic diagram showing chromatography plate marked with lanes and the TLC tank filled with solvent to a level just below the blotted samples to allow samples to move up the plate while left in the tank over time.

6.3.3 Statistical analysis

Results are expressed as means ± standard error of the mean (SEM). In the pilot study, fatty acid oxidation rates were analysed using one way ANOVA with post hoc t-tests between the three groups and paired t-tests comparing before and after the introduction of SSO in the same hearts. The glycolytic rates were analysed using paired t-tests to compare before and after SSO introduction into the same hearts. Cardiac functions were analysed using independent t-test.

Glycolytic rates, fatty acid oxidation and lactate efflux rates of the main study were analysed using two way ANOVA with post hoc t-tests between control, diabetic, control with SSO (Control + SSO) and diabetic with SSO (Diabetic + SSO) groups under the same oxygen concentration. The same two way ANOVA with post hoc t-tests were also carried out on cardiac functions, triglyceride and glycogen concentrations. Difference between the 3 oxygen conditions were not compared as these were previously reported in Chapter 5. $p < 0.05$ was taken as the level of significance.

6.4 RESULTS

6.4.1 Pilot study – Substrate metabolism

Luiken *et al.* showed that 0.4 mM SSO inhibited fatty acid uptake in isolated cardiomyocytes by up to 50% and no further inhibition was observed with double the dose of SSO[60]. Taking the results of the aforementioned study and suggestions from Prof. Jan Glatz and Dr. Luiken, we decided to use 0.5 mM SSO as a standard dose in the pilot study on the perfused heart. Since SSO must be dissolved in DMSO before use, a set of hearts were perfused with KH and 200 µl DMSO only, to ensure that DMSO does not affect fatty acid oxidation in the perfused hearts. Results showed that DMSO did not affect cardiac fatty acid oxidation as there was no difference compared to hearts perfused with KH only (Control) (Figure 6.4). There was a decrease in fatty acid oxidation of 32% compared with control when hearts were perfused with 0.5 mM SSO ($p < 0.05$), and increasing the dose to 1 mM SSO did not further decrease fatty acid oxidation rates.

In addition, hearts perfused with 0.5 mM SSO showed an increase in glycolytic rates by 43% compared to control hearts ($p < 0.05$) (Figure 6.5). This is likely an indirect effect of SSO as it inhibits fatty acid uptake via FAT/CD36 and subsequently fatty acid oxidation, hence it would increase glycolytic rates via the Randle cycle[63].

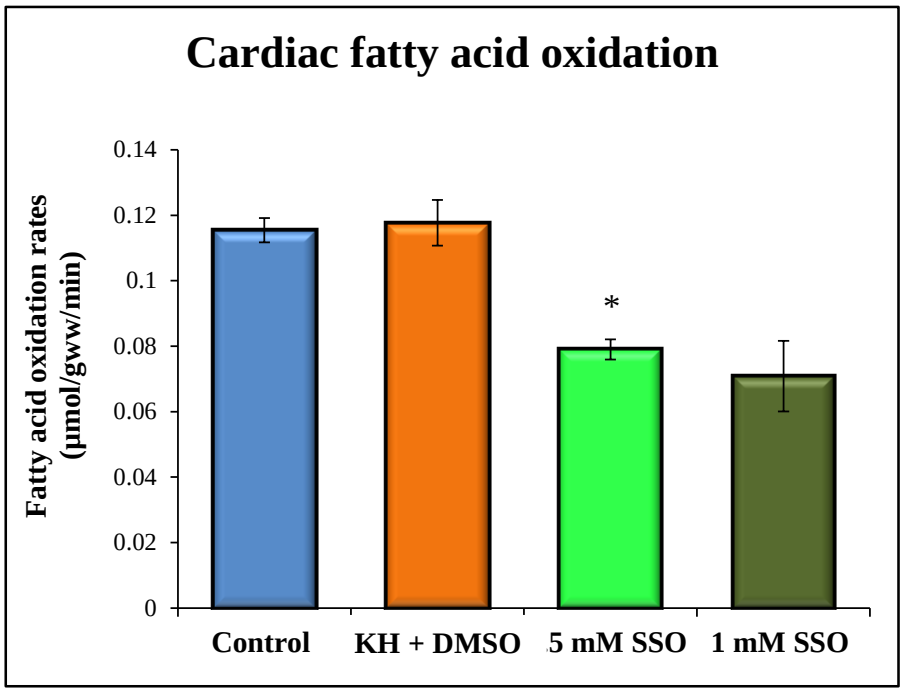

Figure 6.4: Fatty acid oxidation rates of hearts perfused with ^{3}H-palmitate in KH buffer only (Control), KH and 200 µl DMSO (KH + DMSO), 0.5 mM SSO in DMSO and 1 mM SSO in DMSO. *p < 0.05 vs. control and KH + DMSO. n = 2 – 5 per group.

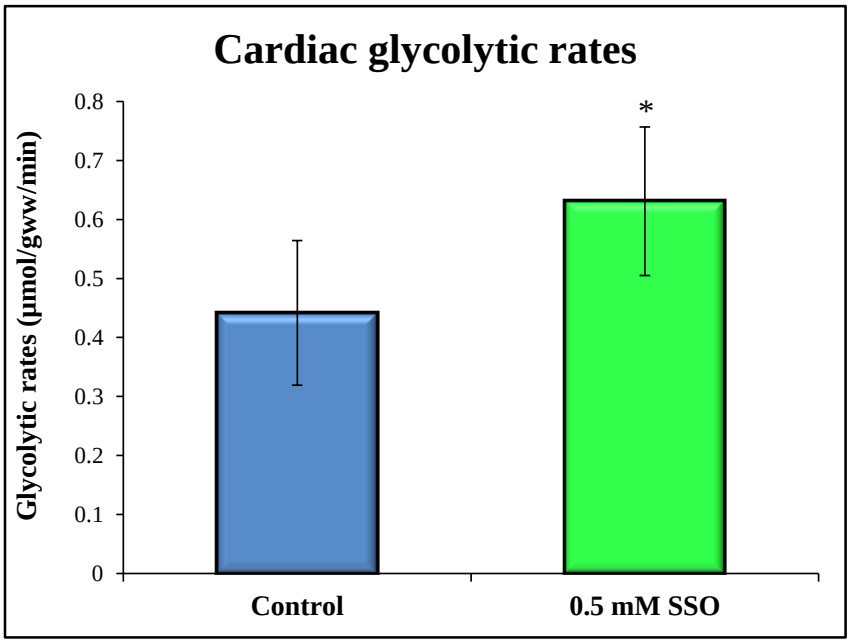

Figure 6.5: Glycolytic rates of hearts perfused with ^{3}H-glucose in KH buffer only (Control), and 0.5 mM SSO in DMSO. *p < 0.05 vs. control. n = 3 per group.

6.4.2 Pilot study – Cardiac function

There were no significant differences in cardiac function between control hearts and hearts perfused with SSO measured by heart rate, developed pressure and rate pressure product (Table 6.1). This shows that 0.5 mM SSO affects substrate metabolism in perfused hearts without having an effect on cardiac function. Therefore the dose of 0.5 mM was determined to be the optimal dose to be used in subsequent experiments in this chapter.

Table 6.1: Cardiac function from isolated control hearts with and without SSO.

	Control	0.5 mM SSO
Heart rate (beats/min)	279 ± 11	287 ± 9
Developed pressure (mmHg)	132 ± 7	125 ± 6
Rate pressure product (mmHg/min x 10^3)	36 ± 1	36 ± 2

n = 5 per group.

6.4.3 Main study – Lipid metabolism

In Chapter 5, untreated control and diabetic hearts were compared. This chapter will focus on the effect of SSO on control and diabetic hearts by comparing between control and diabetic hearts with and without SSO.

There was no significant difference in fatty acid oxidation rates between groups during hypoxia. Upon reoxygenation, SSO significantly decreased fatty acid oxidation rates in SSO-treated control hearts compared to untreated control hearts (Figure 6.6). Interestingly, SSO also decreased fatty acid oxidation rates in diabetic hearts compared to untreated diabetic hearts, to a rate no longer significantly different to control hearts.

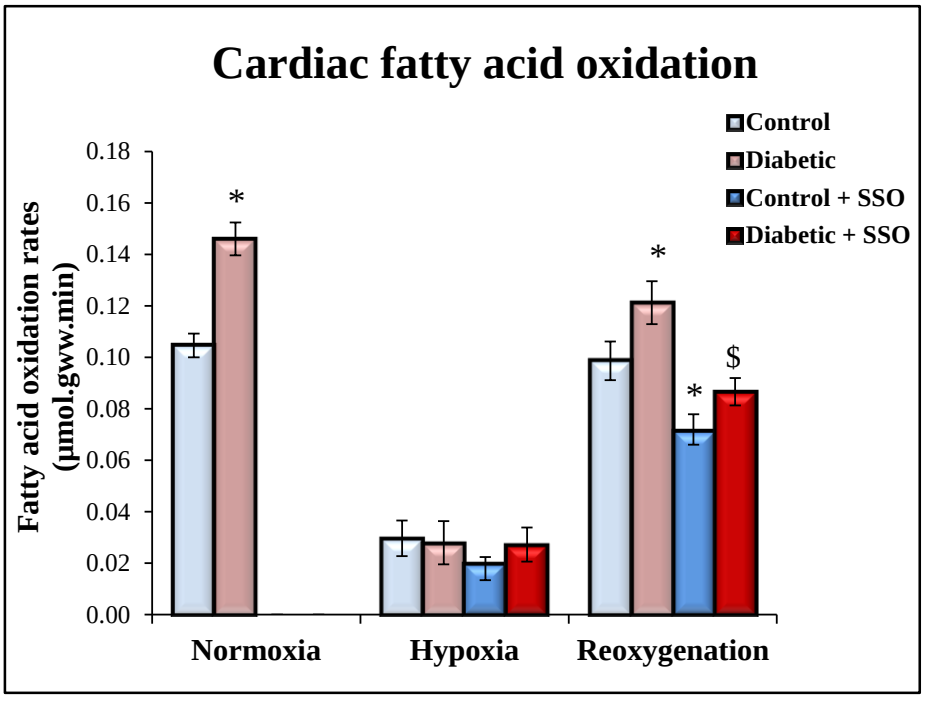

Figure 6.6: Cardiac fatty acid oxidation rates of control, diabetic, control + SSO and diabetic + SSO hearts perfused in normoxia, hypoxia and reoxygenation. * $p < 0.05$ vs. control, , \$ $p < 0.05$ vs. diabetic. n = 6 – 8 per group.

As shown in Chapter 5, the untreated diabetic hearts had increased triglyceride concentrations after reoxygenation compared to untreated control hearts. However SSO inhibited this triglyceride accumulation in the diabetic hearts, resulting in SSO-treated

diabetic hearts having significantly decreased triglyceride concentrations compared to untreated diabetic hearts, and no longer significantly different to control hearts (Figure 6.7).

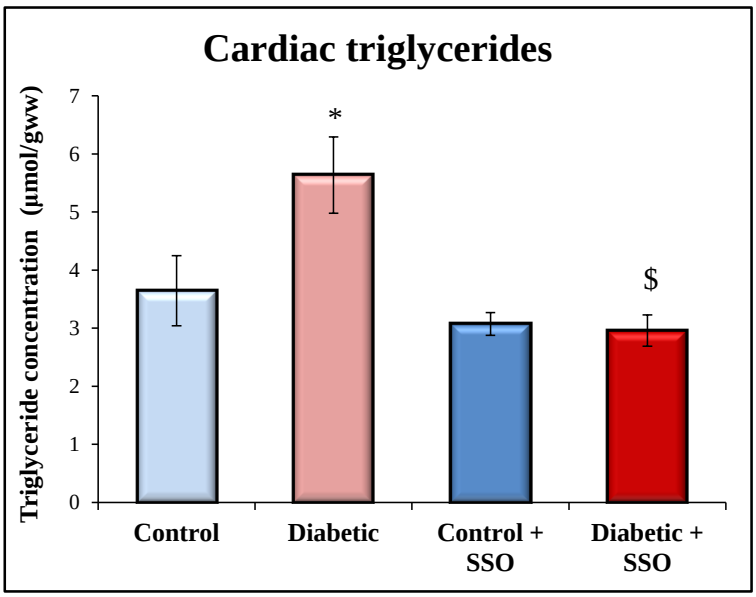

Figure 6.7: Cardiac triglyceride concentrations of control, diabetic, control + SSO and diabetic + SSO hearts at the end of reoxygenation of the acute hypoxic perfusions. * $p < 0.05$ vs. control, $ $p < 0.05$ vs. diabetic. n = 5 – 6 per group.

Incorporation of exogenous ^{3}H-fatty acids into myocardial lipids was measured using TLC, and there were decreases in phospholipids, diacylglycerol and fatty acids in SSO-treated control hearts compared to untreated control hearts (Figure 6.8). There was no difference between SSO-treated and untreated diabetic groups, suggesting that SSO did not have an effect on ^{3}H-fatty acid incorporation into lipids in diabetic hearts. As a result of this, in SSO-treated diabetic hearts, incorporation into phospholipids, diacylglycerol and cholesterol ester were increased compared to SSO-treated control hearts. There was no difference in ^{3}H-fatty acids incorporation into triglyceride concentrations in SSO-treated control and diabetic hearts compared with their respective untreated hearts (Figure 6.9). However, the SSO-treated diabetic hearts had

an increase in ^{3}H-fatty acids incorporation into triglyceride compared to SSO-treated control hearts.

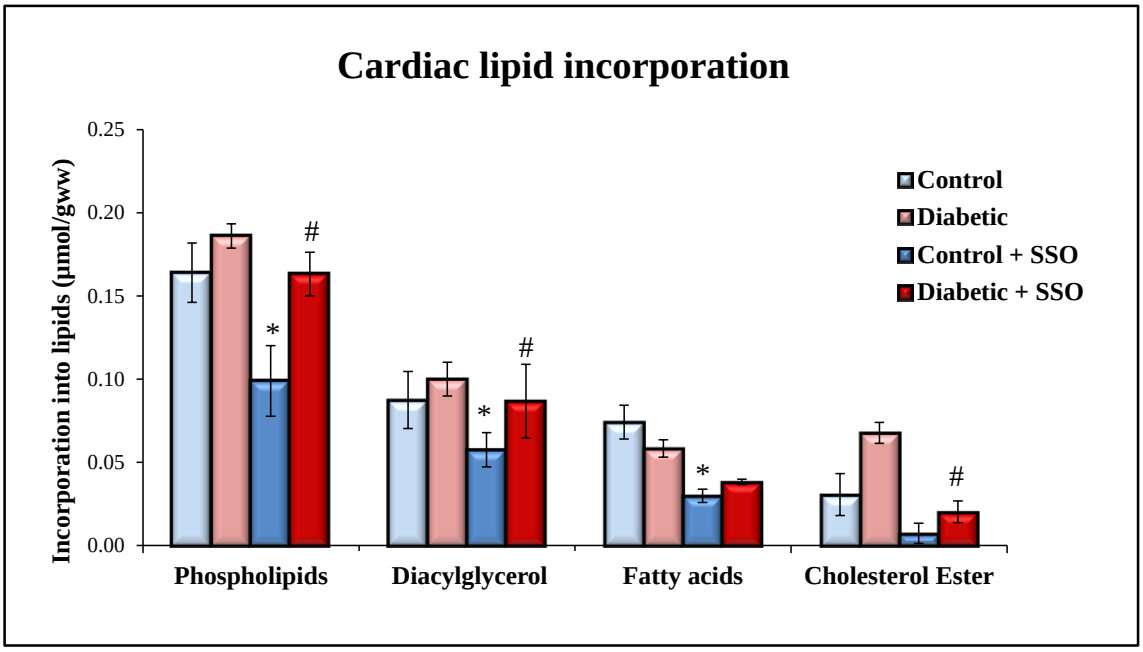

Figure 6.8: Cardiac ^{3}H-lipid incorporation in control, diabetic, control + SSO and diabetic + SSO hearts at the end of reoxygenation of the acute hypoxic perfusions, quantified using TLC technique. * $p < 0.05$ vs. control, # $p < 0.05$ vs. control + SSO. n = 5 – 6 per group. Results are presented in µmol ^{3}H-lipid incorporated per gram wet weight per 72 minutes of experiment.

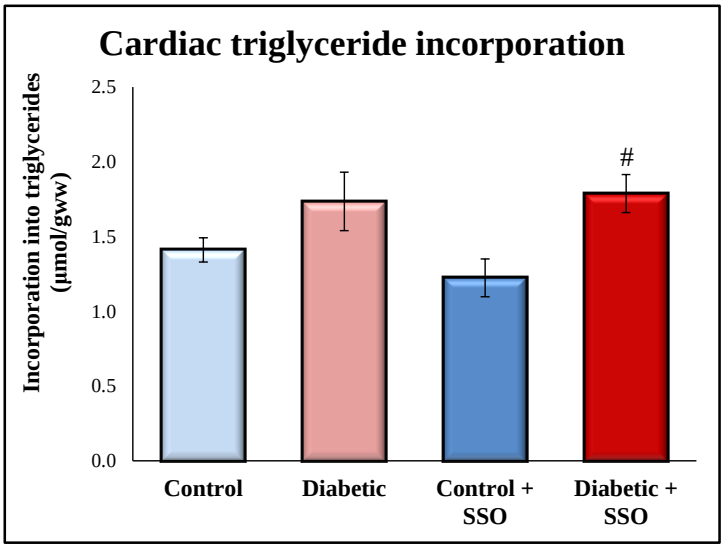

Figure 6.9: Cardiac ^{3}H-triglyceride incorporation in control, diabetic, control + SSO and diabetic + SSO hearts at the end of reoxygenation, quantified using TLC technique. # $p < 0.05$ vs. control + SSO. n = 5 – 6 per group. Results are presented in µmol ^{3}H-lipid incorporated per gram wet weight per 72 minutes of experiment.

6.4.4 Main study – Glucose metabolism

In hypoxia, there was no significant difference in glycolytic rates between control and control + SSO hearts (Figure 6.10). In contrast, SSO increased glycolytic rates in diabetic hearts compared with untreated diabetic hearts during hypoxia. However, this rate was still significantly decreased compared to control hearts treated with SSO. Upon reoxygenation, SSO had no effect compared to their respective untreated groups, with SSO-treated diabetic hearts having decreased glycolytic rates compared to SSO-treated control hearts.

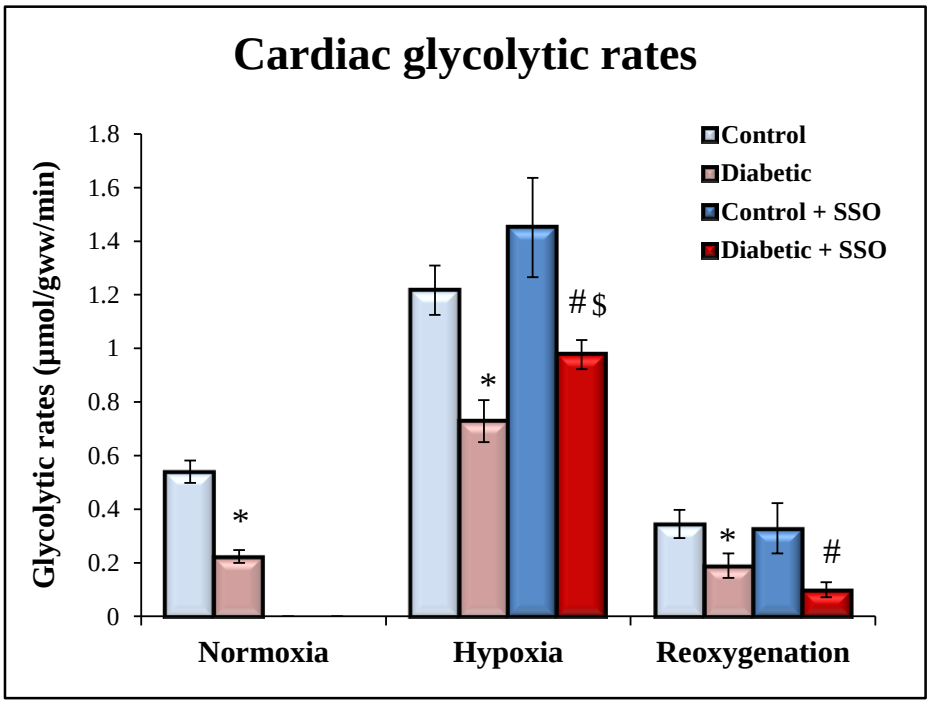

Figure 6.10: Cardiac glycolytic rates of control, diabetic, control + SSO and diabetic + SSO hearts perfused in normoxia, hypoxia and reoxygenation. * $p < 0.05$ vs. control, \$ $p < 0.05$ vs. diabetic, # $p < 0.05$ vs. control + SSO. n = 5 – 7 per group.

SSO did not have a significant effect in lactate efflux rates on control hearts in hypoxia (Figure 6.11). In hypoxia, SSO-treated diabetic hearts had decreased lactate efflux rates compared to SSO-treated control hearts. Upon reoxygenation, the SSO-treated diabetic hearts still had a significant decrease in lactate efflux rates compared to SSO-treated control hearts, and also compared to untreated diabetic hearts.

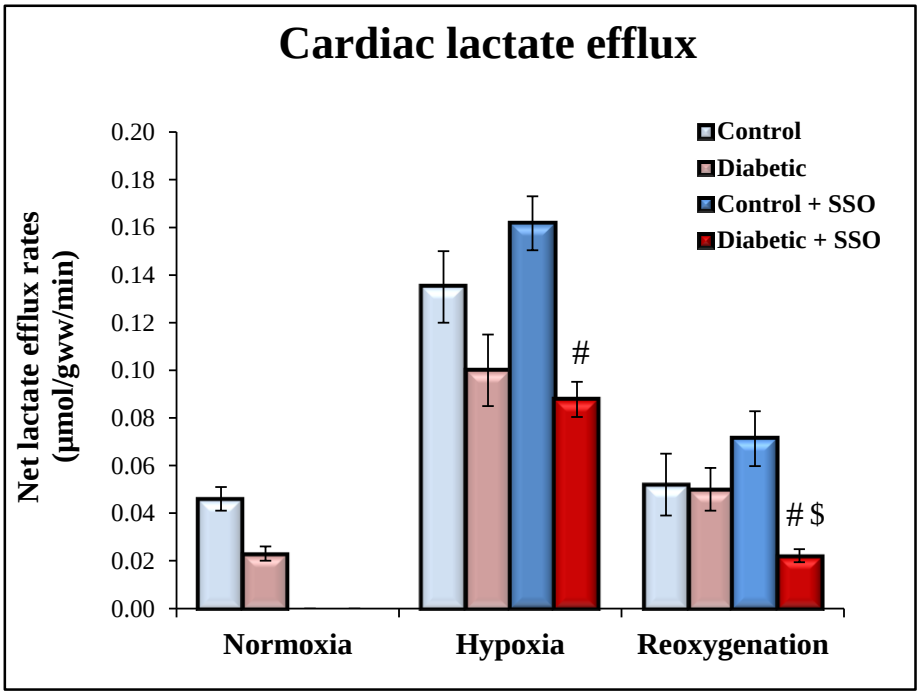

Figure 6.11: Cardiac lactate efflux rates of control, diabetic, control + SSO and diabetic + SSO hearts perfused in normoxia, hypoxia and reoxygenation. $ $p < 0.05$ vs. diabetic, # $p < 0.05$ vs. control + SSO. n = 4 – 6 per group.

Results showed that at the end of reoxygenation, SSO did not have an effect on cardiac glycogen content as both SSO-treated control and diabetic groups were not significantly different compared to their untreated counterparts (Figure 6.12). In addition, SSO-treated diabetic hearts maintained a significantly lower cardiac glycogen concentration compared to SSO-treated control hearts.

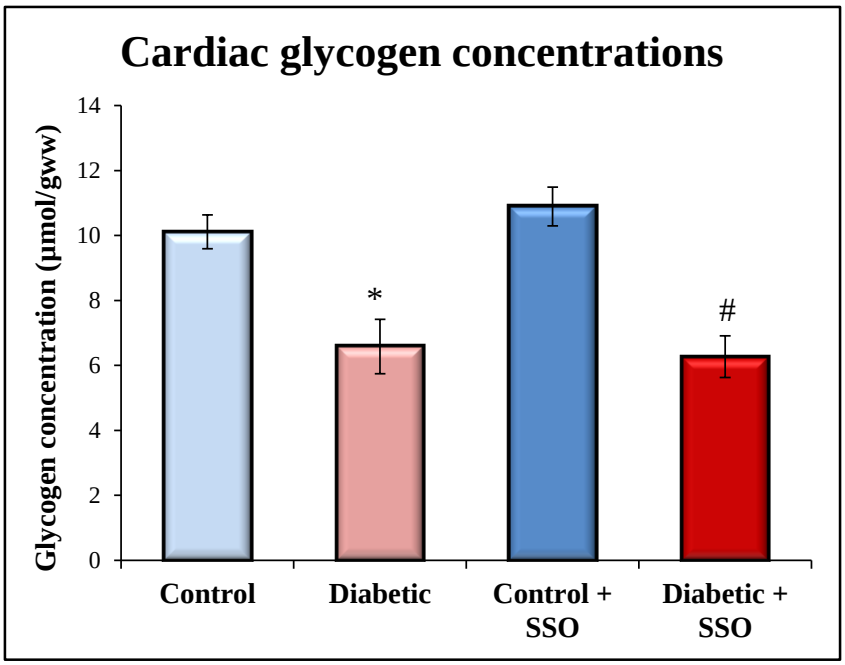

Figure 6.12: Cardiac glycogen concentrations of control, diabetic, control + SSO and diabetic + SSO hearts at the end of reoxygenation of the acute hypoxic perfusions. * $p < 0.05$ vs. control, # $p < 0.05$ vs. control + SSO. $n = 5 – 11$ per group.

6.4.5 Main Study – Cardiac function

Midway through hypoxia, at 32 minutes, SSO-treated control hearts had decreased heart rates compared to untreated control hearts, and this continued throughout the reoxygenation period until the end of perfusion (Figure 6.13). In SSO-treated diabetic hearts, the heart rates were significantly increased only at 44 minutes, and there was no difference during other time points.

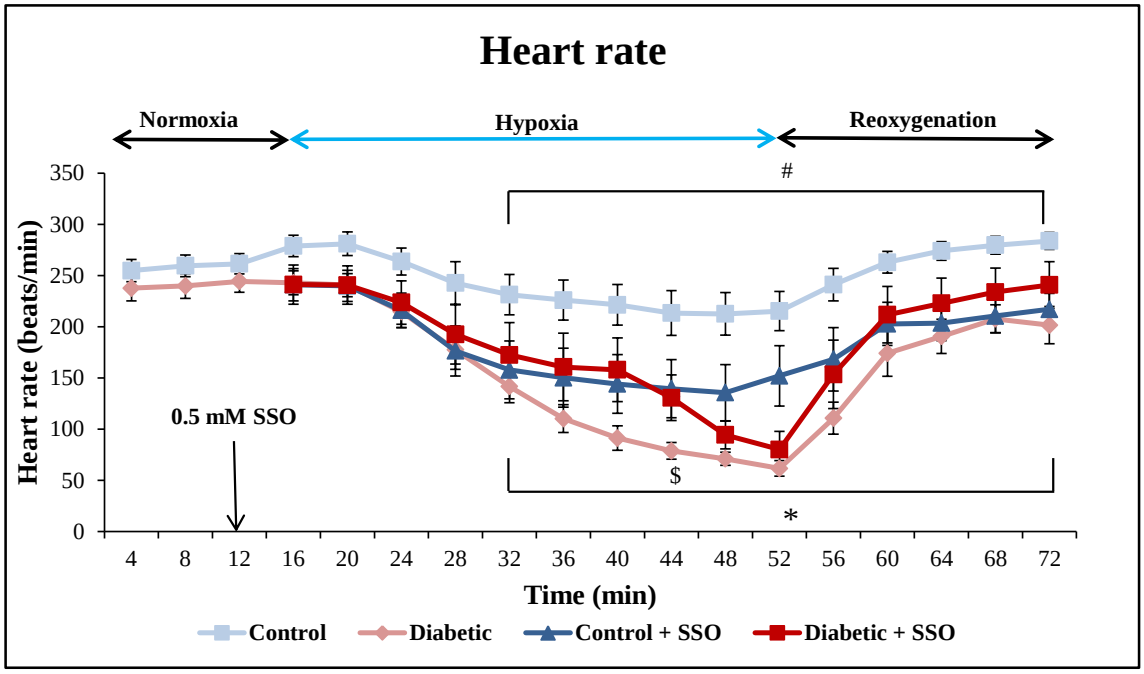

Figure 6.13: Graph showing heart rates of isolated control, diabetic, control + SSO and diabetic + SSO hearts perfused in normoxia, hypoxia and reoxygenation. * $p < 0.05$ control vs. diabetic, # $p < 0.05$ control vs. control + SSO, $ $p < 0.05$ diabetic vs diabetic + SSO. n = 8 – 17 per group.

There was no difference in end-diastolic pressure between SSO-treated control and diabetic hearts compared with their respective untreated hearts (Figure 6.14). Between 16 and 24 minutes (early hypoxia), there was significant increase in end-diastolic pressure in SSO-treated control compared with SSO-treated diabetic hearts. At the end of hypoxia, the significant difference between untreated control and untreated diabetic hearts was no longer present if both groups were pretreated with SSO.

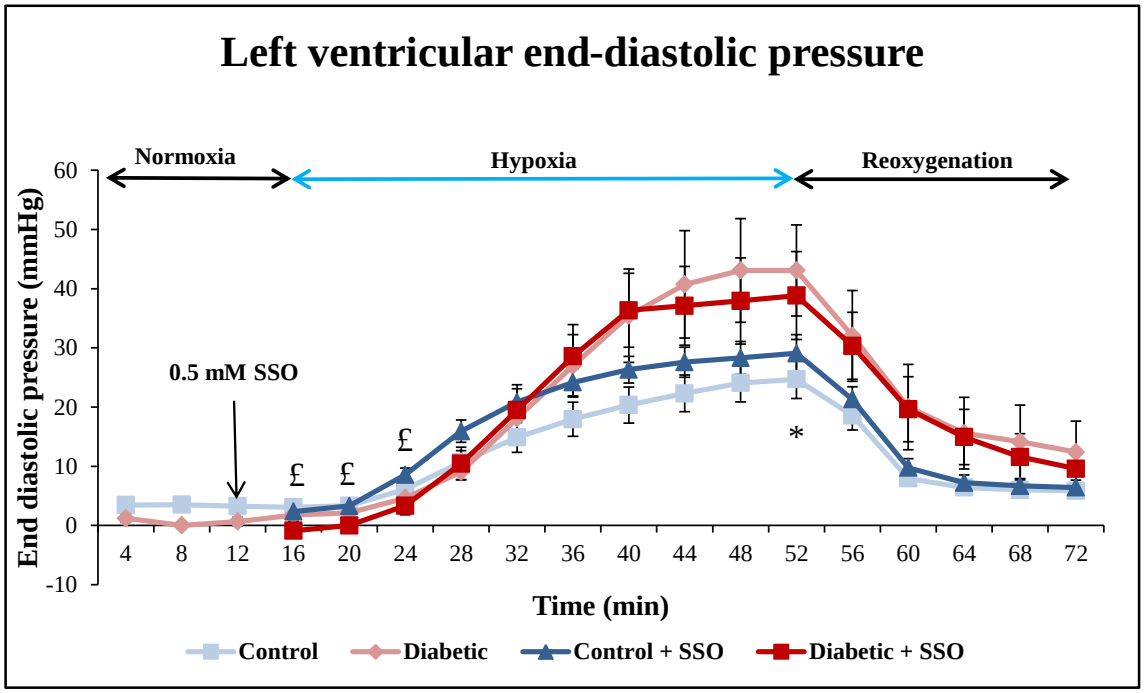

Figure 6.14: Graph showing left ventricular end-diastolic pressure of isolated control, diabetic, control + SSO and diabetic + SSO hearts perfused in normoxia, hypoxia and reoxygenation. * $p < 0.05$ control vs. diabetic, £ $p < 0.05$ control + SSO vs diabetic + SSO. n = 8 – 17 per group.

In terms of left ventricular developed pressure, the SSO-treated control hearts had an increase in pressure compared to untreated control hearts at 20 and 28 minute time points and again at 44 and 48 minute time points (Figure 6.15). The SSO-treated diabetic hearts only exhibited an increase in developed pressure compared to untreated diabetic hearts during the early stage of hypoxia at 20 – 28 minute.

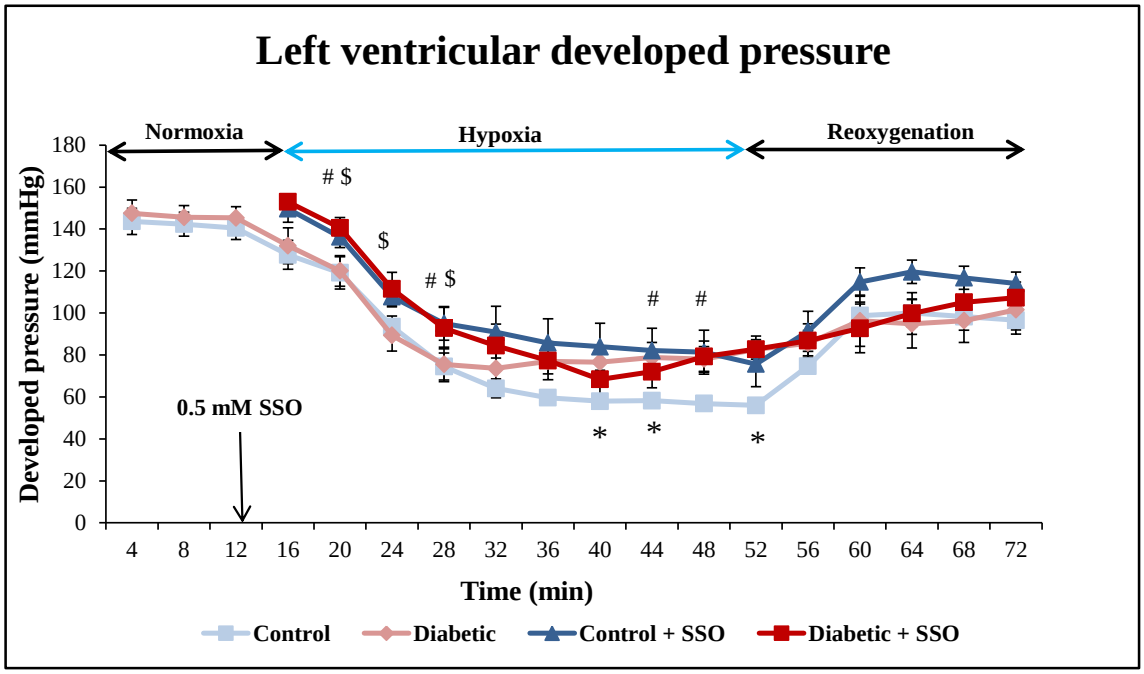

Figure 6.15: Graph showing left ventricular developed pressure of isolated control, diabetic, control + SSO and diabetic + SSO hearts perfused in normoxia, hypoxia and reoxygenation. * $p < 0.05$ control vs. diabetic, # $p < 0.05$ control vs. control + SSO, $ $p < 0.05$ diabetic vs diabetic + SSO. n = 8 – 17 per group.

A striking result was the rate pressure product data, as SSO eliminated the significant decrease in RPP seen in untreated diabetic hearts compared to untreated control hearts following hypoxia and reoxygenation (Figure 6.16). There was no difference between SSO-treated control and diabetic hearts throughout the entire experiment. Most importantly, at the end of reoxygenation there was an increase in RPP in SSO-treated diabetic hearts compared to untreated diabetic hearts. In addition, there was no significant difference between SSO-treated diabetic hearts and untreated control hearts at the end of the experiment, suggesting SSO had normalised the mechanical functions of diabetic hearts to the level of control hearts. The improvement of RPP by SSO was already visible during the short period of reoxygenation with only 4 time points, it is possible that with a longer period of time, the benefit of SSO on cardiac function will be more pronounced.

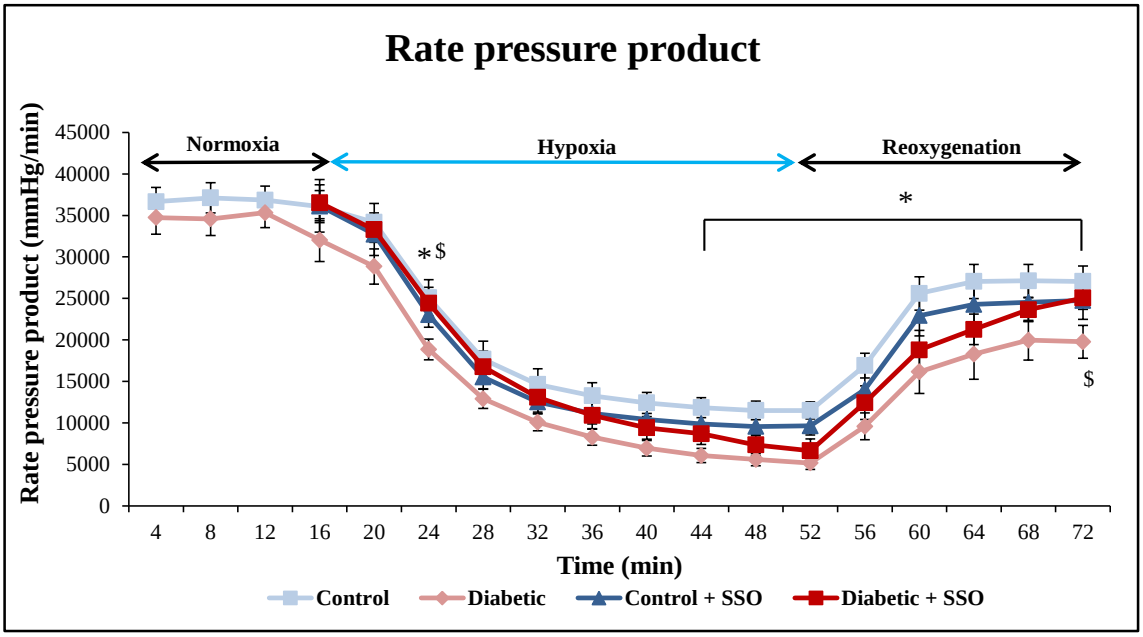

Figure 6.16: Graph showing cardiac rate pressure product of isolated control, diabetic, control + SSO and diabetic + SSO hearts perfused in normoxia, hypoxia and reoxygenation. * $p < 0.05$ control vs. diabetic, $ $p < 0.05$ diabetic vs diabetic + SSO. n = 8 – 17 per group.

6.5 DISCUSSION

As seen in the previous chapter, diabetic hearts had impaired contractile function during acute hypoxia and upon reoxygenation compared with control hearts. This may be associated with the diabetic hearts being metabolically inflexible to acute hypoxia, hence unable to switch substrate metabolism dependency from fatty acid oxidation to glycolysis. Glycolysis is essential in continuously generating ATP anaerobically for survival during hypoxia. In this chapter, we aimed to modulate metabolism in diabetic hearts during hypoxia by using the specific FAT/CD36 inhibitor, SSO. It was shown that SSO decreased lipid metabolism and increased glucose metabolism in diabetic hearts, SSO did not affect RPP in control hearts, it normalised cardiac function of treated diabetic hearts to a level similar to that control hearts by the end of reoxygenation.

6.5.1 SSO alters cardiac substrate metabolism and had no effect on cardiac function in the pilot study

In our pilot study, the optimal dose of SSO used (0.5 mM) was able to decrease fatty acid oxidation rates of control hearts by 32%, with no further decrease with 1 mM SSO. Luiken *et al*. showed that in isolated cardiomyocytes, fatty acid oxidation rates were decreased by 49% with 0.4 mM SSO[60]. It is likely that in an isolated perfused heart, the demand for ATP is greater than in isolated cardiomyocytes, resulting in a higher threshold for fatty acid oxidation rate inhibition. The increase in glucose uptake and glycolysis may be driven by, firstly, the need for more substrates as there was a decrease in fatty acid uptake and oxidation thus producing less ATP, and, secondly, the effect of the Randle cycle decreasing fatty acid oxidation-mediated inhibition of glucose metabolism. The decrease in fatty acid uptake and oxidation will lead to a decrease in acetyl-CoA and citrate production, and both of these molecules inhibits

glucose uptake via GLUT1 and glycolytic enzymes such as PFK-1 and PDH[63]. Although SSO altered cardiac substrate metabolism in the pilot study, it did not have any significant effect on cardiac mechanical function, suggesting that it is indeed an effective modulation of metabolism with no detrimental effects on perfused hearts.

6.5.2 SSO increases glycolytic rates in hypoxia

Most studies performed with SSO measured fatty acid uptake or oxidation in cells, but in this study it was essential to measure the effect of SSO on glycolytic rates and especially during acute hypoxia as this was limited in the diabetic heart. Glycolytic rates were increased in SSO-treated diabetic hearts in hypoxia, suggesting that the inhibition of fatty acid uptake via FAT/CD36 had forced the diabetic hearts to upregulate glycolysis via the Randle cycle during the time when anaerobic ATP would be most needed. This effect, however, was lost upon reoxygenation as the glycolytic rates of the SSO-treated diabetic hearts were not different from that of untreated diabetic hearts. This could be that the high dependency on fatty acid metabolism of the diabetic hearts could only be suppressed when a physiological stressor such as acute hypoxia was also present. In addition, another possible mechanism underlying the decreased glycolytic rates in both treated and untreated diabetic groups upon reoxygenation could be that the depletion of glycogen stores, which was decreased in diabetic hearts, led to the depletion of substrates to form acetyl-CoA needed to from malonyl-CoA by ACC. This led to the lack of regulatory mechanism to limit fatty acid oxidation upon reoxygenation and hence, via the Randle effect, decreased glycolytic rates even further.

On the contrary, there was no significant difference between groups in fatty acid oxidation during hypoxia. This may be due to the severe shortage of oxygen forcing the cardiomyocytes, even in diabetic hearts which were primed to utilise fatty acids, to limit

fatty acid uptake and oxidation in order to minimise oxygen wastage[34] and ROS production[252].

6.5.3 SSO decreases fatty acid oxidation rates in hypoxia

Although acute hypoxia was sufficient to suppress fatty acid oxidation in diabetic hearts, the fatty acid oxidation rates quickly returned to normoxic levels in diabetic hearts upon reoxygenation. However, fatty acid oxidation rates of the SSO-treated diabetic hearts remained suppressed upon reoxygenation to a level similar to that of SSO-treated control hearts. This inhibition of fatty acid oxidation confirmed the irreversible binding of SSO to FAT/CD36 and decreased fatty acid uptake[249]. The total cardiac triglycerides were increased in untreated diabetic hearts compared to untreated control hearts after reoxygenation. This intracellular rise in triglyceride concentrations was previously demonstrated to be positively correlated with insulin resistance development in obese and insulin resistant rats[239, 253, 254]. The accumulation of triglycerides is also an indicator of elevated levels of other lipid intermediates such as diacylglycerol and ceramides, which may also be associated with lipotoxicity and insulin resistance in the diabetic heart[255, 256]. The accumulation of triglycerides was attenuated by SSO in the diabetic hearts and the total concentration was significantly decreased compared with untreated diabetic hearts. This may potentially be beneficial to the diabetic heart and improve recovery upon reoxygenation.

Since both cardiac triglyceride concentration and fatty acid oxidation were decreased in SSO-treated diabetic hearts compared to untreated diabetic hearts, it would be reasonable to assume that ^{3}H-fatty acid incorporation into lipids would be decreased by SSO as well. On the contrary, tissue lipid analysis showed that SSO decreased most exogenous lipid incorporation in SSO-treated control hearts compared to their untreated counterparts but there was no effect on the SSO-treated diabetic hearts. This data

suggests that the lipid incorporation pool was more protected than total intracellular triglycerides and fatty acid oxidation in the diabetic heart. This reaffirms the high reliance of the diabetic heart on lipid metabolism and its requirement to prioritise lipid storage over glycogen accumulation after reoxygenation.

An attempt was made to measure total lipid uptake by summing total lipid incorporation and total fatty acid oxidation throughout the 72 minutes of perfusion protocol. Results showed that SSO decreased total lipid uptake in control hearts compared to untreated control hearts while lipid uptake in SSO-treated diabetic hearts remained elevated compared to SSO-treated control hearts (Figure 6.17). Therefore in control hearts, SSO decreased total lipid uptake, resulting in decrease in lipid incorporation and fatty acid oxidation while in SSO-treated diabetic hearts, total lipid uptake and incorporation remained unchanged while fatty acid oxidation decreased compared to SSO-treated control hearts. This difference may have just been amplified by the decrease in total lipid uptake in SSO-treated control hearts, which highlighted a limitation on this method in measuring total lipid uptake. Since the total fatty acid oxidation throughout the perfusion included pre-SSO oxidation rates, the diabetic hearts increased fatty acid oxidation rates also contributed to the overall greated lipid uptake values which undermined the effect of SSO. Nonetheless, it was evident that SSO decreased fatty acid oxidation and triglyceride deposition in diabetic hearts upon reoxygenation.

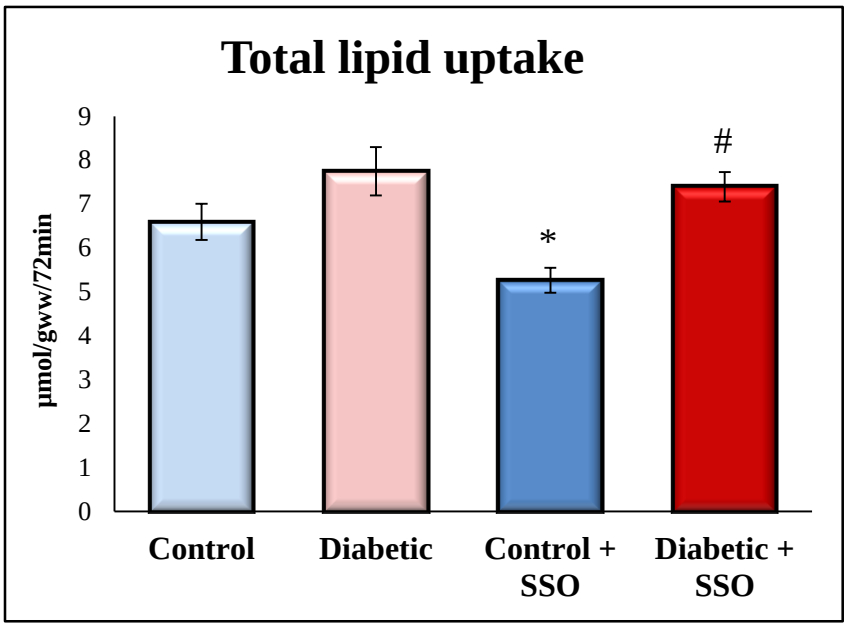

Figure 6.17: Total lipid uptake of isolated control, diabetic, control + SSO and diabetic + SSO hearts perfused in normoxia, hypoxia and reoxygenation summing total lipid incorporation and fatty acid oxidation in 72 minutes. * $p < 0.05$ vs. control, # $p < 0.05$ vs control + SSO. n = 6 per group.

In addition, the lipid incorporation pool is very dynamic as fatty acid continuously becomes esterified and mobilised, hence it was not within the capability of this study to quantify the dynamics of the pool. A pulse-chase experiment such as that conducted by Saddik and Lopaschuk[56] would be needed to measure the turnover rates of these intracellular lipid pools. Most importantly, although superficially SSO does not seem to have an effect on cellular lipid incorporation, after taking all the lipid metabolism data into account, it provides a reasonable explanation. The diabetic hearts were programmed over the duration of the experiment to prefer fat metabolism over glucose metabolism so the diabetic hearts would attempt to increase fatty acid oxidation upon reoxygenation by mobilising fatty acids from the triglyceride stores even following treatment with SSO, but the inhibition of fatty acid uptake via FAT/CD36 forced the decrease in total cardiac triglycerides and subsequently fatty acid oxidation rates. There

was no difference in triglyceride incorporation, suggesting that any fatty acids that enter the cells via other channels such as FABPpm or FATP1, were prioritised to be incorporated into the triglyceride pool in an attempt to replenish the total cardiac triglycerides and restore the high fatty acid oxidation rates.

6.5.4 SSO improves cardiac function in diabetic hearts upon reoxygenation

Data showed that SSO decreased the heart rates and increased developed pressure during hypoxia of SSO-treated control hearts compared to untreated control hearts, but the cardiac function, as defined as RPP, which is a product of heart rate and developed pressure, was not significantly different between the SSO-treated and untreated control hearts. This could be a compensatory reaction of the control hearts after being presented with two physiological stressors (hypoxia and SSO) but overall not having an effect on RPP, the ultimate measurement of cardiac function.

On the other hand, the effect of SSO on cardiac function of diabetic hearts was more pronounced in terms of RPP. The deterioration of RPP in untreated diabetic hearts compared with untreated control hearts from the end of hypoxia until the end of reoxygenation was eliminated by SSO. Most importantly, at 72 minutes, the diabetic hearts had significantly greater RPP compared to untreated diabetic hearts. Coupled with the data on cardiac substrate metabolism, this suggests that firstly, SSO increasing glycolytic rates during hypoxia in diabetic hearts may have provided more anaerobic ATP to maintain cardiac function. Secondly, SSO suppressed fatty acid oxidation rates in diabetic hearts, and triglyceride deposition upon reoxygenation may have minimised the damage from hypoxia-reoxygenation injury and lipotoxicity to the heart. Consequently, the SSO-treated diabetic hearts had improved cardiac function at the end of reoxygenation.

In conclusion, SSO increased glycolytic rates during hypoxia and decreased fatty acid oxidation rates and triglyceride concentrations upon reoxygenation in SSO-treated diabetic hearts compared with untreated diabetic hearts. This may be fundamentally critical to the adaptation to acute hypoxia in diabetic hearts as increasing glycolytic rates may be beneficial in anaerobically generating ATP. The attenuation of the accumulation of triglycerides and decreased fatty acid oxidation may decrease the detrimental effect of lipotoxicity on the heart. The effect of metabolic modulation by SSO was reflected as an improvement in cardiac function in SSO-treated diabetic hearts during hypoxia and upon reoxygenation. Although it appears that SSO is a promising agent to improve diabetic heart function in acute hypoxia, care must be taken as it does bind irreversibly to FAT/CD36 and hence further studies must be carried out to investigate its long term effects. In addition, the short half-life of SSO may be an obstacle to be delivered *in vivo*. Nonetheless, these data provide reaffirming insights that CD36 may be a good therapeutic target to treat type 2 diabetes, although it is still uncertain if its inhibition or activation may be more beneficial. A study has shown that cerivastatin increased aortic CD36 expression, resulting in serum triglyceride concentrations and may have improved insulin resistance syndromes[257]. The decrease in serum triglyceride concentrations may be beneficial in the early stages of diabetes and metabolic syndrome where this may exacerbate the systemic insulin resistance but the inhibition of CD36 to prevent lipid accumulation may be more advantageous as it has been shown that the inhibition of CD36 using anti-CD36 monoclonal antibodies attenuated lipid accumulation and contractile dysfunction in cardiomyocytes[258] while absence of CD36 had cardioprotective effects on Western-type diet-related cardiac dysfunction[259].

CHAPTER 7

GENERAL DISCUSSION

This thesis investigated the relationships between cardiac substrate metabolism, contractile function and hypoxia using a novel type 2 diabetic rat model induced using a low dose STZ in combination with high fat feeding. Overall this thesis showed that the maladaptation of the diabetic hearts to chronic and acute hypoxia may be attributable to cardiac metabolic inflexibility, exacerbated by the elevated fatty acid metabolism associated with upregulation of PPARα targets.

The thesis commenced with the establishment of a novel type 2 diabetic rat model, which exhibited hypercholesterolaemia, hyperglycaemia and adiposity without compromising insulin secretion, resembling human type 2 diabetes (Chapter 3). Subsequently the effect of chronic hypoxia on cardiac function, substrate metabolism and HIF signalling pathway was investigated in type 2 diabetes and glycolysis remained decreased while fatty acid oxidation remained elevated the diabetic hearts compared with control hearts following chronic hypoxia, suggesting metabolic inflexibility (Chapter 4). However there were no defects in the HIF signalling pathway in the diabetic hearts. In acute hypoxia, the diabetic hearts did not upregulate glycolysis to the same extent as control hearts and recovered their elevated fatty acid oxidation rates upon reoxygenation, resulting in the likelihood of cardiac inefficiency, ATP depletion, and reflected in decreased cardiac function during acute hypoxia and reoxygenation (Chapter 5). In the final results chapter, the introduction of SSO prior to acute hypoxia increased glycolytic rates in diabetic hearts compared to untreated diabetic hearts during hypoxia and attenuated myocardial triglyceride deposition upon reoxygenation (Chapter 6). This metabolic modulation resulted in improved cardiac function in diabetic hearts during acute hypoxia and upon reoxygenation.

7.1 THE TYPE 2 DIABETIC RAT MODEL

The first aim of this thesis was to establish our own type 2 diabetic rat model that resembled human type 2 diabetes, was inexpensive yet easy to induce. Our type 2 diabetic rat model used the combination of high fat diet and low dose STZ, which in normoxia, suppressed cardiac glycolysis and increased fatty acid metabolism, coupled with increased oxygen consumption, replicated the diabetic characteristics of the db/db and ob/ob mice models[150, 185, 208, 260]. In addition, our diabetic model did not develop cardiac hypertrophy as seen in ob/ob[150], db/db[149] mice and Zucker diabetic fatty rats[155, 261]. Left ventricular hypertrophy is present in late stages of type 2 diabetes and only seen in approximately half of the patient population in two different studies[262, 263]. Therefore our model was a more suitable platform to investigate the earlier stage of type 2 diabetes. Other important characteristics of our type 2 diabetic model include fasting hyperglycaemia and hyperinsulinaemia, as shown in Chapter 4 and 5, reaffirming that the rats were systemically type 2 diabetic. Characterising cardiac metabolism (Chapter 3) had not been conducted by any other studies before and it was necessary so that the optimal dose of STZ injection to produce a suitable type 2 diabetic model for subsequent studies could be determined.

7.2 HYPOXIA ALTERED CARDIAC SUBSTRATE METABOLISM IN CONTROL HEARTS

In both chronic and acute hypoxia, there were increased glycolytic rates and decreased fatty oxidation rates in control hearts compared to their normoxic counterparts, although the mechanisms involved may be different between chronic and acute hypoxia.

In chronic hypoxia, the increase in glycolysis may be driven by the HIF signalling pathway as a mechanism to decrease oxygen consumption yet maintain ATP production. This has been well documented in hypoxic cancer cells, which upregulated

glycolytic rates and increased lactate production[199, 264]. The upregulation of hypoxia-inducible genes encoding glycolytic enzymes was shown to be specifically activated by HIF-1α in several different cell types[265]. In addition, glycolysis is also uncoupled from the tricarboxylic acid (TCA) cycle via activation of PDK1 by HIF-1α[202] to phosphorylate and inhibit PDH, directing the fate of pyruvate towards lactate production instead of oxidation. There was also a decrease in fat metabolism in the control hearts in chronic hypoxia. Initially it was thought that this decrease in fatty acid oxidation could be due to the increase in glycolysis as according to the Randle cycle, the increased production of glycolytically-derived acetyl-CoA from malonyl-CoA would inhibit CPT1 and fatty acid oxidation[63]. However, this could not be the case given the uncoupling of glycolysis from the TCA cycle. Therefore, the decrease in fatty acid oxidation may be attributable to another mechanism, possibly also regulated by HIF-1α, as HIF-1α was shown to inhibit fatty acid oxidation and energy expenditure in mice and human adipocytes[266]. HIF-1α activation was shown to suppress PPARα DNA binding activity, decreasing PPARα transcriptional activity[194]. This was reaffirmed in Chapter 4 with the decrease of PPARα targets, MCAD activity, PDK4, UCP3 and FATP1 protein levels in control hearts following chronic hypoxia. Taken together, the results suggest that the metabolic adaptation of control hearts to chronic hypoxia involves upregulating of glycolytic pathway and decreasing lipid metabolism.

In acute hypoxia, Chapter 5 illustrated similar increases in glycolytic rates and decreases in fatty acid oxidation rates in control hearts, as seen in response to chronic hypoxia. However, it is not likely that HIF-1α was also the regulator in our acute hypoxic setting as HIF-1α requires time to dimerise, translocate into the nucleus and activate transcriptional and translational changes before exerting an effect on metabolism[97, 267]. Therefore, there must be another mechanism that can produce a

similar significant change in metabolism over a short period of time. Although the metabolic adaptation to acute hypoxia has not been widely studied, the metabolic adaptation to ischaemia has been extensively investigated. AMP-activated protein kinase (AMPK), a serine-threonine kinase sensitive to changes in the AMP to ATP ratios, is an important metabolic regulator, and increases energy production during myocardial ischaemia[217]. It was demonstrated that AMPK activation increases glucose uptake via GLUT4 translocation in rat ventricular muscle tissues[236]. In addition AMPK was also shown to phosphorylate and activate PFK-2[237], increasing glycolytic rates during ischaemia. This was also reaffirmed in another study in mice, demonstrating that AMPK plays an important role in limiting ischaemia-reperfusion injury and apoptosis[268]. However, the role of AMPK in cardiac metabolic adaptation to ischaemia is not limited to the upregulation of glucose uptake and glycolytic rates. While AMPK may still be responsible for the increase in glycolytic rates in acute hypoxia, the mechanism suppressing fatty acid oxidation under these conditions is yet to be determined. This suppressed fatty acid oxidation was also seen in ischaemic rat hearts[139] and it could be postulated that in ischaemia and hypoxia, there is insufficient oxygen to be the final acceptor of electrons in the electron transport chain, resulting in the suppression of fatty acid oxidation.

7.3 METABOLIC INFLEXIBILITY OF DIABETIC HEARTS IN HYPOXIA AND REOXYGENATION

Other studies suggested that the HIF signalling pathway in the type 1 diabetic heart may be impaired[120, 121]. Therefore, it was possible that our type 2 diabetic hearts also had a defective HIF signalling pathway, leading to the abnormal response to chronic hypoxia associated with cardiac metabolic inflexibility. However, the results from Chapter 4 illustrated that there were no defects in the HIF signalling pathway in diabetic hearts.

In all the results chapters, it was demonstrated that the diabetic hearts had decreased glycolysis and increased fatty acid metabolism. Given that PPARα is associated with expression of proteins and enzymes involved in lipid metabolism[88, 269], and the reciprocal relationship between HIF and PPARα[116, 117, 194], PPARα became one of the main investigation areas of this thesis. In Chapter 4, results showed that diabetic hearts had increased MCAD activity, PDK4, UCP3 and FATP1 protein levels, all of which are PPARα targets. This is in agreement with Buchanan *et al.* who demonstrated an increase in PPARα-regulated genes associated with myocardial inefficiency in ob/ob and db/db mouse hearts[185]. In the same study hypoxia brought about the opposite effect on PPARα activation in control hearts by decreasing the PPARα targets. However, the HIF signalling pathway failed to suppress the overactivation of PPARα in diabetic hearts, preventing decrease in fatty acid oxidation and the increase in glycolytic rates, which was observed in hypoxic control hearts.

This elevated fatty acid oxidation and reliance on lipid metabolism in diabetic hearts did not only affect the cardiac adaptation to chronic hypoxia, it also impacted on cardiac metabolism and function in response to acute hypoxia as illustrated in Chapter 5 and 6. Our results showed that diabetic hearts were metabolically inflexible and failed to upregulate glycolysis to the same extent as control hearts during acute hypoxia, most likely resulting in decreased anaerobic ATP production and associated with decreased in cardiac mechanical function. Therefore with acute hypoxia as a physiological stressor, the negative impact of the metabolic inflexibility in diabetic hearts was emphasised and exacerbated. Studies have already established that overactivation of PPARα and increased lipid metabolism in diabetic mouse hearts are associated with decreased cardiac efficiency and post-ischaemic functional loss[185, 190, 208]. Upon reoxygenation, results from Chapter 5 showed that diabetic hearts failed to recover

cardiac function to the same level as control hearts. Kewalramani *et al.* suggested that AMPK activation is compromised in diabetic hearts, resulting in sustained ischaemia-reperfusion injury[270]. The decreased cardiac efficiency in diabetic hearts as discussed earlier may also have increased the severity of the ischaemia-reperfusion injury[185, 190, 208]. In addition the diabetic hearts also exhibited the characteristics of bradycardia and ventricular ectopic beats during hypoxia, followed by cardiac arrhythmia upon reoxygenation. This may also be attributable to the increased fat metabolism in diabetic hearts, specifically the deposition of triglycerides upon reoxygenation, as it has been established that high levels of lipid is associated with arrhythmia[55, 241] as well as accumulation of toxic lipid intermediates such as ceramides and diacylglycerol, which may contribute to lipotoxicity[239, 240]. Table 7.1 summarises the effects of chronic and acute hypoxia on control and diabetic hearts.

Table 7.1: Summary of effect of chronic and acute hypoxia on control and diabetic hearts.

	Chronic hypoxia		Acute hypoxia	
	Control hearts	Diabetic hearts	Control hearts	Diabetic hearts
Glycolysis	↑	↑ ↓	↑	↑ ↓
Lactate efflux	↑	↑ ↓	↑	↑ ↓
Fatty acid oxidation	↓	↔ ↑	↓	↓
IFM fatty acid state 3 respiration	↓	↔		
SSM fatty acid state 3 respiration	↓	↔		
Cardiac triglyceride	↔	↔	↔	↔
Cardiac glycogen	↑	↑ ↓	↓	↓
PPARα targets				
MCAD activity	↓	↓ ↑		
PDK4 protein	↓	↓ ↑		
UCP3 protein	↓	↔ ↑		
FATP1 protein	↓	↔		

↑ = increased compared with normoxic counterpart

↓ = decreased compared with normoxic counterpart

↔ = unchanged compared with normoxic counterpart

↑ = increased compared to hypoxic control hearts

↓ = decreased compared to hypoxic control hearts

7.4 INHIBITION OF FAT/CD36 IMPROVED CARDIAC FLEXIBILITY AND FUNCTION OF DIABETIC HEARTS DURING HYPOXIA AND REOXYGENATION

In Chapter 6, SSO was used to inhibit fatty acid uptake via FAT/CD36 and consequently fatty acid oxidation, in order to upregulate glycolysis of diabetic hearts during acute hypoxia. As was established in Chapter 5, the decreased mechanical function of diabetic hearts during acute hypoxia and upon reoxygenation was associated with metabolic inflexibility. SSO was shown to inhibit fatty acid uptake and oxidation by 50% in cardiomyocytes[60] and therefore via the Randle cycle, the inhibition of fatty acid metabolism should result in an increase in glycolysis. Results from Chapter 6 demonstrated that FAT/CD36 was indeed a potential target for metabolic modulation in improving cardiac function of diabetic hearts during acute hypoxia as it increased glycolysis in hypoxia and attenuated triglyceride deposition upon reoxygenation. The benefit of this metabolic modulation was reflected in improved cardiac function of diabetic hearts at the end of reoxygenation. Therefore, SSO provides a rapid way to selectively modulate metabolism without any adverse effects on the heart. Overall this suggests that targeting lipid metabolism in the diabetic heart is a promising avenue to improve cardiac function and potentially to minimise ischaemia-reperfusion injury.

7.5 LIMITATIONS, FUTURE DIRECTIONS AND CONCLUSION

Since type 2 diabetes is a progressive disease, it has been made aware that the type 2 diabetic model used in this thesis has its limitations representing the exact stage of the disease development. Besides, more experiments such as the glucose tolerance test could have been included to further verify the model. In Chapter 4, a reliable way of measuring PPARα and AMPK levels may elucidate the underlying mechanism of the abnormal response of diabetic hearts to chronic hypoxia. The measurement of AMPK could be especially useful and should be prioritised as future experiments to investigate

if diabetic hearts have impaired AMPK activity, which may lead to decreased cardiac function during acute hypoxia and upon reoxygenation. In addition, it would also be very interesting to measure ROS production in diabetic hearts during hypoxia and upon reoxygenation while adjustments should be made on the glucose and fatty acid concentrations in the perfusion buffer to better represent physiological concentrations instead of a standard concentration across all groups. Chapter 6 could also be expanded using clinically approved drugs such as metformin or thiazolidinediones to investigate if their glycaemic-controlling properties affect cardiac function and metabolism in acute hypoxia.

This thesis has established a novel type 2 diabetic rat model that exhibited hypercholesterolaemia, hyperglycaemia and adiposity, using a combination of low dose STZ and high fat diet. This model was used to demonstrate the effect of chronic and acute hypoxia on the type 2 diabetic heart. Both chronic and acute hypoxia decreased fatty acid metabolism and increased glycolysis via different mechanisms in control hearts. HIF-1α plays a vital role in metabolic regulation and adaptation to chronic hypoxia while AMPK may be responsible for the response to acute hypoxia. However, despite the unaffected HIF signalling pathway in type 2 diabetic hearts, the metabolic response to chronic hypoxia was still blunted and this could be due to the overactivation of PPARα. In acute hypoxia, the elevated fatty acid metabolism associated with impaired upregulation of glycolysis, likely via the Randle cycle, and resulted in increased triglyceride deposition upon reoxygenation. As a result, cardiac function of diabetic hearts started to deteriorate during acute hypoxia and did not recover to the same level as control hearts upon reoxygenation. By inhibiting the first step in cardiac lipid metabolism, FAT/CD36 using SSO, glycolysis was upregulated in diabetic hearts during acute hypoxia and triglyceride deposition upon reoxygenation was attenuated.

The impact of this was translated into the improvement of cardiac function in diabetic hearts at the end of reoxygenation. In summary, this thesis has demonstrated that type 2 diabetic hearts were metabolically inflexible and this is associated with impaired metabolic response and decreased cardiac function following chronic and acute hypoxia. This metabolic and functional maladaptation of type 2 diabetic hearts in hypoxia was improved by the inhibition of FAT/CD36 using SSO. This thesis could also be extended to cover the metabolism and effects of pharmacological agents of type 2 diabetic hearts in ischaemia both *in vivo* and *ex vivo* in isolated heart perfusions. Ultimately should the metabolic modulation be proven as an appropriate therapeutic target, high throughput screenings should be done to identify potential drugs or biologics that can modulate metabolism in type 2 diabetes while conducting further studies to elucidate the mechanism of action of current therapies as they are currently not fully understood.

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APPENDICES

APPENDIX 1 – CHOW DIET COMPOSITION

APPENDIX 2 – HIGH FAT DIET COMPOSITION

APPENDIX 3 – PROTOCOL: PREPARING PALMITATE STOCK SOLUTION AND BINDING OF PALMITATE TO BSA-KH BUFFER

APPENDIX 4 – ^{31}P NMR ACUTE HYPOXIC PERFUSIONS

APPENDIX 5 – PUBLICATION

APPENDIX 1 – CHOW DIET COMPOSITION

Harlan Laboratories

2016

Teklad Global 16% Protein Rodent Diet

Product Description- 2016 is a fixed formula, non-autoclavable diet manufactured with high quality ingredients and designed to support growth and maintenance. 2016 does not contain alfalfa or soybean meal, thus minimizing the occurrence of natural phytoestrogens. Typical isoflavone concentrations (daidzein + genistein aglycone equivalents) range from non-detectable to 20 mg/kg. Exclusion of alfalfa reduces chlorophyll, improving optical imaging clarity. Absence of animal protein and fish meal minimizes the presence of nitrosamines. **Also available certified (2016C) and irradiated (2916). For autoclavable diet, refer to 2016S (Sterilizable).**

Ingredients (in descending order of inclusion)- Ground wheat, ground corn, wheat middlings, corn gluten meal, calcium carbonate, dicalcium phosphate, soybean oil, brewers dried yeast, iodized salt, L-lysine, DL-methionine, choline chloride, magnesium oxide, vitamin E acetate, menadione sodium bisulfite complex (source of vitamin K activity), manganous oxide, ferrous sulfate, zinc oxide, niacin, calcium pantothenate, copper sulfate, pyridoxine hydrochloride, riboflavin, thiamin mononitrate, vitamin A acetate, calcium iodate, vitamin B_{12} supplement, folic acid, biotin, vitamin D_3 supplement, cobalt carbonate.

Standard Product Form: ***Pellet***

Macronutrients		
Crude Protein	%	16.4
Fat (ether extract) [a]	%	4.0
Carbohydrate (available) [b]	%	48.5
Crude Fiber	%	3.3
Neutral Detergent Fiber [c]	%	15.2
Ash	%	4.9
Energy Density [d]	kcal/g (kJ/g)	3.0 (12.6)
Calories from Protein	%	22
Calories from Fat	%	12
Calories from Carbohydrate	%	66
Minerals		
Calcium	%	1.0
Phosphorus	%	0.7
Non-Phytate Phosphorus	%	0.4
Sodium	%	0.2
Potassium	%	0.6
Chloride	%	0.4
Magnesium	%	0.2
Zinc	mg/kg	70
Manganese	mg/kg	100
Copper	mg/kg	15
Iodine	mg/kg	6
Iron	mg/kg	200
Selenium	mg/kg	0.23
Amino Acids		
Aspartic Acid	%	1.0
Glutamic Acid	%	3.3
Alanine	%	0.9
Glycine	%	0.7
Threonine	%	0.6
Proline	%	1.5
Serine	%	0.8
Leucine	%	1.9
Isoleucine	%	0.7
Valine	%	0.8
Phenylalanine	%	0.9
Tyrosine	%	0.5
Methionine	%	0.3
Cystine	%	0.3
Lysine	%	0.8
Histidine	%	0.4
Arginine	%	0.8
Tryptophan	%	0.2

Vitamins		
Vitamin A [e,f]	IU/g	15.0
Vitamin D_3 [e,g]	IU/g	1.5
Vitamin E	IU/kg	110
Vitamin K_3 (menadione)	mg/kg	50
Vitamin B_1 (thiamin)	mg/kg	17
Vitamin B_2 (riboflavin)	mg/kg	15
Niacin (nicotinic acid)	mg/kg	75
Vitamin B_6 (pyridoxine)	mg/kg	18
Pantothenic Acid	mg/kg	33
Vitamin B_{12} (cyanocobalamin)	mg/kg	0.08
Biotin	mg/kg	0.40
Folate	mg/kg	4
Choline	mg/kg	1030
Fatty Acids		
C16:0 Palmitic	%	0.5
C18:0 Stearic	%	0.1
C18:1ω9 Oleic	%	0.7
C18:2ω6 Linoleic	%	2.0
C18:3ω3 Linolenic	%	0.1
Total Saturated	%	0.6
Total Monounsaturated	%	0.7
Total Polyunsaturated	%	2.1
Other		
Cholesterol	mg/kg	--

[a] Ether extract is used to measure fat in pelleted diets, while an acid hydrolysis method is required to recover fat in extruded diets. Compared to ether extract, the fat value for acid hydrolysis will be approximately 1% point higher.

[b] Carbohydrate (available) is calculated by subtracting neutral detergent fiber from total carbohydrates.

[c] Neutral detergent fiber is an estimate of insoluble fiber, including cellulose, hemicellulose, and lignin. Crude fiber methodology underestimates total fiber.

[d] Energy density is a calculated estimate of *metabolizable energy* based on the Atwater factors assigning 4 kcal/g to protein, 9 kcal/g to fat, and 4 kcal/g to available carbohydrate.

[e] Indicates added amount but does not account for contribution from other ingredients.

[f] 1 IU vitamin A = 0.3 µg retinol

[g] 1 IU vitamin D = 25 ng cholecalciferol

For nutrients not listed, insufficient data is available to quantify.

Nutrient data represent the best information available, calculated from published values and direct analytical testing of raw materials and finished product. Nutrient values may vary due to the natural variations in the ingredients, analysis, and effects of processing.

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APPENDIX 2 – HIGH FAT DIET COMPOSITION

Special Diets Services

DIET FORMULATION AND SPECIFICATION DATA

BASIC DIET INFORMATION:

Code:	829197
Name:	RM AFE 60FAT 35CP, 5CHO (M)
Date:	15/10/2013

CALCULATED ANALYSIS:

		FRESH	10% H2O
TOTAL	%	100.00	100.00
MOISTURE	%	4.66	10.00
CRUDE OIL	%	36.01	33.99
CRUDE PROTEIN	%	45.07	42.55
CRUDE FIBRE	%	2.88	2.72
ASH	%	4.02	3.79
NFE	%	6.94	6.55
PECTIN	%	0.00	0.00
HEMICELLULOSE	%	0.08	0.08
CELLULOSE	%	3.85	3.63
LIGNIN	%	0.00	0.00
STARCH	%	0.20	0.19
SUGAR	%	4.83	4.56
GROSS ENERGY	MJ/kg	20.00	18.88
DIGESTIBLE ENERGY	MJ/kg	18.67	17.62
METABOLISABLE ENERGY	MJ/kg	17.64	16.65
AF ENERGY	kcal/kg	5298.10	5001.35
C14 1 MYRISTOLEIC	%	0.02	0.02
C16 1 PALMITOLEIC	%	0.04	0.04
C18 1 W9 OLEIC	%	14.09	13.30
C18 2 W6 LINOLEIC	%	6.95	6.56
C18 3 W3 LINOLENIC	%	0.82	0.77
C20 4 W6 ARICHIDONIC	%	0.02	0.02
C22 5 W3 CLUPANODONIC	%	0.00	0.00
C12:0 LAURIC	%	2.81	2.65
C14:0 MYRISTIC	%	1.13	1.07
C16:0 PALMITIC	%	2.29	2.16
C18:0 STEARIC	%	1.65	1.56
ARGININE	%	1.56	1.47
LYSINE	%	3.84	3.62
S LYS	%	0.00	0.00
METHIONINE	%	1.02	0.96
S METH	%	0.00	0.00
CYSTINE	%	0.96	0.91
S CYST	%	0.18	0.17
TRYPTOPHAN	%	0.71	0.67
S TRYPT	%	0.00	0.00
HISTIDINE	%	0.99	0.93

		FRESH	10% H2O
CL	%	0.30	0.28
S CL	%	0.16	0.15
K	%	0.65	0.61
S K	%	0.50	0.47
MG	%	0.07	0.07
S MG	%	0.05	0.05
FE	mg/kg	55.35	52.25
S FE	mg/kg	43.44	41.01
CU	mg/kg	8.23	7.77
S CU	mg/kg	6.03	5.69
MN	mg/kg	10.98	10.37
S MN	mg/kg	9.48	8.95
ZN	mg/kg	55.79	52.67
S ZN	mg/kg	30.09	28.40
CO	µg/kg	0.01	0.01
S CO	µg/kg	0.00	0.00
I	µg/kg	206.53	194.96
S I	µg/kg	206.50	194.93
SE	µg/kg	159.95	150.99
S SE	µg/kg	159.95	150.99
F	mg/kg	1.01	0.95
VIT A	iu/kg	4000.00	3775.96
S VIT A	iu/kg	4000.00	3775.96
VIT D3	iu/kg	2200.00	2076.78
S VIT D3	iu/kg	1000.00	943.99
VIT E	iu/kg	85.77	80.97
S VIT E	iu/kg	75.00	70.80
VIT B1 THI	mg/kg	4.99	4.71
S VIT B1	mg/kg	4.86	4.59
VIT B2 RIB	mg/kg	5.64	5.32
S VIT B2	mg/kg	4.80	4.53
VIT B6 PYR	mg/kg	5.84	5.51
S VIT B6	mg/kg	5.71	5.39
VIT B12 CY	µg/kg	25.05	23.65
S VIT B12	µg/kg	25.00	23.60
VIT C ASCO	mg/kg	0.00	0.00
S VIT C	mg/kg	0.00	0.00
VIT K MENE	mg/kg	0.77	0.73

This information is intended as a guide only. For actual data we recommend that analysis work is carried out to confirm the nutrient parameters precisely

THREONINE	%	2.22	2.10
S THREO	%	0.00	0.00
ISOLEUCINE	%	2.35	2.22
LEUCINE	%	4.90	4.63
PHENYLALAN	%	1.99	1.88
VALINE	%	2.57	2.43
TYROSINE	%	1.93	1.82
TAURINE	%	0.00	0.00
GLYCINE	%	1.52	1.43
ASPARTIC A	%	1.97	1.86
GLUTAMIC A	%	6.64	6.27
PROLINE	%	2.61	2.46
SERINE	%	2.06	1.94
HYD PROLIN	%	0.00	0.00
HYD LYSINE	%	0.00	0.00
ALANINE	%	2.12	2.00
CA	%	0.54	0.51
S CA	%	0.47	0.44
TOTAL P	%	0.27	0.25
S PHOS	%	0.16	0.15
PHYTATE P	%	0.00	0.00
AVAIL P	%	0.07	0.07
NA	%	0.16	0.15
S NA	%	0.10	0.09

S VIT K	mg/kg	0.77	0.73
FOLIC ACID	mg/kg	1.92	1.81
S FOLIC	mg/kg	1.90	1.79
NICOTINIC	mg/kg	29.88	28.21
S NICOTIN	mg/kg	29.40	27.75
PANTOTHENI	mg/kg	15.53	14.66
S PANTOTH	mg/kg	14.35	13.55
CHOLINE	mg/kg	1027.59	970.03
S CHOLINE	mg/kg	1027.50	969.95
INOSITOL	mg/kg	0.04	0.04
S INOSITOL	mg/kg	0.00	0.00
BIOTIN	µg/kg	200.03	188.83
S BIOTIN	µg/kg	200.00	188.80

INGREDIENTS:

NAME
LACTALBUMIN
CASEIN
OLIVE OIL
SOYA OIL
POTATO PROTEIN
COCONUT OIL
CELLULOSE
MINERALS, VITAMINS, AMINO ACIDS AND TRACE ELEMENTS
DEXTROSE MONOHYDRATE
CHOLINE BITARTRATE
CHOLESTEROL
ANTIOXIDANT

This information is intended as a guide only. For actual data we recommend that analysis work is carried out to confirm the nutrient parameters precisely

APPENDIX 3 – PROTOCOL: PREPARING PALMITATE STOCK SOLUTION AND BINDING OF PALMITATE TO BSA-KH BUFFER

The palmitate stock solution preparation should be conducted only using glass equipment as palmitate may stick to plastic. To make a 100 mM palmitate stock solution, palmitic acid (Sigma) (2.56 g) was dissolved in 2 ml of ethanol in a water bath at 70 ^{o}C. A minimum volume of distilled H_2O was used to dissolve 1.38 g K_2CO_3 and the solution slowly added to the palmitic acid solution. Precipitation occured and it was left to completely dry in the water bath. The dried potassium palmitate salt was dissolved in 100 ml fresh saline to make up a 100 mM stock solution (may need to be left in water bath to fully dissolve the salt).

After 1.5% BSA was fully dissolved in KH buffer, the BSA-KH buffer was heated to 30 ^{o}C. The palmitate stock solution has to be heated in a water bath at 70 ^{o}C to remain in solution. While it is in solution, an amount was pipetted with a heated tip directly into the BSA-KH needed to make up 0.4 mM final concentration of palmitate in the BSA-KH buffer and the palmitate should bind to the albumin immediately.

APPENDIX 4 – ^{31}P NMR ACUTE HYPOXIC PERFUSIONS

A) Trace of cardiac function during normoxia, hypoxia and reoxygenation

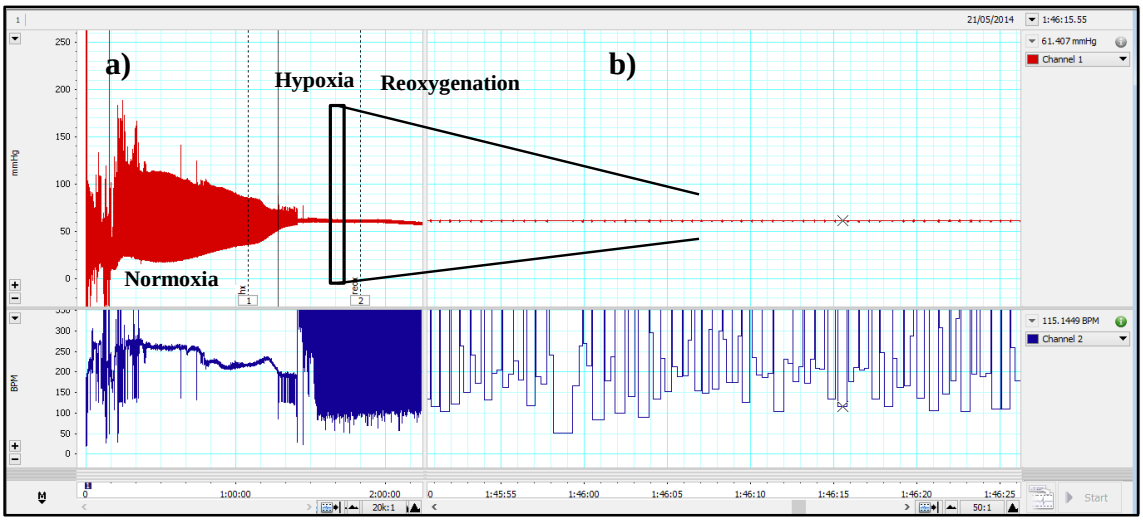

Figure A4.1: Example of cardiac function trace of an isolated perfused control heart. a) Overall cardiac function during experiment. b) Highlighted cardiac function in hypoxia. Due to technical difficulties and airblock formation in the magnet umbilical, cardiac function started to decrease in normoxic conditions and continued to deteriorate during hypoxia, and did not recover upon reoxygenation.

B) ATP concentration of isolated perfused control and diabetic hearts in normoxia, hypoxia and reoxygenation

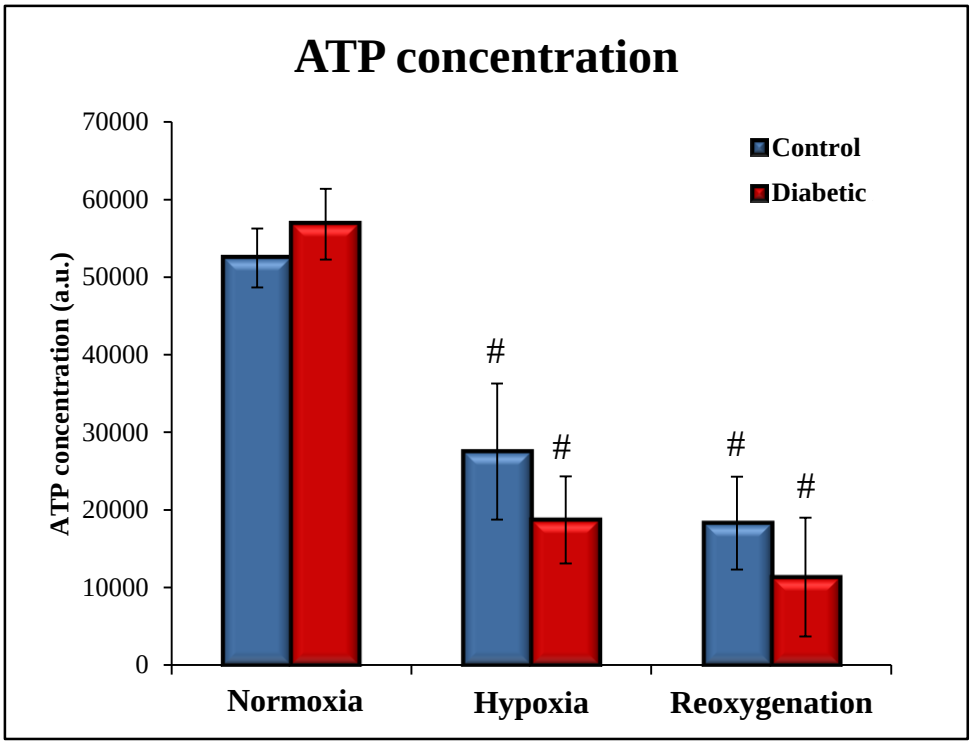

Figure A4.2: ATP concentration in control and diabetic hearts indicating a decrease in ATP production, reflecting the decrease in cardiac function during hypoxia. ATP concentrations of both control and diabetic hearts remained decreased compared to their normoxic levels upon reoxygenation, indicating that the hearts did not recover from acute hypoxia. # $p < 0.05$ vs. same disease state at normoxia. $n = 4 – 6$ per group.